

A BMP-dependent feedback loop regulates *dpp* expression by  
direct and indirect mechanisms in the *Drosophila* wing imaginal  
disc

by

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B.S. The College of William and Mary, 2005

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This dissertation by Maryanna M. Aldrich is accepted in its present form by the Division of Biology and Medicine as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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## Abstract

Morphogen activity gradients assign positional information within fields of cells in a concentration-dependent manner. As transcriptional changes respond to signaling activity thresholds, cells must encounter a particular level of morphogen activity. As such, signal transduction pathways that behave as morphogens must have regulatory mechanisms in place to be robust and accommodate for signaling activity fluctuations. In the *Drosophila* wing imaginal disc, the BMP signaling pathway functions as a morphogen through the graded distribution of phosphorylated transducer, pMad. In this tissue, signaling through the ligand Dpp is important in generating the pMad gradient that directs transcription of target genes required for anterior-posterior patterning. Several mechanisms are known through which this pathway attains robustness; the work presented here identifies and characterizes an additional mechanism of transcriptional regulation of the ligand *dpp*. By examining *dpp* expression with increased or decreased BMP signaling levels, we have demonstrated that transcription of *dpp* within the medial wing pouch is regulated by a negative feedback loop. Mediated through canonical Mad-mediated BMP signaling, regulation of *dpp* occurs in a pMad dose-dependent manner. In the lateral wing pouch, BMP signaling has the ability to positively regulate expression of *dpp*, indicating a domain-specific dual role for BMP signaling in regulating *dpp*. By dissecting *dpp*'s cis-regulatory sequences, we have revealed multiple enhancer regions that mediate feedback regulation of *dpp*. By testing specific binding sites and factors for a requirement in regulating *dpp*, we have determined that Mad plays a direct and indirect role in *dpp* regulation: directly, a Mad binding site influences *dpp* expression in the lateral wing pouch and, indirectly, a factor downstream of the Shn/pMad/Med repressor complex is required for negative feedback of *dpp* in the medial wing pouch. The transcription factor Ci is not required for negative regulation of *dpp*, despite its well-known role in determining the wing disc expression pattern of *dpp*. We propose that this negative feedback mechanism serves to "fine tune" BMP signaling and keep its activity levels within a normal range, accommodating for fluctuations in signaling output and ensuring robust signaling throughout development.



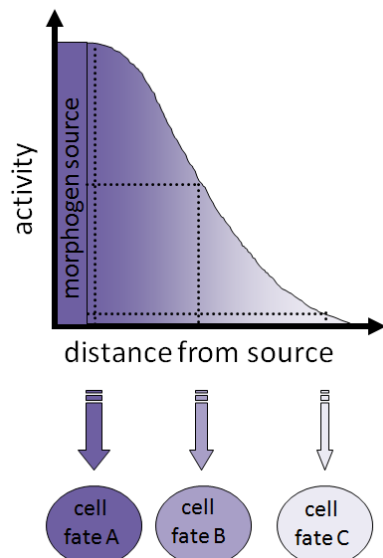
## Chapter 1:

### Introduction

## The Morphogen Gradient Theory

The development of multicellular organisms begins with the fusion of egg and sperm to generate a single-celled zygote. This single cell will undergo a long and complex path of division and differentiation to create the multitude of vastly different and sophisticated cell types found within adult structures. How does this occur? How are these events coordinated? How can so many cell types form from distinct fields of unspecified cells? These questions remain one of the most intriguing phenomena in developmental biology.

Often, tissues are patterned by extracellular signaling cues termed morphogens (Turing, 1952). A morphogen is a factor that can specify cell fates in cells that are exposed to this cue in a concentration-



**Figure 1.1. The morphogen gradient theory.** In this model, a morphogen, emanating from its source on the left, can diffuse across a field of cells, generating a morphogen activity gradient. The morphogen provides position information to direct transcriptional responses and ultimately cell fate patterning across the field of cells. Cells that encounter the high threshold of activity are specified as cell fate A, cells that encounter the moderate threshold are specified as cell fate B, and cells that encounter the low threshold are specified as cell fate C.

dependent manner. The morphogen gradient theory, as originally introduced by Lewis Wolpert, proposed the concept of a diffusible factor being secreted from a localized source that travels to target cells to elicit a response in a concentration-dependent manner (Wolpert, 1969). Once secreted, morphogens are thought to diffuse across a field of cells (Fig 1.1, left), with morphogen concentration decreasing the farther they are from the source. The result is a gradient of morphogen across the tissue with each cell encountering a different level of morphogen (Fig 1.1). Cells may respond to particular thresholds of morphogen, each of which elicits a specific transcriptional response and influences specification of distinct cell fates. In the schematic diagram in figure 1.1, the cells in this field can respond to three distinct thresholds of morphogen that influence the specification of cells to fates A, B, and C.

Many different factors have been found to elicit responses in a concentration-dependent manner (Tabata and Takei, 2004). These include, but are not limited to, secreted ligands associated with the Hh,

Wnt, FGF, and TGF- $\beta$  signal transduction pathways. While the original model proposed by Wolpert was concerned with the concentration of secreted ligand across a field of cells, it has become increasingly clear that the concept of a gradient of one component is a simplistic view of what actually occurs *in vivo*. The ability of the receiving cell to respond to a morphogen is shaped not only by the concentration of ligand it's exposed to, but also factors such as receptor availability, receptor affinity, availability of intracellular components, and the presence or absence of negative regulators of the pathway. When considering all of these variables, the morphogen gradient theory as explained by a gradient of ligand is inadequate. We must, therefore, consider a model in which a field of cells is patterned by a morphogen "activity" gradient which takes into account all of these factors.

For a secreted ligand to participate in the generation of a morphogen activity gradient, it must reach its target cells within a tissue and initiate a signal transduction cascade. The source of ligand is often many cells away from its target and must somehow travel to these cells. There is no definitive explanation that exists to explain how all ligands move in all tissues and it is likely that one or several mechanisms of ligand delivery play a role in each particular morphogen gradient system observed. In *Drosophila* alone, several models have been proposed to explain how ligand secreted from a source can travel to its target cells, including: passive diffusion, restricted diffusion, planar transcytosis, lipoprotein transfer, facilitated diffusion, and cytonemes (Cadigan, 2002; Yan and Lin, 2009).

The passive diffusion model states that ligands secreted from their source diffuse passively and form a gradient as they reach their target cells. This model may explain some morphogen systems, such as the intracellular distribution of Bicoid and Nanos in the syncytial *Drosophila* embryo (Gregor et al., 2005), but it is not sufficient to explain the extracellular distribution of most secreted ligands. The restricted diffusion model, instead, poses that the extracellular environment, including extracellular matrix proteins and receptors, encountered by secreted ligands will influence ligand movement (Lander et al., 2002; Lin and Perrimon, 2002). Extracellular factors such as heparan sulfate proteoglycans (HSPGs) have been found to play critical roles in determining the range of ligand action within many signaling contexts, including in generating the activity gradient of the Wg, BMP, and Hh pathways in the *Drosophila* wing imaginal disc (Princivalle and de Agostini, 2002). HSPGs, bound to the membrane either by anchoring through GPI

linkage or through a transmembrane domain, contain a protein core with attached HS (heparan sulfate), glycosaminoglycan (GAG), or chondroitin sulfate (CS) side-chains. These side-chains can interact with secreted ligands to facilitate their movement, stabilize them, or act as a co-receptor.

The planar transcytosis model argues that factors can travel across fields of cells through a series of exocytic and endocytic events (Entchev and González-Gaitán, 2002; Entchev et al., 2000). The lipoprotein transfer model is based on the idea that secreted lipids are carried to their target cells through lipid-dense particles. There is evidence that this mechanism may aid in the travel of Hh and Wg ligands (Panakova et al., 2005). In facilitated diffusion, ligands are bound by carrier proteins that aid in their movement. This form of ligand movement has been found to play an important role in generating the BMP activity gradient in the early *Drosophila* embryo (Shimmi et al., 2005). In the final model of ligand movement, long actin-based cellular projections called cytonemes allow direct interaction between ligand producing cells and target cells (Affolter and Basler, 2011; Ramirez-Weber and Kornberg, 1999). Recent work has elucidated a role for cytonemes in BMP and EGFR signaling in the development of *Drosophila* imaginal discs (Hsiung et al., 2005; Roy et al., 2011)

Many different examples of morphogen gradients have been recognized throughout development that play roles in an assortment of cell types and developmental contexts. For example, in early *Xenopus* patterning, Wnt signaling, originating from an area of the embryo known as the Nieuwkoop center, plays a role in specifying cell fate along the dorsal-ventral axis of the embryo (Funayama et al., 1995; Guger and Gumbiner, 1995). High levels of Wnt activity specifies dorsal mesoderm tissue through activity of the Wnt transducer,  $\beta$ -catenin. Further from the Nieuwkoop center, where  $\beta$ -catenin levels are low, ventral tissue is induced. Morphogen gradients can also have a combinatorial effect, such that the relative activity of two different signaling pathways determines cell fate. Combining two activity gradients serves to increase the variety and number of cellular responses and potential cell fates to a level unattainable with just one gradient. A classic example of two signaling pathways behaving as morphogen gradients in concert involves Shh and BMP signaling in patterning of the vertebrate neural tube. In this tissue, Shh and BMP form reciprocal gradients along the dorsal-ventral axis of the neural tube. BMP signaling originating from the overlying dorsal epidermis is important in specifying dorsal neural tube

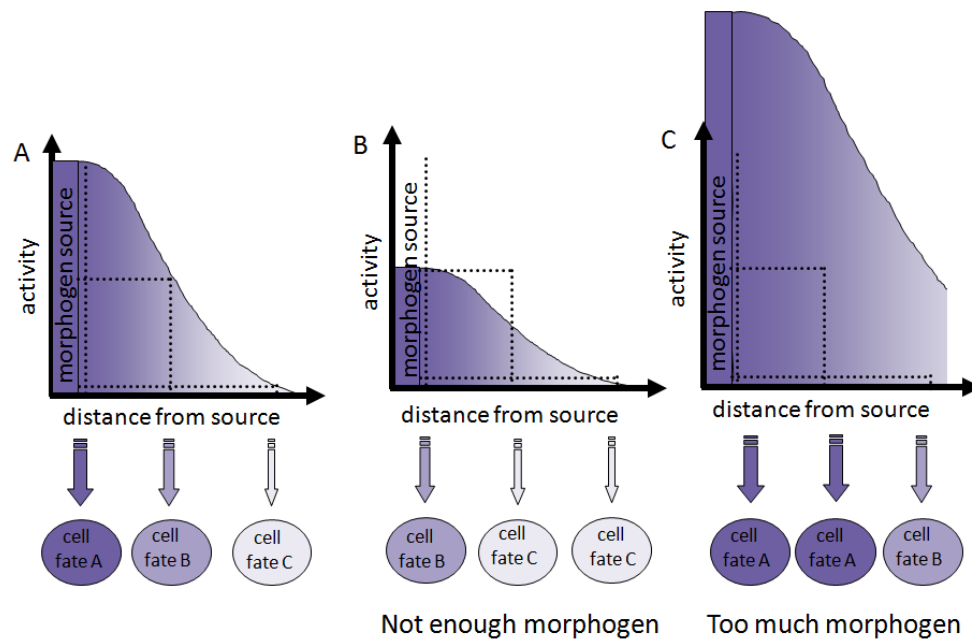
fates. Shh signaling and BMP antagonists originating from the ventrally located notochord are important in specifying ventral neural tube cell fates (McMahon et al., 1998; Patten and Placzek, 2002; Selleck et al., 1998).

### **Regulation and robustness of morphogen gradients**

In a system like that of a morphogen gradient where discrete thresholds of activity are important, it is critical that the activity gradient be capable of accommodating for fluctuations in activity. This idea that morphogen gradients can remain stable in the face of fluctuations in activity is known as robustness, and a robust signaling pathway can effectively accommodate for changes in signaling levels (Barkai and Leibler, 1997; Barkai and Shilo, 2009; Eldar et al., 2004; Freeman, 2000; Kerszberg, 2004; Kerszberg and Wolpert, 2007). Proper thresholds of activity must be met at the appropriate time in order to ensure correct transcriptional responses and cell specification. In the event of aberrant signaling, the expected activity threshold may not be met in all regions of the tissue and specific cell fates may be lost or gained, leading to compromised development (Fig 1.2). If, for example, signaling levels are reduced then cells may never encounter the higher thresholds of activity, cellular responses that respond to this threshold may not occur, and high-threshold fates may not be realized (Fig 1.2B). Conversely, an inappropriately high level of morphogen activity could result in more cells encountering a high threshold of activity, resulting in specification of more high-threshold fates at the expense of the low-threshold fates (Fig 1.2C). In a robust signaling system, a decrease (Fig 1.2B) or an increase (Fig 1.2C) in signaling would be accommodated for, bringing signaling levels back to normal (Fig 1.2A) and development would proceed normally.

During development, a multitude of events could occur that can lead to perturbations in signaling levels. Cells are subjected to a variety of external conditions, such as environmental or physical stresses, as well as genetic effects, such as hypomorphic alleles or genetic backgrounds, that could lead to fluctuations in activity of a signaling pathway. Furthermore, processes intrinsic to development, such as tissue growth and proliferation, generate an ever-changing field of cells and can influence the level and range of a morphogen gradient. In any given system, the specific morphogen gradient may be dynamic

across developmental time, but the system likely still requires distinct activity thresholds to be met at particular times in development. With these considerations in mind, it is clear that mechanisms must be in place to attain robust signaling and accommodate for and buffer against fluctuations in signaling that occur in response to environmental, genetics, and developmental events as, without such mechanisms, there could be disastrous results on development overall. We expect developmentally important signaling pathways to have an extensive network of regulatory mechanisms in place to respond to any signaling changes that may occur.



**Figure 1.2. Altered morphogen levels influence cell fate patterning.** As cellular responses depend on discrete thresholds of morphogen activity, it is critical that these thresholds are properly attained during development. Fluctuations in activity may result in failure to meet thresholds and a subsequent loss or gain of cell fates. **A.** Proper morphogen thresholds are met in this field of cells, leading to specification of fates A, B, and C. **B.** Morphogen activity levels are improperly reduced. Under these signaling conditions, the high threshold of activity is not met, resulting in a loss of cell fate A and an overabundance of cell fate C. **C.** Morphogen levels have been increased above endogenous levels, resulting in increased specification of cell fate A at the expense of cell fate C.

There are several ways that a pathway could attain robustness, but one particularly intriguing mechanism is the autoregulatory feedback loop (Freeman, 2000). In this system, a signal transduction pathway can affect its own signaling output, in either a positive or negative manner. A positive feedback loop occurs when an initially low level of activity amplifies its own output by initiating a stronger response over time, achieved by increasing the level or activity of some pathway component in response to signaling. A negative feedback loop, on the other hand, is a compensatory mechanism that acts to

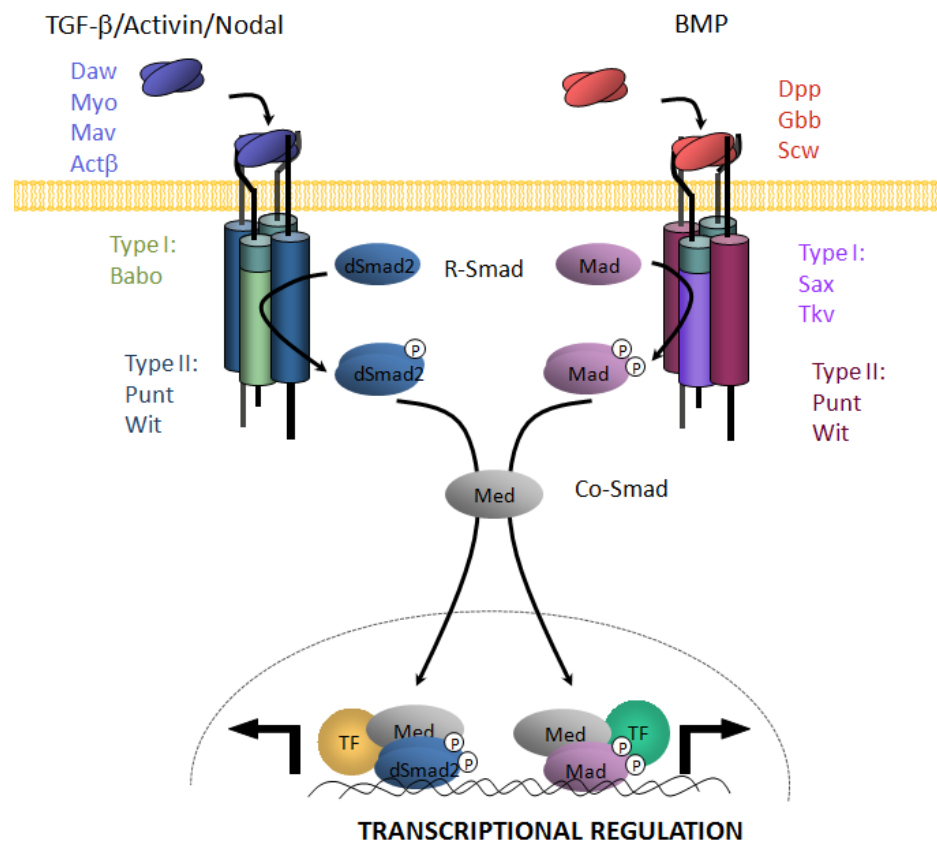
maintain signaling levels. This mode of feedback can lead to a decrease in a pathway's own activity if too much signaling occurs or it can cause an increase in a pathway's own activity if there is insufficient signaling. In the examples presented in figure 1.2, a negative feedback loop could accommodate for these changes in signaling levels by increasing signaling in figure 1.2B and decreasing signaling in figure 1.2C, bringing the gradient back to its appropriate level. This may be realized by altering the expression of either a key component in the pathway or a negative regulator of the pathway. Negative feedback loops, therefore, act as sensing mechanisms of signaling output that can modulate the signal accordingly and allow robust signaling activity. Given this, it is not surprising that many developmentally important signaling pathways are regulated by one or several negative feedback loops (Barkai and Shilo, 2009; Freeman, 2000; Kerszberg and Wolpert, 2007; Perrimon and McMahon, 1999).

Crosstalk between other signaling pathways represents another mechanism often used to regulate signaling levels. This effect can be either positive or negative, but in this case, one pathway regulates the output of another pathway. Often, feedback loops will incorporate both internal and external mechanisms, such that a pathway can affect its own activity, but may rely on a second pathway to complete the loop. Interaction between two or several signal transduction pathways results in an extensive and complex signaling network capable of accommodating for a variety of signaling fluctuations that cells encounter.

### ***Drosophila* TGF- $\beta$ Signaling**

The TGF- $\beta$  superfamily of signal transduction plays many important roles during development, affecting processes such as tissue growth, cell division, pattern formation, and apoptosis (Kitisin et al., 2007; Wu and Hill, 2009). This superfamily consists of a collection of closely related and highly conserved signaling molecules that function through similar mechanisms. These signaling molecules can be classified into three branches: BMP signaling, TGF- $\beta$  signaling, and activin/Nodal signaling. Only two of these branches are found in *Drosophila*: BMP signaling and activin/TGF- $\beta$ /Nodal signaling (Raftery and Sutherland, 1999).

In many developmental contexts, these pathways behave as morphogen gradients and elicit a concentration-dependent response in tissue patterning. As previously mentioned, the BMP pathway behaves as a morphogen in development of the vertebrate neural tube. In addition, BMP signaling plays a critical and conserved role in dorsal-ventral patterning of *Drosophila*, *Xenopus*, and Zebrafish embryos, as well as in anterior-posterior patterning of the *Drosophila* wing imaginal disc (Affolter and Basler, 2007; Dale and Wardle, 1999; Ferguson and Anderson, 1992; Schier and Talbot, 2005; Schmidt et al., 1995; Wharton et al., 1993). The BMP and activin signaling pathways have been extensively studied and are well understood, but there is still much to learn about the mechanisms in place that maintain robustness of these pathways in different developmental contexts.



**Figure 1.3. The *Drosophila* TGFβ/BMP signaling pathway.** In *Drosophila* the TGF-β superfamily of signaling molecules consists of two branches: TGF-β/activin/nodal signaling and BMP signaling. Signaling is initiated through the activity of a secreted ligand dimer which binds a heterotetrameric complex of two type II and two type I kinase receptors. Upon complex formation, the type II receptors phosphorylate the type I receptors which will phosphorylate the intracellular transducer, the R-Smad. pSmad forms a complex with its Co-Smad, accumulates in the nucleus, and affects target gene transcription. The activin pathway signals through four distinct ligands *daw*, *mav*, *myoglianin*, and *Actβ*; the distinct type I receptor, *babo*, and the R-Smad *dSmad2*. The BMP pathway signals through the ligands *gbb*, *dpp*, and *scw*; the type I receptors *tkv* and *sax*; and the R-Smad *Mad*. The type II receptors *punt* and *wit* are shared, as is the Co-Smad, *Med*. (Wharton and Derynck, 2009)



The central architecture and signaling mechanism is the same between BMP and activin signaling, but many of the specific molecular players of the pathway differ (Fig 1.3). Signaling is initiated by binding of a ligand dimer to the receptor complex in the receiving cell. In BMP signaling, these ligands are Decapentaplegic (Dpp), Glass bottom boat (Gbb), and Screw (Scw) and in activin signaling, they are Dawdle (Daw), Myoglianin (Myo), Maverick (Mav), and Activin- $\beta$  (Act $\beta$ ) (Arora et al., 1994; Gelbart, 1989; Lo and Frasch, 1999; Nguyen et al., 2000; Parker et al., 2006; Wharton et al., 1991). Ligands are translated as long precursor proteins that are cleaved and processed by furin type convertases (Cui et al., 2001; Cui et al., 1998) to form the mature ligand from the C terminal portion of the pro-protein. Ligands are secreted into the extracellular space as dimers where they can travel to their target cells.

Upon ligand binding, a heterotetrameric receptor complex is assembled that consists of two type II and two type I serine/threonine kinase receptors (Shi and Massagué, 2003). The type II receptors are constitutively active kinases that phosphorylate and activate the type I receptors in response to ligand binding. Phosphorylation of the type I receptor occurs in its intracellular GS domain, a glycine and serine rich region adjacent to the transmembrane domain (Wrana et al., 1994). *Drosophila* has two type II receptors called Punt (Put) and Wishful thinking (Wit), both of which can facilitate either BMP or activin, depending on the tissue (Aberle et al., 2002; Childs, 1993)(Nir Yakoby, unpublished poster presentation). Two BMP- specific type I receptors exist in *Drosophila*, Thick veins (Tkv) and Saxophone (Sax), and one activin specific type I receptor exists, Baboon (Babo) (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Wrana et al., 1994; Xie et al., 1994).

The signal is then propagated to the nucleus through a class of proteins known as Smads (Raftery and Sutherland, 1999). Once phosphorylated, the type I receptors phosphorylate the Receptor-mediated-Smad (R-Smad). The BMP and activin pathways signal through distinct R-Smads: Mad in BMP signaling and dSmad2 (or Smox) in activin signaling (Brummel et al., 1999; Henderson and Andrew, 1998; Sekelsky et al., 1995). The vertebrate homologs of these proteins are the Smads-1/5/8 and Smads-2/3, respectively. Phospho-Smad will then form a complex with the Co-Smad, accumulate in the nucleus, and affect target gene expression. *Drosophila* has one Co-Smad, known as Medea (Med), which is shared between the BMP and activin pathways (Wisotzkey et al., 1998). Vertebrates have one Co-Smad as well, known as Smad4.

Several mechanisms exist to negatively regulate activin and BMP signaling activity (Moustakas and Heldin, 2009). In vertebrates, the inhibitory Smads (I-Smads), Smad6 and Smad7, can influence TGF- $\beta$  signaling by blocking activity of the type I receptors, enhancing degradation of the type I receptors, blocking phosphorylation of the R-Smads, or preventing R-Smad/Co-Smad complex formation (Bai et al., 2000; Ebisawa et al., 2001; Hayashi et al., 1997; Imamura et al., 1997; Nakayama et al., 1998; Park, 2005; Yan et al., 2009). The *Drosophila* I-Smad homolog, Dad, has been found to inhibit BMP signaling by binding the type I receptors Tkv and Sax and interfering with phosphorylation and nuclear translocation of Mad. However, Dad has not been observed to interact with or inhibit signaling through the activin type I receptor, Babo (Inoue et al., 1998; Kamiya et al., 2008). Furthermore, *dad* is a target of BMP signaling activity and, thus, serves as a negative feedback modulator to allow for robust signaling (Ogiso et al., 2011; Tsuneizumi et al., 1997).

Extracellular regulators also play a major role in determining the activity level and range of signaling (Umulis et al., 2009). Many such factors exist, including Noggin, Chordin, and Follistatin which play a critical role in early *Xenopus* organizer function (Fainsod et al., 1997; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994; Zimmerman et al., 1996). These factors often influence the availability of ligand to receptor by directly binding the ligand and blocking access to receptor. One particularly interesting example of extracellular regulation is that of Short gastrulation (Sog) in establishing the dorsal activity of pMad in the *Drosophila* embryo (Shimmi et al., 2005). Sog is found in the lateral regions of the embryo and directly binds the ligands Dpp and Scw, displaying higher affinity for Dpp/Scw heterodimers over homodimers of either ligand. Once bound, Sog inhibits ligand activity in the lateral embryo while facilitating transport of these ligands to the dorsal embryo. Once these complexes reach the dorsal midline, the protease Tolloid (Tld) cleaves Sog and releases Dpp and Scw, allowing the BMPs to signal. Through this process, Sog both inhibits BMP signaling in the lateral regions of the embryo and facilitates BMP signaling in the dorsal midline of the embryo.

Interestingly, despite strikingly similar mechanisms of signal transduction, BMP and activin/TGF- $\beta$  signaling often have opposing effects on cellular responses. In osteoblast maturation, BMP2 induces

expression of differentiation markers, while TGF- $\beta$  antagonizes the effect of BMP2 treatment (Spinella-Jaegle et al., 2001), possibly by up-regulating I-Smads (Maeda et al., 2004; Shen et al., 2007). Knock-down of TGF- $\beta$  at particular times during osteoblast differentiation has the ability to enhance BMP2-induced differentiation (de Gorter et al., 2011; Shen et al., 2007). TGF- $\beta$  and BMP signaling have also been found to have opposite effects in stimulating cell migration. In the epithelial-to-mesenchymal transition (EMT) that occurs during renal development, TGF- $\beta$  promotes EMT while BMP7 reverses TGF- $\beta$ -induced EMT (Zeisberg et al., 2003). In contrast, in the migration of endothelial cells, TGF- $\beta$  signaling through ALK5, the homolog of *Drosophila baboon*, inhibits migration and proliferation of these cells while BMP signaling through Alk1, the homolog of *Drosophila sax*, facilitates these processes (Goumans et al., 2002). Furthermore, BMP and TGF- $\beta$  have been found to have opposing effects on chick interdigital cell death. BMP treatment enhances interdigit apoptosis while TGF- $\beta$ 1 or TGF- $\beta$ 2 treatment inhibits interdigit cell death and causes ectopic chondrogenesis or digit formation (Ganan et al., 1996).

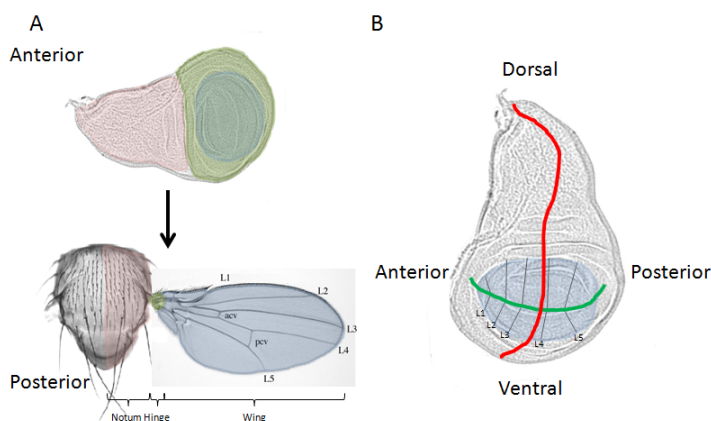
Importantly, there are also examples of crosstalk between BMP and TGF- $\beta$  signaling. In cell culture, stimulation by TGF- $\beta$  can lead to phosphorylation of the BMP-specific R-Smads, Smad1/5 (Daly et al., 2008). In this system, TGF- $\beta$  signaling also results in signaling-competent mixed-Smad complexes consisting of the BMP specific R-Smads, Smads1/5, and the TGF- $\beta$  specific R-Smads, Smad2/3. These mixed Smad complexes were found to be required for anchorage-independent cell growth. Studies in *Drosophila* cell culture have demonstrated phosphorylation of Mad by the activin specific receptor, Babo but not of dSmad2 by Tkv or Sax (Gesualdi and Haerry, 2007).

### **Development of the wing imaginal disc**

In *Drosophila* wing development, BMP signaling plays an important role in pattern formation along the anterior-posterior axis of the wing imaginal disc. The entire exoskeleton of the adult fly is derived from just a few types of cells. The abdominal structures form from histoblasts and all other epidermal structures form from imaginal discs. Imaginal discs arise from embryonic ectodermal cells and will generate all adult appendages, including the mouth parts, antennae, eyes, legs, wings, halteres, and

genitalia, as well as all trunk structures that correspond to each appendage (Cohen, 1993). They are ideal systems in which to study the development of adult structures because they are easily located and manipulated. During larval development these tissues will respond to extensive growth and patterning cues to allow for proper specification of cell fates.

The imaginal discs are sac-like structures composed of a single-celled epithelium which remain attached to the larval wall by stalk cells. Two contiguous epithelial layers are separated by a central lumen. One of these layers, called the disc proper, is characterized by a columnar organization and will become the associated adult structure. The other layer, known as the peripodial epithelium, is a flattened squamous layer that will contribute little to the adult appendage, but is important in maintaining disc integrity and in later metamorphosis of the disc. During pupal development, the discs will undergo an extensive metamorphosis where they evert and elongate, eventually fusing before becoming their final adult structure. Pertinent to this work is the development of the wing imaginal disc, as a role for BMP signaling in patterning of this tissue is well-characterized. The wing disc is responsible for not only generating the adult wing but also the notum, which is the dorsal portion of the thoracic segment of the



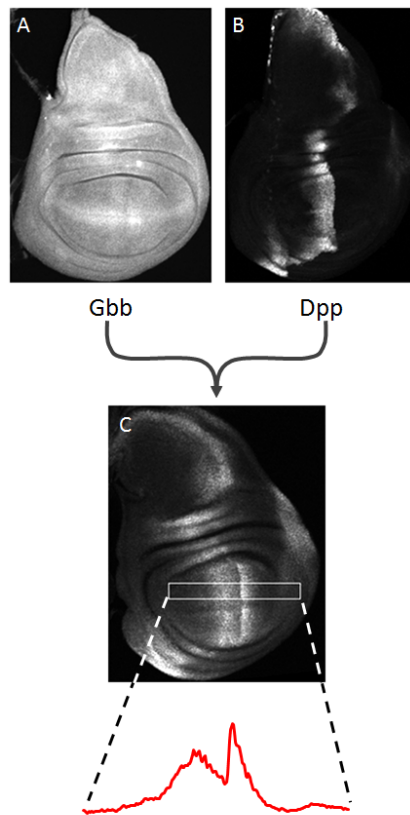
**Figure 1.4. The *Drosophila* wing imaginal disc.** **A.** Cells within specific regions of the wing imaginal disc are patterned and specified to become the adult notum (in pink), hinge (in green), and wing (in blue). **B.** The wing imaginal disc has a distinct morphology with defined anterior/posterior and dorsal/ventral axes, the boundaries of which are shown in red and green, respectively. Within the wing pouch (shown in blue), precursors to the adult veins, L1-L5, are specified, the approximate locations of which are labeled.

adult, and the hinge, where the wing attaches to the notum (Fig 1.4A).

The wing imaginal disc has distinct anterior, posterior, dorsal, and ventral compartments (Fig 1.4B), which are established early in development in response to the cues that exist in the surrounding embryonic

segments and parasegments. During pupal development, the disc everts with the dorsal-ventral boundary leading, resulting in apposition and fusion of the cells of the dorsal and ventral compartments (Aldaz et

al., 2010). The resulting adult wing is two cells thick, one from the dorsal compartment and one from the ventral compartment of the wing disc. The dorsal-ventral boundary of the wing disc becomes the adult wing margin.



**Figure 1.5. Gbb and Dpp contribute to BMP signaling in the wing disc.** *gbb*, visualized by *gbb-lacZ* courtesy of Takuya Akiyama, is broadly expressed across the wing disc (**A**) while *dpp*, visualized by *dpp<sup>p10638</sup>-lacZ*, displays a restricted expression pattern along the anterior-posterior boundary of the wing disc (**B**). Signaling through both ligands contributes to the pMad activity gradient in the wing disc (**C**). **C.** pMad is observed in two peaks, one high peak just posterior to the anterior-posterior compartment boundary and one lower peak in the anterior compartment. The area between the peaks is known as the trough.

### BMP signaling patterns the wing imaginal disc

The wing imaginal disc has been extensively used as a model system to study BMP morphogen function. In this tissue, BMP signaling provides positional information across the anterior-posterior axis important for specifying wing vein fate (Khalsa et al., 1998; Lecuit et al., 1996; Nellen et al., 1996; Singer et al., 1997). The adult wing displays a stereotypical pattern of five longitudinal veins that run proximodistally along the wing, designated as L1 to L5 (Fig 1.4B). Later during pupal development, these precursors will differentiate into vein tissue.

Both Dpp and Gbb are required for patterning the wing imaginal disc (Fig 1.5) (Bangi and Wharton, 2006; Ray and Wharton, 2001; Spencer et al., 1982). Together, signaling through both ligands generates the wildtype activity gradient of BMP signaling (Bangi and Wharton, 2006; Khalsa et al., 1998), which can be visualized by observing the phosphorylated form of the Mad protein, pMad (Fig 1.5C). In a normal disc, the pMad gradient displays a characteristic profile consisting of two peaks, a moderate anterior and a high posterior peak. Outside of the peaks, pMad displays a

graded distribution towards the lateral regions of the wing disc, while between them is a region of lower pMad known as “the trough.” The broad distribution of pMad differs considerably from *dpp* expression,

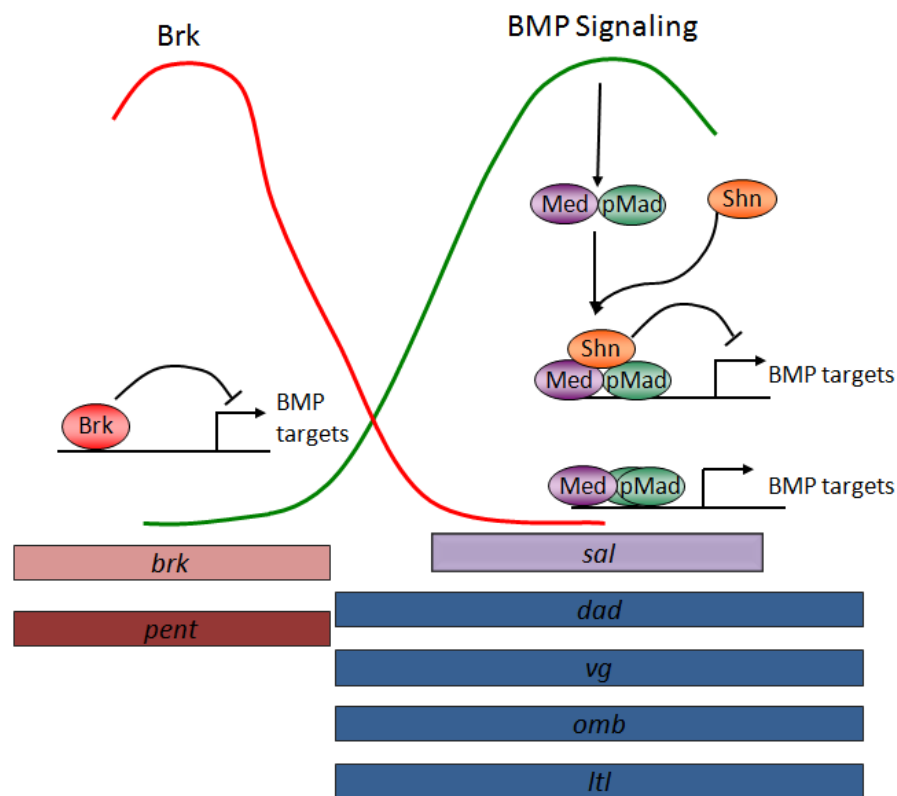
which is narrowly expressed (Masucci et al., 1990; Teleman and Cohen, 2000), suggesting that the concentration of Dpp itself may not explain or account for the BMP activity gradient. Recent work has indicated that it is unlikely that Dpp acts alone in the wing disc and it appears, instead, that Dpp works in combination with Gbb (Bangi and Wharton, 2006; Ray and Wharton, 2001).

*dpp* is expressed in the anterior compartment of the wing disc in a stripe along the anterior-posterior boundary while *gbb* displays a broader expression domain (Fig 1.5A,B). Despite having overlapping expression domains, Gbb acts over a long-range in the wing disc while Dpp acts over a short-range (Bangi and Wharton, 2006). The importance of each of these ligands in patterning was elucidated through analysis of phenotypes associated with *dpp* or *gbb* mutant wing discs. A reduction in *dpp* alone leads to patterning defects of the adult wing veins L2 and L4, derived from cells located centrally in the wing disc, whereas loss of *gbb* influences the specification of L5, derived from distal posterior cells. However, anterior expression of *gbb* is critical for specification of L5. This indicates that *gbb* has a much greater effective range than *dpp*. It is the combination of a long-range effect of Gbb and a short-range effect of Dpp that generates the pMad gradient and leads to proper patterning of the wing disc (Bangi and Wharton, 2006; Khalsa et al., 1998; Ray and Wharton, 2001).

Proper formation of the pMad gradient in the wing disc is also impacted by key downstream signaling components. Both type I receptors, Tkv and Sax, are critical in mediating the BMP signal in the wing disc (Bangi and Wharton, 2006; Haerry et al., 1998). In addition, the inhibitory Smad, Dad, is required for robustness of pMad gradient formation in the face of altered *tkv* expression (Ogiso et al., 2011). Furthermore, a secreted feedback regulator of BMP signaling has recently been identified that functions in generating proper wing patterning. *pentagone* is a target of BMP signaling that is secreted from the lateral regions of the wing disc and appears to play a role in distribution of ligand across the wing disc in collaboration with the glypican Dally. In the absence of *pent*, pMad levels are increased in the center of the disc and lose their graded distribution towards the lateral regions of the wing disc (Hamaratoglu et al., 2011; Vuilleumier et al., 2010).

BMP signaling has several target genes that are important for cell fate specification in the wing disc. Some of these include *spalt-major* (*salm*), *spalt-minor* or *spalt-related* (*salr*), *optomotor-blind* (*omb*),

and *brinker* (*brk*) (Barrio and de Celis, 2004; Barrio et al., 1996; Grimm and Pflugfelder, 1996; Minami et al., 1999)(Fig 1.6). Both *sal* genes are expressed in a moderately-wide domain in both the anterior and posterior compartments of the wing disc. *omb* displays a wider expression pattern and is expressed throughout most of the wing pouch. *brk* is expressed in the lateral regions of the wing disc in both the anterior and posterior compartments where pMad levels are low or absent. The locations of the primordia for L2 and L5 correspond to the boundaries between expression domains of these BMP target genes. At the anterior edge of the *sal* expression domain, factors required for specification of the L2 primordia, particularly *knirps*, are activated (de Celis and Barrio, 2000). In the posterior compartment, factors required for L5 specification, such as *abrupt*, are expressed at the boundary of the *omb* and *brk* expression domain (Cook et al., 2004).



**Figure 1.6. BMP signaling in the wing imaginal disc.** pMad activity in the medial region of the wing disc generates an inverse gradient of Brinker (Moser and Campbell, 2005) through repression by a Shn/pMad/Med complex (Pyrowolakis et al., 2004). Together, pMad, Brk, and Shn/pMad/Med determine the expression domains of BMP target genes. *dad*, *Itl*, *omb*, *sal*, and *vg* are expressed in the medial region of the wing disc while *brk* and *pent* are expressed in the lateral region of the wing disc.

### Generating the expression domains of BMP targets in the wing disc

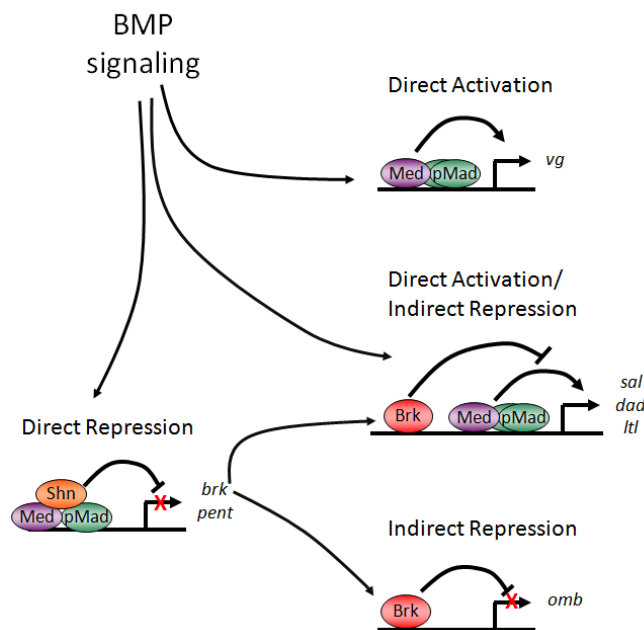
BMP signaling elicits positive and negative transcriptional responses that, together, are important in generating the expression domains of genes critical for patterning the wing disc. These responses can occur through both direct and indirect mechanisms (Fig 1.7). First, a pMad/Med complex can directly activate the expression of target genes. Second, pMad can directly repress target genes in a complex with Med and a co-repressor, Schnurri (Shn). Third, pMad can indirectly affect target gene expression through the action of direct target. Through the efforts of one or several of these mechanisms, the expression domains of BMP targets is established, generating nested domains of target gene expression in the wing disc (Fig 1.6)

A gene that is directly activated by BMP signaling in the wing disc is *vestigial (vg)*. *vg* is broadly expressed in the presumptive wing pouch along the anterior-posterior axis and is absolutely required for proper wing development (Williams et al., 1991). Expression of *vg* is under the control of several enhancers, one of which, the *vg quadrant enhancer*, is positively regulated by BMP signaling. pMad directly binds this enhancer sequence and activates expression of *vg* in the wing blade (Kim et al., 1997).

Another direct BMP target, *brk*, is directly repressed by BMP signaling. *brk* is expressed in the lateral regions of the wing pouch and is absent from the center of the wing disc in a pattern complementary to the gradient of pMad (Jazwinska et al., 1999; Moser and Campbell, 2005; Muller et al., 2003). Repression of *brk* is accomplished through a complex consisting of pMad, Med, and a third component that behaves as a repressor, Schnurri (Shn) (Marty et al., 2000). This complex binds to a DNA silencing element (SE) for which the consensus sequence has been identified as GNCGNCNNNNGTCT (Gao et al., 2005; Pyrowolakis et al., 2004). In this complex, Shn itself does not bind DNA but, instead, relies on Mad and Med to interact with DNA at the 5' and 3' ends of the sequence, respectively. The spacing between these two contact points, denoted as 5 Ns in the consensus binding sequence, is essential for proper complex formation and for subsequent repression of target genes. In addition to *brk*, the Shn/pMad/Med complex has been observed to repress *pent* by acting on a SE found within its regulatory sequences (Vuilleumier et al., 2010).



Many targets of BMP signaling are indirect targets, regulated by Brk (Campbell and Tomlinson, 1999; Kirkpatrick et al., 2001). *omb*, as previously mentioned, is expressed throughout most of the wing pouch in both the anterior and posterior compartments. The expression pattern of *omb* is largely determined by a combination of activation, most likely from Wingless signaling, and by direct repression by Brk (Grimm and Pflugfelder, 1996; Sivasankaran et al., 2000). For some target genes, the final



**Figure 1.7. BMP signaling directly and indirectly influences expression of target genes.** The final expression patterns of all BMP target genes in the wing disc is determined through a combination of direct and indirect activation and repression events. BMP signaling directly activates expression of *vg* through the activity of pMad (Kim et al., 1997). Through a repressive Shn/pMad/Med complex, BMP signaling can directly repress *brk* and *pent* (Gao et al., 2005; Pyrowolakis et al., 2004; Vuilleumier et al., 2010). Through Brk, BMP signaling exerts an indirect effect on target genes. Brk represses the expression of *omb* (Sivasankaran et al., 2000). Expression of *sal*, *dad*, and *ltl* is determined in response to a combination of activation by pMad and repression by Brk (Barrio and de Celis, 2004; Szuperák et al., 2011; Weiss et al., 2009; Wilson and Chuang, 2010). Figure modified from (Affolter et al., 2001).

Brk. Regulation of *dad* expression responds to positive input from pMad and negative input from Brk on a sequence called the activation element (AE) with direct competition between Brk and pMad on these sequences (Weiss et al., 2009). The *sal* genes are also regulated by both pMad and Brk, but these factors bind in different enhancer regions and likely do not compete for binding (Barrio and de Celis, 2004). A final target, *ltl*, is likely regulated by both Brk and Mad as binding sites for both factors have been identified within its regulatory sequences (Szuperák et al., 2011).

### Regulation of *dpp* expression

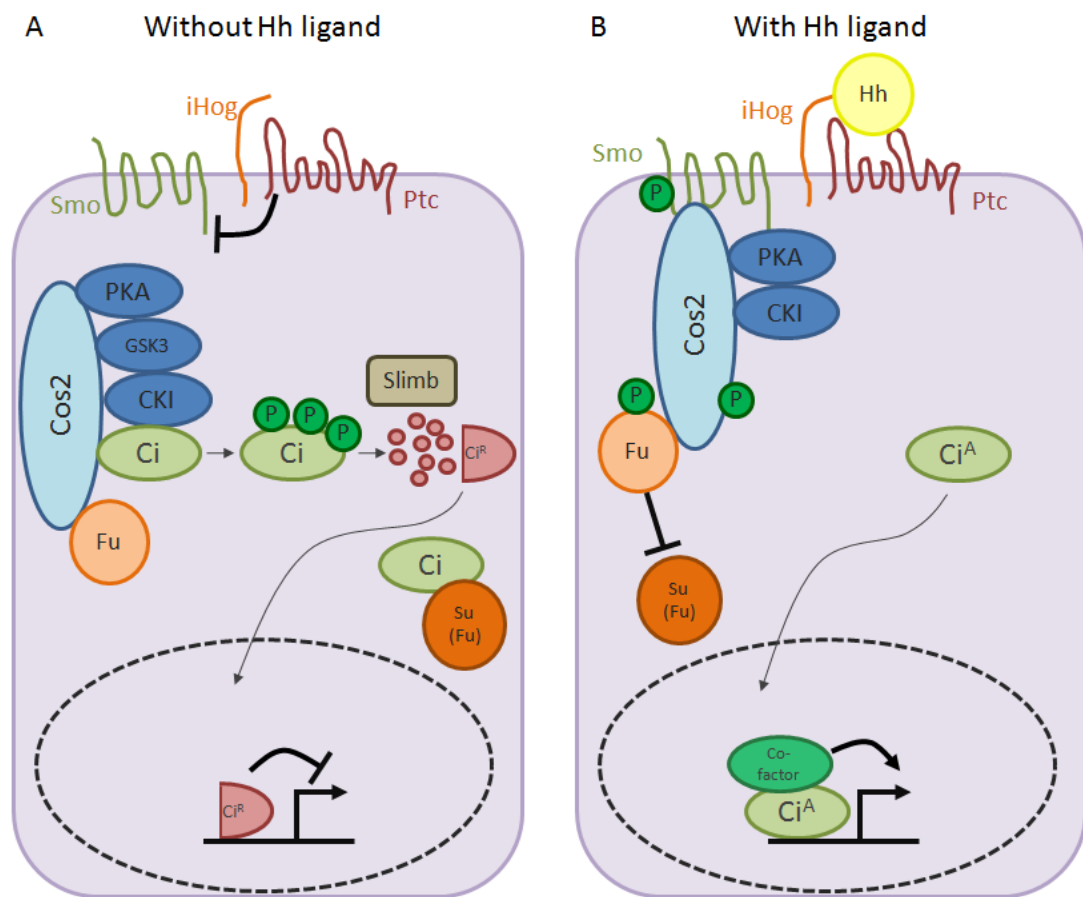
A major step in generating the BMP activity gradient in the wing disc is first establishing the expression domains of the BMP ligands, *gbb* and *dpp* (Fig 1.5). *dpp* expression in the wing disc is highly

localized and confined to a stripe of anterior cells along the anterior-posterior compartment boundary. As the first *Drosophila* BMP to be identified, *dpp* has been especially well-characterized and much is known about the factors and pathways that determine its expression pattern in the wing disc. *Gbb*, on the other hand, displays a much broader expression pattern in the wing pouch and little is known regarding the regulation of *gbb* expression in the wing disc. Based on the strikingly different expression patterns of these two ligands, it is likely that the expression of *dpp* and *gbb* is regulated by different mechanisms.

Activation of *dpp* expression in the anterior compartment of the wing disc is largely under the control of the hedgehog (Hh) signal transduction pathway (Alexandre, 1996). The Hh signaling pathway (Fig 1.8), like BMP signaling, is a well conserved signaling pathway that plays an important role in many developmental processes (Varjosalo and Taipale, 2008). In *Drosophila*, hedgehog signaling is initiated through activity of the ligand Hh and it regulates the processing and activation of the hedgehog transducer, Cubitus interruptus (Ci) (Jia and Jiang, 2006; Osterlund and Kogerman, 2006; Tabata, 1992; Wilson and Chuang, 2010). Ci is the *Drosophila* homolog of the vertebrate Gli proteins, Gli1, Gli2, and Gli3, all of which are zinc finger-containing transcription factors that act as either transcriptional activators or repressors. In *Drosophila*, the single Ci protein achieves both of these functions depending on how it has been processed. In the absence of Hh signaling, Ci is cleaved and functions as a repressor (Ci<sup>R</sup> or Ci-75), while in response to Hh signaling, Ci is protected from cleavage, activated, and translocated into the nucleus where it functions as an activator (Ci<sup>A</sup> or Ci-155). (Aza-Blanc et al., 1997; Chen et al., 1999; Müller and Basler, 2000; Ohlmeyer and Kalderon, 1998; Wang and Holmgren, 2000).

In the absence of Hh ligand (Fig 1.8A), the receptor Patched (Ptc) blocks the activity of Smoothened (Smo), a seven pass transmembrane G protein coupled receptor (Denef et al., 2000; Taipale et al., 2002). When blocked by Ptc, Smo is inactive, allowing an intracellular Ci processing complex to form. This complex contains the scaffolding protein, Costal2 (Cos2), and the kinases, Fu, PKA, CK1, and GSK3 (Jia et al., 2003; Méthot and Basler, 2000; Robbins et al., 1997; Wang et al., 2000; Wang and Jiang, 2004; Wang and Holmgren, 2000). Within this complex, Ci is phosphorylated by PKA which primes it for subsequent phosphorylation events by CK1 and GSK3 (Jia et al., 2002; Jia et al., 2004; Price and Kalderon, 1999; Price and Kalderon, 2002; Wang et al., 1999; Zhang et al., 2005). When fully phosphorylated, it is

recognized by SCF E3 ubiquitin ligase, Slimb/b-TRCP, for proteasome-mediated proteolysis (Jia et al., 2005; Jiang and Struhl, 1998; Smelkinson and Kalderon, 2006; Smelkinson et al., 2007). Truncated Ci, called Ci-75 or Ci<sup>R</sup>, retains its Zn finger DNA binding domain, but not its dCBP co-activator interaction domain (Akimaru, 1997; Aza-Blanc et al., 1997). The result is a protein that behaves as a transcriptional repressor of Hh target genes; it can bind DNA but cannot activate transcription. Any un-processed Ci present in these cells will be retained in the cytoplasm by association with the protein Suppressor of Fused (Su(fu)) (Méthot and Basler, 2000; Monnier et al., 1998; Monnier et al., 2002; Sisson et al., 2006;



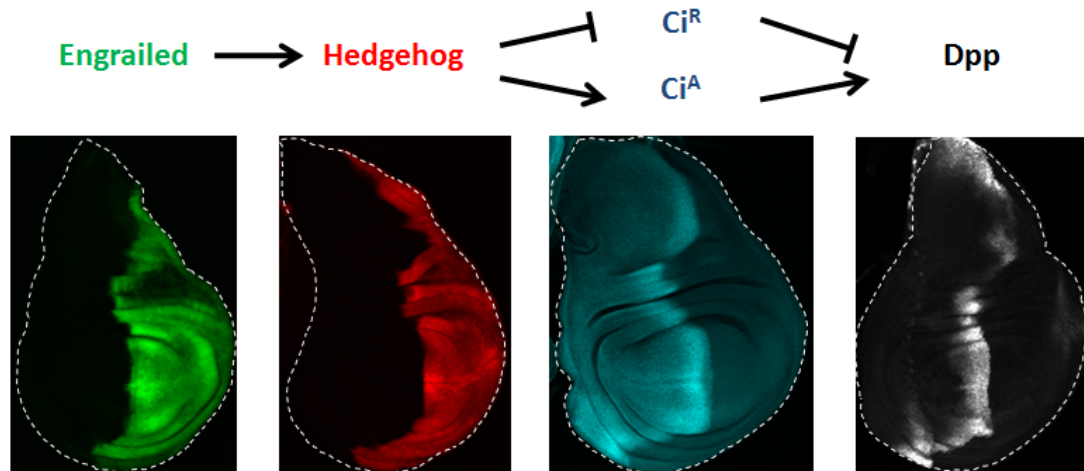
**Figure 1.8. *Drosophila* Hedgehog signaling.** **A.** In cells that do not receive Hh signaling, Ci is cleaved into its truncated, repressor form known as Ci<sup>R</sup>. In these cells, Ptc blocks the activity of the transmembrane component Smo. In the absence of Smo activity, an intracellular complex of Cos2, Fu, PKA, CK1, and GSK3 forms and initiates a series of phosphorylation events on Ci. Once phosphorylated, it is targeted by Slimb for proteasome-mediated partial proteolysis. Truncated Ci, lacking its transactivation domain, enters the nucleus and behaves as a transcriptional repressor. Su(Fu) prevents any remaining full-length Ci from entering the nucleus. **B.** In cells that receive Hh ligand, Ci is protected from cleavage and acts as an activator through its active form, known as Ci<sup>A</sup>. Hh ligand binds the receptor Ptc and the repression of Smo is relieved. Smo recruits Cos2 and Fu to the membrane, disrupting the Ci processing complex and stabilizing Ci. Fu is activated by Smo and antagonizes Su(Fu), allowing full-length Ci to enter the nucleus. In cooperation with co-factors, it can now activate transcription of target genes. Figure modified from (Jia and Jiang, 2006; Wilson and Chuang, 2010).

Wang et al., 2000).

In cells that encounter Hh ligand, Hh is received by a receptor complex consisting of Ptc and the co-receptors, Interference hedgehog (iHog) and Brother of interference hedgehog (Boi) (Camp et al., 2010; Chen, 1996; Marigo, 1996; Yan et al., 2010). The binding of Hh to Ptc relieves the repression that Ptc had imposed on Smo (Khaliullina et al., 2009; Taipale et al., 2002). Now active, Smo recruits Cos2 and Fu to the membrane, efficiently disrupting the Ci processing complex (Ruel et al., 2007; Ruel et al., 2003). Without phosphorylation by PKA, CK1, and GSK3, Ci is stabilized. At the highest levels of Hh signaling, Fu bound to Smo is activated which, in turn, antagonizes Su(fu) (Monnier et al., 1998; Murone et al., 2000; Sisson et al., 2006). This allows for activation and nuclear translocation of full-length Ci, called Ci<sup>A</sup>, leading to up-regulation of Hh target genes (Ohlmeyer and Kalderon, 1998; Wang et al., 2000; Wang and Jiang, 2004; Wang and Holmgren, 2000).

In the wing disc, Hh acts as a short range signaling molecule (Strigini and Cohen, 1997) emanating from the posterior compartment (Fig 1.9), where it is highly expressed in response to activation by a factor called Engrailed (En) (Zecca et al., 1995). En also represses expression of *ptc* and *ci* in the posterior compartment, precluding transmission of the Hh signal in these cells (Chanas et al., 2004; Eaton and Kornberg, 1990; Saenz-Robles et al., 1995; Sanicola et al., 1995). Ptc is expressed in a narrow stripe of cells just anterior to the anterior-posterior boundary, efficiently restricting Hh signaling activity to only these cells in the anterior compartment. Ci is expressed throughout the anterior compartment, but it is only stabilized and activated in the cells closest to the anterior-posterior compartment boundary that receive Hh. The result is a high ratio of Ci<sup>A</sup>:Ci<sup>R</sup> in the cells closest to the anterior-posterior boundary, with Ci<sup>R</sup> as the predominate form throughout the rest of the anterior compartment (Aza-Blanc et al., 1997).

Hh signaling behaves as morphogen gradient in this tissue and elicits a concentration-dependent transcriptional response of its target genes which include: *en*, *ptc*, *dpp*, *iroquois (iro)*, and *collier (Col)* (Hooper and Scott, 2005; Strigini and Cohen, 1997). *dpp* is expressed in response to moderate levels of Hh signaling and is sensitive to both the activator and repressor forms of Ci (Hepker et al., 1999; Méthot and Basler, 1999; Müller and Basler, 2000; Parker et al., 2011; Tanimoto et al., 2000). Along the anterior-posterior boundary of the wing disc, activation by Ci<sup>A</sup> is required to attain endogenous *dpp* expression



**Figure 1.9. *dpp* expression in the wing disc is determined by Hh signaling.** Posterior *engrailed* expression, visualized in green by *en-Gal4>UAS-GFP*, allows expression of the signaling molecule, *hedgehog*, visualized in red by *hh-Gal4>UAS-GFP*, specifically in the posterior compartment. Hh travels from the posterior compartment to the anterior compartment and initiates signaling within a stripe of cells along the anterior-posterior boundary. The result of Hh signaling is the stabilization, activation, and nuclear translocation of the Hh signal transducer, Ci, visualized in cyan by antibody staining against the full-length form of Ci, Ci<sup>A</sup> (Aza-Blanc et al., 1997; Chen et al., 1999; Wang and Holmgren, 2000). Anterior cells that do not receive Hh signal generate a truncated form of Ci which behaves as a repressor. *dpp* responds to both Ci<sup>R</sup> and Ci<sup>A</sup> (Méthot and Basler, 1999), and is, thus, expressed in the stripe of cells containing high levels of Ci<sup>A</sup> and repressed throughout the remainder of the anterior compartment, visualized by *dpp<sup>p10638</sup>lacZ* expression in white.

levels, whereas throughout the remainder of the anterior compartment Ci<sup>R</sup> represses *dpp*. In the absence of either Ci protein or Ci binding to the *dpp* cis-regulatory regions, *dpp* is expressed at a low level throughout the anterior compartment of the wing pouch, indicating the presence of an un-identified “basal” activator of *dpp* (Hepker et al., 1999; Méthot and Basler, 1999; Müller and Basler, 2000). Expression of *dpp* is precluded from the posterior compartment due to repression by the transcription factors En and Invected (Inv) (Sanicola et al., 1995; Zecca et al., 1995).

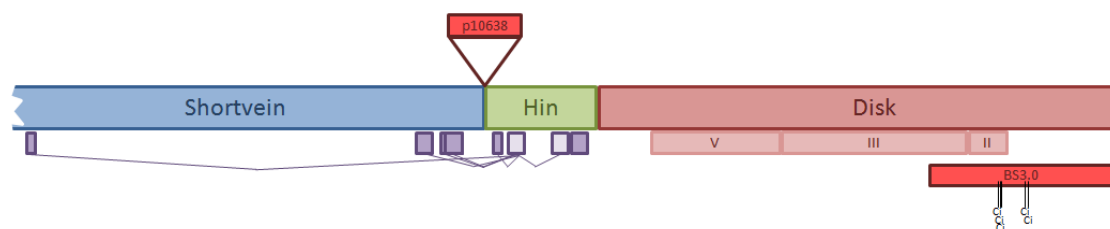
### Molecular organization of the *dpp* locus

The *dpp* locus in *Drosophila* contains extensive regulatory regions devoted to spatial and temporal regulation of *dpp* during embryonic, larval, and pupal development (Blackman et al., 1991; Jackson and Hoffman, 1994; Kopp et al., 1999; Masucci et al., 1990). The entire locus contains at least 56kb of sequence that is regulated by complex and varied mechanisms throughout development. This locus can be separated into three main sections: the *shortvein*, *Hin*, and *disk* regions (Fig 1.10) (St

Johnston et al., 1990). The *shortvein* and *disk* regions primarily contain enhancer sequences, while the *Hin* region contains coding regions for the *dpp* gene as well as enhancer sequences.

The *Hin* domain is approximately six kb in length and is thus named because of the haploinsufficient nature of the *dpp* mutations mapped to this region (Irish and Gelbart, 1987; Spencer et al., 1982). This region contains the coding sequences for all five transcripts (transcripts A-E) of *dpp* and null mutations within the coding sequence are haploinsufficient. Also located within this region is the 3' non-coding exon shared between all five transcripts of *dpp* as well as a non-coding exon specific to the *dpp* transcript A (St Johnston et al., 1990). Mutations within the *dpp* coding sequence were characterized based upon the degree of dominant lethality that they exhibit. Those that display >50% dominant lethality are classified as null *dpp<sup>hin</sup>* alleles while those that display <50% dominant lethality are classified as hypomorphic *dpp<sup>hr</sup>* alleles (Wharton et al., 1993). Most of the lesions found within this region are point mutations that affect the function of Dpp. Also within the *Hin* domain are *cis*-regulatory sequences required for embryonic expression of *dpp*.

The *shortvein* (*shv*) region of the *dpp* locus is an extensive *cis*-regulatory fragment consisting of over 25kb of sequence that is located 5' to the *Hin* region of *dpp* (Segal and Gelbart, 1985; Stultz et al., 2005). This region was named for the distally truncated veins observed in mutations isolated from this region, and sequences present in this region direct expression of *dpp* required for pupal vein formation



**Figure 1.10. Molecular organization of the *dpp* locus.** The *dpp* locus is comprised of three large regions: *shortvein* (*shv*), *hin*, and *disk* (St Johnston et al., 1990). The *hin* region (in green) is approximately six kb in length and contains the coding sequence for all five *dpp* isoforms as well as some non-coding exons (exons are displayed in purple). The 25kb *shv* region (in blue) contains several non-coding exons as well as regulatory sequences for *dpp* expression in vein primordia, larval tissues, and the head capsule. The *disk* region (in pink) is 23kb in length and contains regulatory sequences for imaginal disc expression of *dpp*. It is separated into the V, III, and II regions based on alleles whose breakpoints fall in these regions and the severity of these alleles. *dpp<sup>p10638</sup>lacZ* (in red) is a *dpp* enhancer trap in which the *lacZ* coding sequence has been inserted upstream of the *dpp* coding sequence (Twombly et al., 1996). *dpp<sup>BS3.0</sup>lacZ* (in red) is a *dpp* reporter construct created from 10kb of *dpp<sup>disk</sup>* sequence. Both recapitulate *dpp* expression in the wing imaginal disc (Blackman et al., 1991). Within the sequences contained in *dpp<sup>BS3.0</sup>lacZ* are five binding sites for Ci important in wing disc expression of *dpp*. (Hepker et al., 1999; Müller and Basler, 2000).

(Sotillos and de Celis, 2006). Mutations that lead to lethality during larval development have also mapped to the *shv* region. In addition, this region contains much of the *cis*-regulatory sequences required for expression of *dpp* in formation of the adult head capsule. The 5' non-coding exons and promoter sequences required for proper expression of the *dpp* transcripts B, C, D, and E are also found within the *shortvein* region (St Johnston et al., 1990).

The *disk* region of the *dpp* locus contains much of the *cis*-regulatory sequence required for imaginal disc expression of *dpp* (Blackman et al., 1991). This region is approximately 23kb in length and is located 3' to the *Hin* sequences. Lesions associated with this region affect the development of imaginal disc derived structures and are often large chromosomal inversions, translocations, or deletions which effectively move *cis*-regulatory sequence out of their wildtype context. Mutations were classified and mapped based upon the severity of the resulting phenotype as either class V, III, or II alleles (Spencer et al., 1982; St Johnston et al., 1990). Class V mutations are the most severe and remove the most *cis*-regulatory sequence. Their breakpoints map closest to the *Hin* region and result in the loss or severe reduction of imaginal disc tissue. Class III mutations are less severe, with breakpoints that map to the central portion of the *disk* region and that result in loss of distal tissue from most imaginal disc derived structures. The least severe mutations belong to class II and these breakpoints map close to the 3' end of the *disk* region. These mutations result in the loss of tissue within the wing blade, haltere, and male genitalia.

### **Observing expression of *dpp***

To determine the function of *dpp* enhancer sequence and visualize *dpp* expression patterns, *dpp* reporter constructs have been generated. These constructs were created by inserting a region of the *dpp*<sup>disk</sup> sequence upstream of a minimal promoter and an observable reporter gene, such as *lacZ*. Expression of these constructs leads to beta-galactosidase protein production that can easily be visualized by an enzymatic reaction, in which beta-galactosidase cleaves the X-gal substrate into a blue precipitate, or by immunostaining using a commercially available antibody directed against  $\beta$ Gal. Interestingly, specific pieces of the *dpp*<sup>disk</sup> sequence direct expression of *lacZ* in particular imaginal discs or particular

locations within imaginal discs (Blackman et al., 1991; Kopp et al., 1999; Masucci et al., 1990). Reporters constructed from the 5'-most *dpp*<sup>disk</sup> sequence direct expression of *lacZ* in all or most imaginal discs, while reporter constructs containing sequences farther downstream displayed variable expression in various imaginal discs. It appears, then, that *cis*-regulatory sequences of *dpp* are modular, with different regions directing expression in specific tissue. Together, all generate the endogenous expression pattern of *dpp*.

One reporter construct in particular has been extensively used as a read-out for *dpp* expression in the imaginal discs, particularly in the wing disc. This reporter, known as *dpp*<sup>BS3.0</sup>*lacZ*, is constructed from 10kb of the *dpp*<sup>disk</sup> region spanning the class III and class II regions (Fig 1.10) (Blackman et al., 1991). It directs strong expression of *lacZ* along the anterior-posterior boundary of the wing imaginal disc. Often used in studies of *dpp*, *dpp*<sup>BS3.0</sup>*lacZ* is considered to faithfully recapitulate the expression pattern of *dpp* in the wing imaginal disc.

A second commonly used reporter for *dpp* expression is known as *dpp*<sup>p10638</sup>*lacZ* (Fig 1.10). Unlike *dpp*<sup>BS3.0</sup>*lacZ*, this reporter is an enhancer trap rather than a transgene, with the *lacZ* gene inserted 5' to the *dpp* transcript A start site (Twombly et al., 1996). In this line, expression of *lacZ* is expected to respond to all *dpp* enhancer sequences and accurately reflect the endogenous expression domains of *dpp*. Indeed, expression of this reporter fully recapitulates the expression pattern of *dpp* throughout development, including embryonic and imaginal disc expression and, importantly, along the anterior-posterior boundary of the wing disc. Additionally, *dpp*<sup>p10638</sup>*lacZ* is a weak hypomorphic allele of *dpp*, but this does not seem to affect its pattern of expression in the wing disc, suggesting that it is too weak of an allele to influence patterning or that there are compensatory mechanisms in place to accommodate for it.

### **Sequences required for expression of *dpp* in the wing disc**

In the wing disc, *dpp*<sup>BS3.0</sup>*lacZ* and *dpp*<sup>p10638</sup>*lacZ* express in the endogenous expression domain of *dpp* in a stripe of cells just anterior to the anterior-posterior compartment boundary. Since initially observed, the *cis*-regulatory sequences contained within *dpp*<sup>BS3.0</sup>*lacZ* have been dissected for their ability to mediate expression of *dpp* in these cells. Within these sequences, many smaller regions are also capable of directing expression of *lacZ* in the wing pouch, indicative of the presence of binding sites for



factors that mediate expression in this tissue (Hepker et al., 1999; Masucci et al., 1990; Müller and Basler, 2000).

Much work has been done to elucidate the mechanisms that direct expression of *dpp* to this specific set of cells in the wing imaginal disc. As mentioned previously, expression in this region is largely due to a balance of positive input from  $Ci^A$  and negative input from  $Ci^R$ . *Ci* binding sites in the *dpp cis*-regulatory sequences are found in two clusters about 1.6kb apart: a 5' cluster containing three *Ci* binding and a 3' cluster contains two *Ci* binding sites. These clusters have been independently tested and each has been found to play an essential role in generating the endogenous expression domain of *dpp* in the wing imaginal disc (Hepker et al., 1999; Müller and Basler, 2000).

The 5' cluster of *Ci* binding sites was tested by Konrad Basler's lab (Müller and Basler, 2000). In these experiments, they examined a subclone of *dpp<sup>BS3.0</sup>lacZ* containing a 3.8kb BamHI fragment that is sufficient to drive expression of *lacZ* in the wing disc. A series of truncations of this fragment were generated and analyzed for their expression in the wing pouch until they were able to identify a minimal sequence for activation in the wing disc. This reporter, called fragment 10, was approximately 800bp in length and contains the 5' cluster of three *Ci* binding sites. Further analysis of fragment 10, through a series of deletions and mutations, revealed the importance of these *Ci* binding sites in restricting expression of *dpp* to the stripe.  $Ci^A$  binds these sites to activate *dpp* expression along the anterior-posterior stripe and  $Ci^R$  binds to repress *dpp* throughout the remainder of the anterior wing pouch. In the absence of *Ci* binding, *dpp* is broadly expressed in the anterior compartment, highlighting the importance of  $Ci^R$  in restricting *dpp* expression to the anterior-posterior stripe. In addition, these experiments identified a small region (~100bp) within fragment 10 that is required for general activation in the wing, eye, leg, and antennal imaginal discs. It was inferred that these sites must mediate activation by a "basal" activator, but the identity of the factor was not investigated.

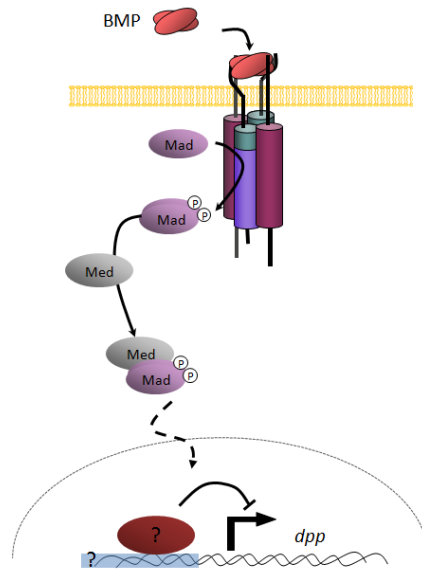
The importance of the 3' cluster of *Ci* binding sites was independently tested in a report from the Holmgren lab (Hepker et al., 1999). In these experiments they tested factors responsible for wing disc expression of a small *dpp* reporter called *dpp<sup>BS3.23</sup>lacZ* (referred to as *dppho-lacZ* in their experiments). This reporter contains a 358 bp fragment of *dpp<sup>disk</sup>* sequence contained within *dpp<sup>BS3.0</sup>lacZ*. Through gel

mobility shift assays they confirmed direct binding of Ci protein to this fragment. Additionally, mutational analysis of these binding sites confirmed a requirement for Ci binding to generate the *dpp*<sup>BS3.23</sup>*lacZ* expression pattern. However, unlike the reporter analyzed by the Basler lab (Müller and Basler, 2000), this mutant reporter did not express ubiquitously throughout the anterior compartment, indicating the absence of additional activating sequences in this region.

### **Feedback regulation of *dpp***

While much is known about the mechanisms in place that establish expression of *dpp*, less is known about maintenance of *dpp* expression in the wing imaginal disc. It has been observed in several other developmental contexts that expression of *dpp* responds to a positive autoregulatory loop in BMP signaling. Positive regulation of *dpp* plays a role in maintaining expression of *dpp* in the eye imaginal disc, important for initiation and progression of the morphogenetic furrow (Chanut and Heberlein, 1997; Lee et al., 2007; Pignoni and Zipursky, 1997; Wiersdorff et al., 1996). *dpp* is also positively regulated by BMP signaling during development of the early embryo, the embryonic visceral mesoderm, the embryonic midgut, and the pupal veins (Biehs et al., 1996; Hursh et al., 1993; Yu et al., 1996a; Yu et al., 1996b). There is limited evidence for positive regulation of *dpp* in the wing imaginal disc (Hepker et al., 1999; Nahmad and Stathopoulos, 2009).

Several observations have suggested that *dpp* may be negatively regulated by BMP signaling as well. For example, flies that contain a modest increase in *dpp* or, specifically, up to eight copies of *dpp* show few wing patterning defects, suggesting that the animal can compensate for the increase in *dpp* (Morimura et al., 1996; Yu et al., 1996a). Furthermore, negative transcriptional regulation of *dpp* has been observed in the wing disc in response to increased levels of *tkv* (Crickmore and Mann, 2007; Haerry et al., 1998; Ogiso et al., 2011) and reduced levels of the downstream target of BMP signaling, *omb* (Umemori et al., 2007). However, all such observations have only been briefly mentioned and have never been well characterized. In addition, several lines of evidence in our lab had hinted of a negative relationship between *gbb* levels and Dpp activity, suggesting to us that *dpp* could be regulated by a negative feedback loop. It has been unclear whether negative regulation of *dpp* represents a true negative feedback loop



**Figure 1.11. Expression of *dpp* is negatively regulated by BMP signaling.** We hypothesize that transcription of *dpp* is regulated through a BMP-signaling-dependent negative feedback loop. The components of the BMP pathway that generate repression of *dpp*, as the specific factors and *dpp* regulatory sequences that mediate this response will be addressed in this work.

that signals through the entire BMP pathway.

Furthermore, no attempts have been made to determine the mechanisms by which *dpp* is negatively regulated.

Negative feedback of *dpp* presents as a particularly exciting mechanism for maintenance of the pMad gradient in the wing disc. For this reason, we were interested in taking a closer look at this process and determining the underlying mechanisms. We hypothesized that *dpp* is, indeed, negatively regulated by a BMP-dependent feedback loop (Fig 1.11) and that this process contributes to

robustness of BMP signaling in the wing imaginal disc. Chapter two describes our experiments

characterizing this process in the wing imaginal disc. We have determined that negative regulation of *dpp* is Mad dose-dependent and represents a true negative feedback loop through canonical BMP signaling.

Furthermore, we have addressed some of the spatial implications of this process and demonstrated that, within the imaginal disc, it serves to regulate *dpp* only within its endogenous expression domain. In

Chapter three we address the specific molecular players of this process. Our results have indicated that many independent sequences within the *dpp cis*-regulatory sequences mediate feedback regulation of

*dpp*. In addition, we have determined that Ci is dispensable for this negative feedback loop while Mad and Shn play a more active role in this process.

## Chapter 2:

Expression of *dpp* is regulated by a BMP-dependent negative feedback loop in the wing imaginal disc

I performed all experiments presented here, with the exception of the following data:  
Figure 2.4D (Juliane Kellner), figure 2.5D (Juliane Kellner), figure 2.6A (Lorena Soares),  
figure 2.7 (Lorena Soares), figure 2.9 (Lorena Soares), figure 2.10C-D (Lorena Soares),  
figure 2.11B (Lorena Soares), and Table 2.1 (Juliane Kellner)

## Abstract

Gradients of morphogen activity assign positional information within a tissue by affecting cells in a concentration-dependent manner. As cells must encounter and respond to the correct dosage of morphogen activity, regulatory mechanisms must exist to ensure robustness within signal transduction pathways that generate morphogen activity gradients. In the *Drosophila* larval wing disc, the Bone Morphogenetic Protein (BMP) signaling pathway generates a morphogen activity through the graded distribution of the phosphorylated transducer, phospho-Mad (pMad), that directs distinct transcriptional responses of target genes important in patterning along the anterior-posterior axis of the wing disc. The ligands Gbb and Dpp cooperate to generate the pMad gradient and pattern the wing disc, but several lines of evidence have indicated that *gbb* cannot compensate for reduced *dpp* function. Further analysis of this observation has revealed that transcription of *dpp* is negatively regulated by BMP signaling in the wing imaginal disc. The work presented here examines the effect of manipulating components of the BMP signaling cascade on *dpp* feedback regulation. We have determined that endogenous expression of *dpp* along the anterior-posterior boundary of the wing pouch is regulated by a BMP-dependent negative feedback loop. The strength of the transcriptional response in *dpp* is pMad dose-dependent, with larger increases in pMad levels correlating with stronger decreases in *dpp* expression. In addition, expression of *dpp* is regulated in response to both increased and decreased BMP signaling. While this negative feedback mechanism is only active within the endogenous expression domain of *dpp* in the wing disc, we believe that this effect is not limited to the wing pouch as we have also found that *gbb* levels can influence the viability of *dpp*<sup>disk</sup> mutant combinations in a manner consistent with negative transcriptional regulation of *dpp* levels. We propose that this negative feedback mechanism serves to “fine tune” BMP signaling and keep its activity level within its normal range, ensuring robust signaling throughout development.

## Introduction

During the development of multi-cellular organisms, cells are patterned in response to extracellular signaling cues and signaling transduction pathways which generate morphogen activity gradients. Through cellular responses to distinct activity thresholds, morphogen activity gradients assign positional information to cells within a tissue. BMP signaling is well known to play a critical role in patterning the *Drosophila* larval wing imaginal disc where it influences expression of key components important for specification of cell fates. In the wing disc tissue, a graded distribution of the BMP transducer, pMad, acts as a morphogen and affects target gene transcription in a concentration-dependent manner.

In this tissue, BMP signaling is initiated by signaling through the ligands Dpp and Gbb. Dpp is expressed from a narrow stripe of cells along the anterior-posterior compartment boundary of the wing pouch while Gbb is more broadly expressed. Ligand dimers signal through a heterotetrameric receptor complex of two type II and two type I serine/threonine-protein kinase receptors (Raftery and Sutherland, 1999). Upon complex formation, the constitutively active type II receptors phosphorylate the type I receptors which, in turn, phosphorylate the intracellular transducer of BMP signaling, Mad. pMad, in complex with its Co-Smad, Med, can now accumulate in the nucleus and affect target gene transcription. Cooperation of Dpp and Gbb generates a gradient of pMad activity along the anterior-posterior boundary of the wing pouch. Patterning the wing pouch requires the action of both ligands, as a reduction in level of either results in improper pMad gradient formation and adult wing phenotypes (Bangi and Wharton, 2006; Khalsa et al., 1998; Ray and Wharton, 2001).

As cells respond to thresholds of morphogen activity, it is important that specific levels of activity are met at appropriate times in development. Perturbations in signaling output in response to environmental or genetic fluctuations could impact the ability of this to occur. In addition, the imaginal disc is dynamic during development as it undergoes extensive tissue growth and proliferation. These signaling pathways must be highly robust to accommodate for fluctuations in activity and allow scaling of the gradient as the tissue grows. One particularly intriguing and efficient mechanism by which a signaling

pathway could accomplish this is through negative feedback regulation of transcription or activity of some component of the pathway. Negative autoregulatory loops not only provide a mechanism for a pathway to sense its final output, but they feed directly back into the pathway to compensate for any changes in signaling level (Freeman, 2000).

Many factors are known to interact with BMP pathway components and regulate signaling activity (Moustakas and Heldin, 2009). However, specific autoregulatory mechanisms that contribute to robustness in response to fluctuations in pMad levels are just beginning to become elucidated. Recently, the BMP target and inhibitory Smad, Dad, has been implicated to function in an autoregulatory feedback loop that maintains robust BMP signaling levels (Ogiso et al., 2011). In addition, two BMP targets, *pentagone (pent)* and *larval translucida (Itl)*, have recently been identified and characterized as secreted feedback regulators of the pathway (Hamaratoglu et al., 2011; Szuperák et al., 2011; Vuilleumier et al., 2010). As expression of *dad*, *pent*, and *Itl* are all sensitive to BMP signaling levels and influence signaling activity, they present as potential mechanisms by which the BMP signaling pathway could accommodate for fluctuations in signaling levels. Work in our lab has identified an additional mechanism through which BMP signaling could mediate its activity output, that of negative regulation of *dpp* transcription. We propose that feedback regulation of *dpp*, an upstream component, is part of a negative feedback loop that allows for “fine tuning” of the pathway.

Tight regulation of *dpp* is not unexpected as *dpp* is one of the few loci that exhibit haplolethality in *Drosophila*, indicating sensitivity to a reduction in *dpp* levels (Irish and Gelbart, 1987; Segal and Gelbart, 1985; Spencer et al., 1982; Wharton et al., 1993). Curiously, the animal is less sensitive to increases in *dpp* as little change in wing patterning is produced by either a modest increase in *dpp* levels (Morimura et al., 1996) or by eight genomic copies of *dpp* (Yu et al., 1996). This raises the possibility that efficient feedback mechanisms are in place to compensate for increased BMP signaling. Several studies have briefly noted that *dpp* expression can be negatively regulated in response to BMP signaling (Crickmore and Mann, 2007; Haerry et al., 1998; Ogiso et al., 2011; Umemori et al., 2007). The work presented here is the first report to systematically and specifically characterize this negative feedback regulation.

Our experiments have revealed that *dpp* expression is reduced in response to increased levels of canonical BMP signaling, mediated through increased expression or activity of BMP ligands, receptors, and of the transducer Mad. The strength of the transcriptional response of *dpp* correlates to pMad levels, with the greatest increase in pMad leading to the strongest reduction in *dpp* expression, suggesting that this effect is pMad dose-dependent. Furthermore, expression of *dpp* is increased in response to reduced BMP signaling levels, indicating that this feedback loop can efficiently accommodate for inappropriate decreases as well as increases in signaling output. We have also found that feedback is spatially restricted within the developing wing imaginal disc, such that the effect of *dpp* negative feedback is most prominent in the medial domain where endogenous *dpp* expression is at its highest and absent in the lateral-most region of the wing disc where *dpp* is normally not expressed. It is likely that a similar negative feedback mechanism exists in other tissues, as we have also identified a negative genetic interaction between specific alleles of *dpp* and *gbb*, such that *gbb* copy number influences lethality associated with *dpp* alleles. Future experiments will characterize the specific mechanisms of negative *dpp* transcriptional regulation and will seek to determine the particular sequences and factors that mediate this response.



## Materials and Methods

### *Drosophila melanogaster* strains, crosses, and husbandry

Flies were raised on standard cornmeal/yeast medium at 25°C in plastic bottles or glass vials. Flies used for virgin collection were raised at 18°C or 22°C in plastic bottles. Crosses were performed using virgin females. Unless otherwise stated, all alleles used are described in flybase (<http://flybase.org/>)

### RNA *in situ* Hybridization

Probe generation:

Probe template was generated by PCR amplification of a 1kb region of *dpp* or *lacZ* cDNA using Pfu polymerase. Primers were designed to contain Sp6 polymerase binding sites (5' ATTTAGGTGACACTATAGA-3') in the forward direction and T7 polymerase binding sites (5'-TAATACGACTCACTATAGG-3') in the reverse direction and were: *lacZ* forward ATTTAGGTGACACTATAGATTACTTTGACCAACAAGAACG; *lacZ* reverse TAATACGACTCACTATAGGAGCAGACCATTTTCAATCC; *dpp* forward ATTTAGGTGACACTATAGAATTCA TCGCCGCCATAGC; *dpp* reverse TAATACGACTCACTATAGGTCCACCAGCAGTCCGTAGTTG. Probes were generated using the Roche DIG RNA labeling kit (SP6/T7) according to manufacturer's directions for a 20µl reaction. *lacZ* probe was created with 2.6µl template and *dpp* probe was created with 5.2 µl template. Probes were treated with 2µl DNAase for 15 minutes at 37°C then treated with 2µl 0.2M EDTA (pH8.0) to stop the reaction. To precipitate the probes: add 2.5 µL 4M LiCl and 75 µL chilled 100% EtOH, precipitate 30 min at -80°C, spin 13,000g for 15min at 4°C, discard supernatant, wash with 50µl cold 70% EtOH, spin 13,000g for 5min at 4°C, discard sup, air dry, elute in 100µl DEPC-H<sub>2</sub>O and store at -80°C until use. To make DEPC-H<sub>2</sub>O add DEPC at 0.05% DEPC (v/v) to distilled water and let sit overnight at RT, autoclave. Alkaline hydrolysis was performed to generate smaller probe fragments of approximately 250bp. To 10µl RNA add 10ml DEPC-H<sub>2</sub>O, and 20µL carbonate buffer (60mM Na<sub>2</sub>CO<sub>3</sub>, 40mM NaHCO<sub>3</sub>, pH 10.2), incubate 60°C for 28 min (time = [length initial transcript in kb – desired probe length in kb]/[0.11kb/min X length initial transcript in kb X desired probe length in kb]), stop reaction with 40µL hydrolysis-neutralization buffer (3M sodium acetate, 1% acetic acid (v/v), pH6.0), add 240µl cold EtOH, mix and incubate 30 min at

-80°C, spin 13,000 for 15 min at 4°C, remove supernatant, wash with 100µL cold 70% EtOH, spin 13,000 for 5min at 4°C, dry, resuspend in 50µL DEPC-H<sub>2</sub>O, store at -80°C.

Size and relative concentration of probes was confirmed through formaldehyde gel electrophoresis: pour RNA gel (1% agarose melted in water, cooled, add 0.1 volumes 10X MOPS buffer, 0.16volumes formalin), load 2.5µL RNA in 2.5µL 4X sample buffer (2 volumes 10X MOPS, 3 volumes formalin) and 5µL formamide heated to 68°C for 5 min, transfer to nitrocellulose membrane overnight by wicking, the following day wash 3X quickly in TBST (50mL 1M Tris-HCl pH7.6, 30mL 5M NaCl, 0.1% w/v Tween20, up to 1L water), block 1hr in 1% BSA in TBST, incubate in antibody (1:3000 anti-DIG-AP in block) 2hr at RT, wash 3X 10-15 min TBST, wash quickly in AP staining buffer (100m Tris pH 9.5, 50mM MgCl<sub>2</sub>, 100mM 5M NaCl), then develop in 10mL AP staining buffer with 66µL NBT and 33µL BCIP.

To obtain tissue for in situ hybridization, we crossed:

*dpp* RNA probe: *w* ; *UAS-tkv<sup>QD</sup>* males to *w<sup>1118</sup>* females to generate *w/+* ; *UAS-tkv<sup>QD</sup>/+* control larvae  
*w* ; *UAS-tkv<sup>QD</sup>* males to *w*; *ap-Gal4 UAS-GFP* ; *+ / T(2;3)SM5-TM6b* females to generate  
*w*; *ap-Gal4 UAS-GFP/+* ; *UAS-tkv<sup>QD</sup>/+* larvae  
*w* ; *UAS-tkv<sup>QD</sup>* males to *w MS1096-Gal4* females to generate *w MS1096/+* ; *UAS-tkv<sup>QD</sup>/+*  
 larvae

*lacZ* RNA probe: *w* ; *dpp<sup>p10638</sup>lacZ* ; *UAS-tkv<sup>QD</sup> / T(2;3)SM5-TM6b* males to *w<sup>1118</sup>* females to generate  
*dpp<sup>p10638</sup>lacZ /+* ; *UAS-tkv<sup>QD</sup>/+* control larvae  
*w* ; *dpp<sup>p10638</sup>lacZ* ; *UAS-tkv<sup>QD</sup> / T(2;3)SM5-TM6b* males to *w*; *ap-Gal4 UAS-GFP* ; *+ /*  
*T(2;3)SM5-TM6b* females to generate *w*; *ap-Gal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ* ; *UAS-tkv<sup>QD</sup>/+*  
 larvae  
*w* ; *dpp<sup>p10638</sup>lacZ* ; *UAS-tkv<sup>QD</sup> / T(2;3)SM5-TM6b* males to *w MS1096-Gal4* females to  
 generate *w MS1096/+* ; *dpp<sup>p10638</sup>lacZ/+* ; *UAS-tkv<sup>QD</sup>/+* larvae

In Situ hybridization:

Protocol modified from Ethan Bier and chapter 12.5 from Ashburner (Ashburner, 1989; Bier and Sturtevant, 1997).

**Tissue collection and fixation:** Third instar larvae were raised at 25°C, dissected in PBS for 5 min, and fixed in 4% formaldehyde/PBS at RT for 30 min. Carcasses were then dehydrated by 8 X 5min washes in MeOH followed by 5 X 5min washes in EtOH, and stored at -20 in EtOH until use.

**Prehybridization:** Carcasses underwent a series of washes consisting of: 3X 5min washes in EtOH, 1 hr in 50/50 xylenes/EtOH, 5X 5 min washes in EtOH, 2X 5 min washes in MeOH, 5 min 50/50 MeOH/5% formaldehyde in PBTx (0.1% Tween-20 in PBS ), 30 min in 5% formaldehyde in PBTw, and 5X 10 min washes in PBTw. They were then treated with proteinase K for 3 min at 10µg/mL in PBTw, following by 2X 2 min glycine washes (PBTw with 2mg/mL glycine), and 2X 10 min PBTw washes. Carcasses were post-fixed for 30 min in 5% formaldehyde in PBTw and then washed 5X 10 min in PBTw.

**Hybridization:** Everted carcasses were washed once in 50/50 PBTw/hybridization buffer (HB: 50% formamide, 5X SSC, 100µg/ml sonicated herring sperm DNA, 50 µg/ml heparin, 0.1% Tween-20), followed by 3X 10 min HB washes, and prehybridization overnight at 55°C. Probes were denatured by boiling for 10 min and diluted 4µL probe into 60uL HB. 60µL HB + probe was added to each sample and incubated at 55°C for 22 hours, with periodic flicking to mix (hourly).

**Washes and detection:** wash once quickly in HB, incubate 1 hour in HB at 55°C, wash 6X 20 min in HB at 55°C. Work gradually back to PBT at RT with 3X 15 washes containing 750 HB/250 PBTw, 500 HB/500 PBTw, 250 HB/750 PBTw , then wash 5X 10 min at RT in PBTw. Incubate overnight in 500 µL PBTw with alkaline phosphatase conjugated anti-DIG Ab at 1:2000 at 4°C. Wash 5X 10min in PBTw then treat with AP staining buffer (100mM NaCl, 50mM MgCl<sub>2</sub>, 100mM Tris-HCl pH 9.5, 0.1% Tween-20, 1mM levamisole) 3X 10 min with shaking. Stain in dark with shaking 20min-2 hr in 500µl staining solution with 4.5 µL NBT and 3.5 µL BCIP. Wash 2X 10 min in staining buffer and 4X 10 min in PBTw before mounting in 80% glycerol.

### **Dissections and immunostaining**

Unless otherwise stated, larvae were dissected as third instar crawlers. All steps were carried out in glass wells without rocking. Briefly, larvae were collected and the anterior 2/3 of the larvae was everted in ice cold PBS and transferred to PBS on ice for no longer than 30 minutes. Tissue was fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature (RT). For the pMad staining shown in figure

2.7, discs were fixed in 4% formaldehyde diluted in water for five minutes at RT. After fixation, discs were washed 3X 20 minutes in PBST (0.1% Triton-X in PBS) at RT. Samples were blocked for one hour in PBST + 1% NGS at RT and then incubated overnight at 4°C with the appropriate primary antibody concentration in PBST + 1% NGS + 0.02% sodium azide. Following incubation, discs were washed 3X 20 minutes in PBST at RT, followed by a one hour block in PBST + 1% NGS at RT. Discs were incubated for 2 hours at RT in the appropriate secondary antibody in PBST + 1% NGS + 0.02% sodium azide. When used, Hoechst-33432 was added to the secondary antibody solution. Tissue was next blocked for one hour in PBST + 1% NGS at RT and then stored in mounting solution (80% glycerol, 0.1% N-propyl-gallate in water) at 4°C until mounting. Discs were dissected away from cuticles and mounted on glass slides in 8µL mounting solution and covered with a #1.5 coverslip.

The following antibodies were used: anti-pMad (PS1, rabbit, 1:2000. Used in figure 2.7, a generous gift from P. ten Dijke; rabbit monoclonal Smad3 phospho (pS423/425), 1:1000, Epitomics (Kamimura et al., 2011; Miller et al., 2011)), anti-βGal (mouse monoclonal, 1:3000, Promega; rabbit polyclonal, 1:3000, Cappel). Secondary antibodies used were Alexa Fluor 633 goat anti-rat IgG, Alexa Fluor 633 goat anti-mouse IgG, Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 555 goat anti-rabbit IgG, Alexa Fluor 555 donkey anti-mouse IgG, Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 405 goat anti-mouse IgG, Molecular probes. All were used at a 1:3000 concentration. Nuclei were labeled with Hoechst-33432 at a 1:1000 dilution.

#### **Confocal microscopy and image analysis**

Images were taken with a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope (figures 2.4D, 2.5D, 2.6A, 2.7, 2.9, 2.10A,C,D, 2.11B), a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope (figures 2.2, 2.3, 2.4A-C, 2.5A-C, 2.6B, 2.8, 2.10B, 2.11A,D), and a Nikon Microphot-FXA (figure 2.1). Confocal images were taken as z-stacks with one 1µM (Leica) or optimal (Zeiss) slice sizes at either 512X512 or 1024X1024 pixels. All images were taken sequentially. Scan speed of 6 was used (Zeiss).

Unless noted in the figure legend, images shown represent a collapsed z-projection of the sum of slice intensities, generated in Fiji.

Intensity profiles were created using Fiji software using the “plot profile” function. For each experiment, profiles were plotted from a rectangular selection, which gives the vertically averaged pixel intensity at each x-position. The rectangular selection size varied between experiments, but was kept constant within an experiment. Rectangular selections were placed at approximately the same location in all disc images within an experiment. Location was determined by relative position to the anterior-posterior border or to location of intensity peaks. In experiments in which average profiles were created, Microsoft Excel was used to average the intensity value at each position of all profiles for each genotype. In all intensity plots, the X axis represents each pixel and the Y axis represents pixel intensity.

**Analysis of  $dpp^{p10638}lacZ$  and  $dpp^{B53.0}lacZ$  expression and pMad levels with over-expression of ligand or receptor by various *Gal4* drivers**

GFP expression in different *Gal4* domains and  $dpp^{p10638}lacZ$  expression in these backgrounds was examined by performing the following crosses:

*omb-Gal4/Y ; UAS-GFP/+* males to *yw ; dpp^{p10638}lacZ/SM6a* virgin females to generate *omb-*

*GAL4/+ ; UAS-GFP/ dpp^{p10638}lacZ* larvae

*MS1096-Gal4/Y ; UAS-GFP/+* males to *yw ; dpp^{p10638}lacZ/SM6a* virgin females to generate

*MS1096-GAL4/+ ; UAS-GFP/ dpp^{p10638}lacZ* larvae

*ap-Gal4 UAS-GFP/T(2;3) SM5-TM6b* males to *yw ; dpp^{p10638}lacZ/SM6a* virgin females to generate

*ap-GAL4 UAS-GFP/ dpp^{p10638}lacZ* larvae

Expression patterns of  $dpp^{p10638}lacZ$  with over-expression of *UAS-tkv<sup>QD</sup>* were examined by performing the following crosses:

*omb-Gal4/Y ; UAS-GFP/+* males to *w ; dpp^{p10638}lacZ ; UAS-tkv<sup>QD</sup>/T(2;3) SM5-TM6b* virgin females

to generate *omb-GAL4/+ ; UAS-GFP/ dpp^{p10638}lacZ ; UAS-tkv<sup>QD</sup>/+* larvae

*MS1096-Gal4/Y ; UAS-GFP/+* males to *w ; dpp^{p10638}lacZ ; UAS-tkv<sup>QD</sup>/T(2;3) SM5-TM6b* virgin

females to generate *MS1096-GAL4/+ ; UAS-GFP/ dpp^{p10638}lacZ ; UAS-tkv<sup>QD</sup>/+* larvae

*ap-Gal4 UAS-GFP/T(2;3) SM5-TM6b* males to *w ; dpp^{p10638}lacZ ; UAS-tkv<sup>QD</sup>/T(2;3) SM5-TM6b*

virgin females to generate *ap-GAL4 UAS-GFP/ dpp^{p10638}lacZ ; UAS-tkv<sup>QD</sup>/+* larvae

Expression patterns of  $dpp^{BS3.0}lacZ$  with over-expression of  $UAS-tkv^{QD}$  were examined by performing the following crosses:

$w$   $MS1096-Gal4/Y$ ;  $dpp^{BS3.0}lacZ/+$  males to  $UAS-tkv^{QD}$  females to generate  $w$   $MS1096-Gal4/Y$ ;

$dpp^{BS3.0}lacZ / UAS-tkv^{QD}$  larvae.

$w$ ;  $ap-Gal4$   $UAS-GFP$ ;  $dpp^{BS3.0}lacZ / T(2;3)SM5-TM6b$  males to  $UAS-tkv^{QD}$  females to generate  $w$ ;

$ap-Gal4$   $UAS-GFP/+$ ;  $dpp^{BS3.0}lacZ/UAS-tkv^{QD}$  larvae.

Expression patterns of  $dpp^{p10638}lacZ$  with over-expression of  $UAS-gbb^{9.7}$  were examined by performing the following crosses:

$omb-Gal4/Y$ ;  $UAS-GFP/+$  males to  $yw$   $UAS-gbb^{9.7}$ ;  $dpp^{p10638}lacZ/CyO$   $Wg-lacZ$  virgin females to

generate  $omb-GAL4/yw$   $UAS-gbb^{9.7}$ ;  $UAS-GFP/dpp^{p10638}lacZ$  larvae

$MS1096-Gal4/Y$ ;  $UAS-GFP/+$  males to  $yw$   $UAS-gbb^{9.7}$ ;  $dpp^{p10638}lacZ/CyO$   $Wg-lacZ$  virgin females

to generate  $MS1096-GAL4/yw$   $UAS-gbb^{9.7}$ ;  $UAS-GFP/dpp^{p10638}lacZ$  larvae

$ap-Gal4$   $UAS-GFP/T(2;3)$   $SM5-TM6b$  males to  $yw$   $UAS-gbb^{9.7}$ ;  $dpp^{p10638}lacZ/CyO$   $Wg-lacZ$  virgin

females to generate  $yw$   $UAS-gbb^{9.7}$ ;  $ap-GAL4$   $UAS-GFP/dpp^{p10638}lacZ$  larvae

Expression patterns of  $dpp^{p10638}lacZ$  with over-expression of  $UAS-dpp^w$  were examined by performing the following crosses:

$omb-Gal4/Y$ ;  $UAS-GFP/+$  males to  $w$ ;  $dpp^{p10638}lacZ$ ;  $UAS-dpp^w/T(2;3)$   $SM5-TM6b$  virgin females

to generate  $omb-GAL4/+$ ;  $UAS-GFP/dpp^{p10638}lacZ$ ;  $UAS-dpp^w/+$  larvae

$MS1096-Gal4/Y$ ;  $UAS-GFP/+$  males to  $w$ ;  $dpp^{p10638}lacZ$ ;  $UAS-dpp^w/T(2;3)$   $SM5-TM6b$  virgin

females to generate  $MS1096-GAL4/+$ ;  $UAS-GFP/dpp^{p10638}lacZ$ ;  $UAS-dpp^w/+$  larvae

$ap-Gal4$   $UAS-GFP/T(2;3)$   $SM5-TM6b$  males to  $w$ ;  $dpp^{p10638}lacZ$ ;  $UAS-dpp^w/T(2;3)$   $SM5-TM6b$

virgin females to generate  $ap-GAL4$   $UAS-GFP/dpp^{p10638}lacZ$ ;  $UAS-dpp^w/+$  larvae

Expression patterns of  $dpp^{BS3.0}lacZ$  with over-expression of  $UAS-dpp^w$  were examined by performing the following crosses:

$omb-Gal4$  to  $UAS-dpp^w$   $dpp^{BS3.0}lacZ/TM6b$  to generate  $omb-Gal4/+$ ;  $UAS-dpp^w$   $dpp^{BS3.0}lacZ/+$

larvae

#### FLP-out clone over-expression studies

Clones of cells over-expressing a particular UAS construct were generated by the FLP-out method (Struhl and Basler, 1993)

*tkv<sup>QD</sup>* FLP-out clones were generated by crossing:

*w/Y; AyGal4 UAS-GFP; dpp<sup>BS3.0</sup>lacZ /T(2;3) SM5-TM6b* males to *y w hsFLP122/y w; UAS-tkv<sup>QD</sup>* females to generate *y w hsFLP122/w or Y; AyGal4 UAS-GFP/UAS-tkv<sup>QD</sup>; dpp<sup>BS3.0</sup>lacZ /+* larvae.  
*y w hsFLP122/Y; AyGal4 UAS-GFP/+* males were crossed to *w; dpp<sup>p10638</sup>lacZ; UAS-tkv<sup>QD</sup>/T(2;3)* females to generate *y w hsFLP122/+; AyGal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ; UAS-tkv<sup>QD</sup>/+* larvae.

*sax<sup>QD</sup>* FLP-out clones were generated by crossing:

*w/Y; AyGal4 UAS-GFP; dpp<sup>BS3.0</sup>lacZ /T(2;3) SM5-TM6b* males to *y w hsFLP122/y w; UAS-sax<sup>QD</sup>* females to generate *y w hsFLP122/w or Y; AyGal4 UAS-GFP/UAS-sax<sup>QD</sup>; dpp<sup>BS3.0</sup>lacZ /+* larvae.

*gbb* FLP-out clones were produced by crossing:

*w/Y; AyGal4 UAS-GFP; dpp<sup>BS3.0</sup>lacZ /T(2;3) SM5-TM6b* males to *y w hsFLP122/y w; UAS-gbb<sup>9.9</sup>* females to generate *y w hsFLP122/w or Y; AyGal4 UAS-GFP/UAS-gbb<sup>9.9</sup>; dpp<sup>BS3.0</sup>lacZ /+* larvae.

*dpp* FLP-out clones were produced by crossing:

*w/Y; AyGal4 UAS-GFP; dpp<sup>BS3.0</sup>lacZ /T(2;3) SM5-TM6b* males to *y w hsFLP122/y w; UAS-dpp<sup>w</sup>* females to generate *y w hsFLP122/w or Y; AyGal4 UAS-GFP/ UAS-dpp<sup>w</sup>; dpp<sup>BS3.0</sup>lacZ /+* larvae.

*Mad* FLP-out clones were generated by Lorena Soares. Materials and methods for this experiment could not be found.

Crosses were pre-mated in glass vials and then moved to apple juice plates with yeast paste and brooded every 24 hours. To induce the expression of FLP recombinase, apple juice plates containing larvae were sealed and heat shocked at 37°C by submersion in a water bath for 10-15 minutes at either 48-72 or 54-76 hours post lay to generate early or mid second instar clones. At least one hour after heat shock, larvae were transferred to vials. Clones were identified by the expression of GFP.

#### Loss-of-function clone studies

Clones of genetically identical cells were generated by the FLP-mediated recombination method (Xu and Rubin, 1993)

*gbb<sup>2</sup>* null clones were generated by crossing:

*w/Y; FRT<sup>G13</sup> gbb<sup>2</sup>; dpp<sup>BS3.0</sup> lacZ / T(2;3) SM5-TM6b* males to *hsFLP1; FRT<sup>G13</sup> GFP/CyO GFP* females to generate *hsFLP1; FRT<sup>G13</sup> gbb<sup>2</sup>/FRT<sup>G13</sup> GFP; dpp<sup>BS3.0</sup> lacZ /+* larvae.

*sax<sup>4</sup>* null clones were generated by crossing:

*w/Y; FRTG13 sax<sup>4</sup>; dpp<sup>BS3.0</sup> lacZ / T(2;3) SM5-TM6b* males to *hsFLP1; FRT<sup>G13</sup> GFP/CyO GFP* females to generate *hsFLP1; FRTG13 sax<sup>4</sup>/FRT<sup>G13</sup> GFP; dpp<sup>BS3.0</sup> lacZ /+* larvae.

*tkv<sup>a12</sup>* hypomorph clones were generated by crossing:

*y w hsFLP122; FRT<sup>40A</sup> tkv<sup>a12</sup> / CyO* males to *y w; FRT<sup>40A</sup> GFP; dpp<sup>BS3.0</sup> lacZ / T(2;3)* females to generate *y w hsFLP122/; FRT<sup>40A</sup> tkv<sup>a12</sup> / FRT<sup>40A</sup> GFP; dpp<sup>BS3.0</sup> lacZ /+* larvae.

Crosses were pre-mated in glass vials and then moved to bottles and brooded every 24 hours. To induce the expression of FLP recombinase, bottles were heat shocked at 37°C by partial submersion in a water bath for 2 hours at 24-48 hours post lay to generate first instar clones. Larvae were selected and dissected as third instar crawlers. Clones were identified by the absence of GFP.

#### **Analysis of *hh<sup>MRT</sup>* phenotypes with increased BMP signaling**

The original *hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ* stock used by Lorena had broken down so a new recombinant was generated.

Animals over-expressing *UAS-tkv<sup>QD</sup>* were generated by crossing:

*hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ/TM3 Sb* males to *Ore-R* virgin females to generate *hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ/+* control larvae

*hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ/TM3 Sb* males to *w; UAS-tkv<sup>QD</sup>* virgin females to generate *hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ/ UAS-tkv<sup>QD</sup>* control larvae

*w MS1096-Gal4/Y; +/+; hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ /+* males to *w; +/+; UAS-tkv<sup>QD</sup>* females to generate *w MS1096-Gal4/w; +/+; hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ / UAS-tkv<sup>QD</sup>* larvae and *hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ / UAS-tkv<sup>QD</sup>* control larvae

*w A9-Gal4/Y; +/+; hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ /+* males to *w; +/+; UAS-tkv<sup>QD</sup>* females to generate *w A9-Gal4/w; +/+; hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ / UAS-tkv<sup>Q199D</sup>* larvae and *hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ / UAS-tkv<sup>QD</sup>* control larvae

Statistical significance of these results was tested by X<sup>2</sup> analysis.



To generate *tkv<sup>QD</sup>* FLP-out clones:

*y w/Y ; AyGal4 UAS-GFP/+ ; hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ* males were crossed to *y w f hsFLP122 ; +/+ ; UAS-tkv<sup>QD</sup>* females to generate *y w f hs-FLP122/+ ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/+ ; hh<sup>MRT</sup> dpp<sup>BS3.0</sup> lacZ / UAS-tkv<sup>QD</sup>* larvae.

Crosses were pre-mated in glass vials and then moved to apple juice plates with yeast paste and brooded every 24 hours. To induce the expression of FLP recombinase, apple juice plates containing larvae were sealed and heat shocked at 37°C by submersion in a water bath for 10-15 minutes at either 48-72 or 54-76 hours post lay to generate early or mid second instar clones. Clones were identified by the expression of GFP.

### Viability studies of *dpp<sup>hr56</sup>/dpp<sup>d5</sup>* and *dpp<sup>hr4</sup>/dpp<sup>d5</sup>* animals with *p[6.8-gbb<sup>+</sup>]*3.3

To determine control viabilities of *dpp<sup>hr</sup>* and *dpp<sup>d5</sup>*:

*dpp<sup>d5</sup>/CyO* males were crossed to *y w ; wg<sup>Sp</sup>/Sm6a* females to generate *y w/Y ; dpp<sup>d5</sup>/wg<sup>Sp</sup>* progeny.

*y w/Y ; dpp<sup>d5</sup>/wg<sup>Sp</sup>* males were crossed to *dpp<sup>hr56</sup>/Sm6a* or *dpp<sup>hr4</sup>/Sm6a* females to generate the following genotypes, scored for viability:

(1)*dpp<sup>hr</sup>/dpp<sup>d5</sup>*                      (2)*dpp<sup>hr</sup>/wg<sup>Sp</sup>*                      (3)*dpp<sup>d5</sup>/CyO*                      (4)*wg<sup>Sp</sup>/Sm6a*

Viability of each class was calculated as a percentage of the expected, based on the *wg<sup>Sp</sup>/Sm6a* progeny class. The expected ratio of *dpp<sup>hr</sup>/Wg<sup>Sp</sup>* to *dpp<sup>d5</sup>/CyO* was used in the next step.

To test viability of *dpp<sup>hr56</sup>/dpp<sup>d5</sup>* or *dpp<sup>hr4</sup>/dpp<sup>d5</sup>* with *p[6.8-gbb<sup>+</sup>]*3.3:

*dpp<sup>hr56</sup>/Sm6a* or *dpp<sup>hr4</sup>/Sm6a* males were crossed to *y w ; dpp<sup>d5</sup>/CyO ; p[6.8-gbb<sup>+</sup>]*3.3/*p[6.8-gbb<sup>+</sup>]*3.3 females to generate *y w/Y ; dpp<sup>hr</sup>/dpp<sup>d5</sup> ; p[6.8-gbb<sup>+</sup>]*3.3/+ progeny.

*y w/Y ; dpp<sup>hr</sup>/dpp<sup>d5</sup> ; p[6.8-gbb<sup>+</sup>]*3.3/+ males were crossed to *y w ; dpp<sup>d5</sup>/CyO ; p[6.8-gbb<sup>+</sup>]*3.3/*p[6.8-gbb<sup>+</sup>]*3.3 females to generate the following genotypes, scored for viability:

(1)*y w ; dpp<sup>hr</sup>/dpp<sup>d5</sup> ; p[6.8-gbb<sup>+</sup>]*3.3 / *p[6.8-gbb<sup>+</sup>]*3.3

(2)*y w ; dpp<sup>hr</sup>/dpp<sup>d5</sup> ; p[6.8-gbb<sup>+</sup>]*3.3 / +

(3)*y w ; dpp<sup>hr</sup>/CyO ; p[6.8-gbb<sup>+</sup>]*3.3 / *p[6.8-gbb<sup>+</sup>]*3.3 AND *y w ; dpp<sup>d5</sup>/CyO ; p[6.8-gbb<sup>+</sup>]*3.3 / *p[6.8-gbb<sup>+</sup>]*3.3

(4)  $y\ w ; dpp^{hr} / CyO ; p[6.8-gbb^+]3.3 / +$  AND  $y\ w ; dpp^{d5} / CyO ; p[6.8-gbb^+]3.3 / +$

For classes (3) and (4), the expected number of each genotype was estimated based on the ratio observed in the control crosses. The viabilities of classes (1) and (2) were determined as a percentage of expected, which in this case is the estimated number of  $dpp^{d5} / CyO ; p[6.8-gbb^+]3.3 / +$

**Viability studies of  $dpp^{hr}/dpp^d$  animals with  $gbb^1$ :**

Reciprocal crosses were performed between either  $dpp^{d6}/CyO$  or  $dpp^{d5}/CyO$  and either  $dpp^{hr27}\ cn\ bw/Sm6a$  or  $dpp^{hr4}\ cn\ bw/Sm6a$  to generate:

$dpp^d/dpp^{hr}\ cn\ bw$        $dpp^d/Sm6a$        $dpp^{hr}\ cn\ bw/ CyO$

Reciprocal crosses were performed between either  $dpp^{d6}\ bw\ gbb^1/Sm6a$  or  $dpp^{d5}\ bw\ gbb^1/Sm6a$  and either  $dpp^{hr27}\ cn\ bw/Sm6a$  or  $dpp^{hr4}\ cn\ bw/Sm6a$  to generate:

$dpp^d/dpp^{hr}\ cn\ bw$        $dpp^d\ bw\ gbb^1/Sm6a$        $dpp^{hr}\ cn\ bw/Sm6a$

For every cross, all progeny were scored. Viabilities were determined as the number of  $dpp^d/dpp^{hr}$  as a percentage of expected, which was the number of  $dpp^d/CyO$ , and the number of  $dpp^d\ gbb^1/dpp^{hr}$  as a percentage of expected, which was the number of  $dpp^d\ gbb^1/Sm6a/$

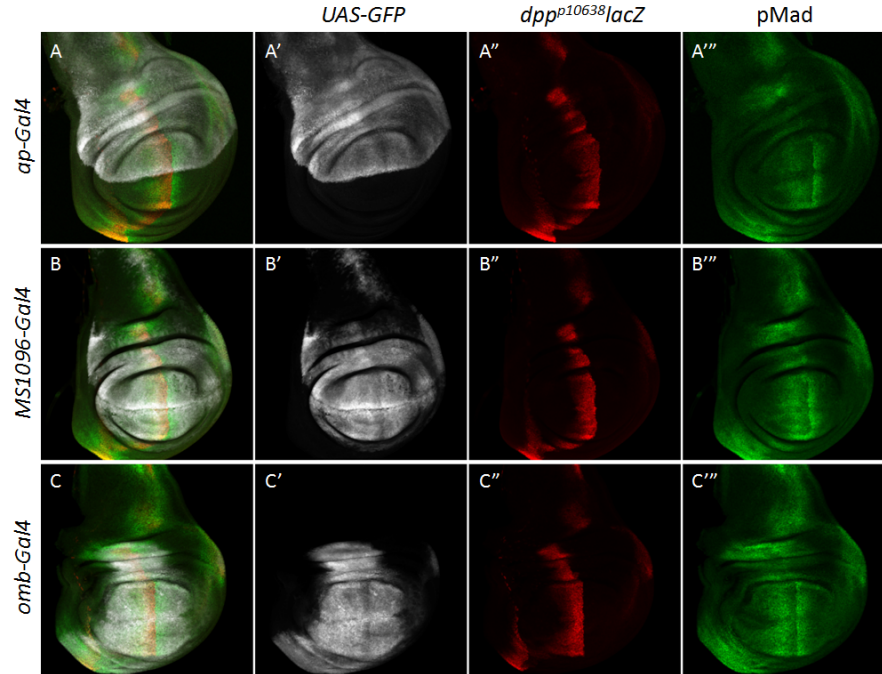
## RESULTS

### Increased BMP Type I receptor activity reduces *dpp* transcription in the wing imaginal disc

We had several lines of evidence, both from observations we had made in the lab (described below, tables **2.1** and **2.2**) and from published observations (Crickmore and Mann, 2007; Haerry et al., 1998; Ogiso et al., 2011; Umemori et al., 2007), that suggested to us that *dpp* could be negatively regulated by components of the BMP signaling pathway. If, indeed, this is occurring then we would expect to see a negative relationship between BMP signaling levels and *dpp* transcription levels. To test this, we asked whether *dpp* levels are reduced in response to increased BMP signaling.

We took advantage of the *Gal4-UAS* expression system to increase the expression of particular components of the BMP pathway in specific regions of the wing disc and examined the resulting *dpp* expression. To avoid potential non cell-autonomous effects of over-expressing BMP ligand that might complicate interpretation, we activated BMP signaling cell-autonomously by expressing constitutively active forms of the BMP type I receptors, Tkv<sup>OD</sup> or Sax<sup>OD</sup> (Wieser et al., 1995). *UAS-tkv<sup>OD</sup>* was specifically expressed in particular regions of the wing disc using a variety of different *Gal4* lines: *ap-Gal4* which is restricted to the dorsal compartment of the wing imaginal disc, *MS1096-Gal4* which is expressed in the entire wing pouch as well as in parts of the hinge and notum, and *omb-Gal4* which is expressed broadly in the wing pouch. *ap-Gal4* is a particularly useful *Gal4* driver as its expression is restricted to the dorsal compartment of the wing disc and allows for comparison against wildtype tissue in the ventral compartment, therefore serving as an internal control. Figure **2.1** displays the expression domain of each of these *Gal4* lines in the wing imaginal disc, which is marked by expression of *UAS-GFP*, in relation to the pMad activity gradient and expression of the *dpp* reporter construct, *dpp<sup>p10638</sup>lacZ*.

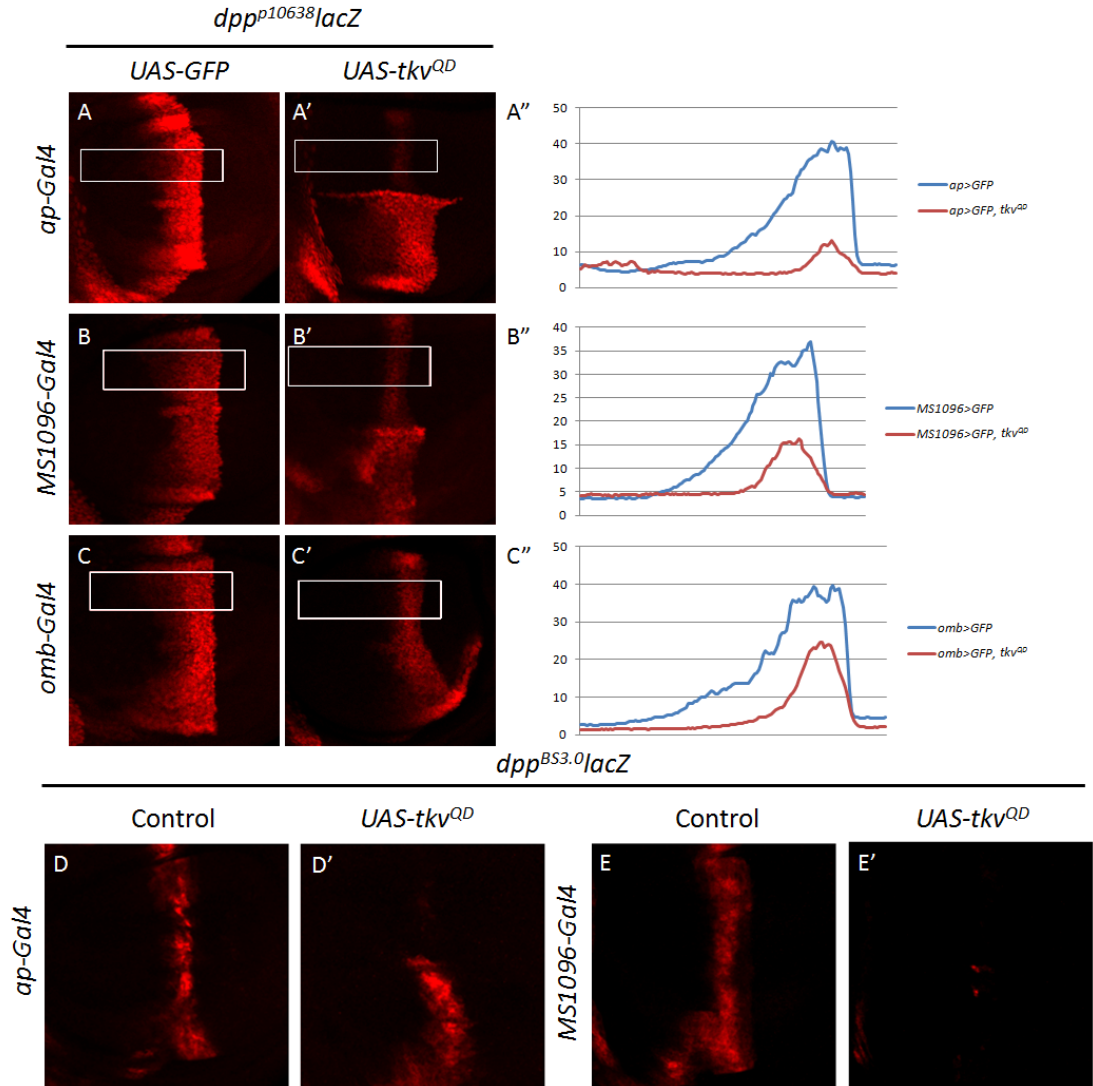
As a read-out for *dpp* transcription, we monitored the expression level of two *dpp* reporters: *dpp<sup>BS3.0</sup>lacZ*, a transgene comprised of 10kb *dpp<sup>disk</sup>* enhancer sequence directly upstream of the minimal *heat shock* promoter and the *lacZ* coding sequence (Blackman et al., 1991), and *dpp<sup>p10638</sup>lacZ*, an enhancer trap in which the *lacZ* coding sequence has been inserted downstream of the *dpp* start site (Twombly et al., 1996). Both of these reporters express in a stripe of anterior cells along the anterior-posterior



**Figure 2.1: Expression patterns of several *Gal4* drivers used to over-express components of the BMP signaling pathway.** *Gal4* expression domains are visualized by expression of *UAS-GFP*, displayed in white (second panel). The expression pattern of *dpp<sup>p10638</sup>lacZ* is shown in red (third panel) and pMad staining is shown in green (fourth panel). The overlay (left panel) demonstrates the relative overlap between each *Gal4* driver and the endogenous expression domain of *dpp* and activity of BMP signaling. *Gal4* drivers used in our studies include: **A.** *ap-Gal4*, which is expressed in the dorsal compartment of the wing imaginal disc, **B.** *MS1096-Gal4*, which is expressed throughout the wing pouch and hinge and in some regions of the notum, and **C.** *omb-Gal4*, which is expressed broadly across the wing pouch and in some hinge tissue, but is not expressed in the notum. All wing discs are oriented with anterior to the left, posterior to the right, dorsal to the top, and ventral to the bottom.

compartment boundary of the wing disc, with *dpp<sup>BS3.0</sup>lacZ* expressed in a narrower domain than *dpp<sup>p10638</sup>lacZ*. They are widely accepted to recapitulate the endogenous expression of *dpp*. Figure 2.1 displays the expression pattern of *dpp<sup>p10638</sup>lacZ* relative to the *Gal4* drivers used and to pMad.

For all over-expression experiments, a representative example of a control and an over-expression wing pouch are shown. As a second method to display our results, we generated intensity profiles of *lacZ* expression from the boxed regions in the displayed discs, as well as from comparable regions in other discs of the same genotype. For each experiment, the size of the rectangular selection used to generate the profiles and the approximate location within the disc from which the profiles were generated was consistent. When noted, average profiles were generated from intensity profiles from two or more samples, which include the displayed sample, to more accurately reflect the expression behavior between samples.



**Figure 2.2. Over-expression of activated Type I BMP receptor, *tkv<sup>QD</sup>*, in the wing pouch leads to a reduction in *dpp-lacZ* expression.** *UAS-tkv<sup>QD</sup>* was over-expressed in the wing disc using various *Gal4* drivers and the resulting expression patterns of *dpp<sup>p10638</sup>lacZ* (A-C) and *dpp<sup>BS3.0</sup>lacZ* (D,E) were examined. For all experiments unless otherwise noted, for each experiment control and *tkv<sup>QD</sup>* over-expression images were taken at the same confocal settings to allow level comparison between samples. Boxed areas in the wing pouches represent the areas used to generate intensity profiles of *dpp-lacZ* expression levels as shown in the right panels (A-C). Intensity profiles displayed represent data from a single sample or from an average of several samples, as noted. **A.** *ap-Gal4* was used to drive *UAS-GFP* alone (A, n=3) and in combination with *UAS-tkv<sup>QD</sup>* (A', n=1). **A''.** Expression of *dpp<sup>p10638</sup>lacZ* was significantly reduced in the dorsal compartment with *tkv<sup>QD</sup>* expression. **B.** *MS1096-Gal4* was used to drive *UAS-GFP* alone (B, n=3) and in combination with *UAS-tkv<sup>QD</sup>* (B', n=2). **B''.** The presence of *tkv<sup>QD</sup>* leads to a substantial reduction in expression of *dpp<sup>p10638</sup>lacZ*. **C.** *omb-Gal4* was used to drive *UAS-GFP* alone (C, n=1) and in combination with *UAS-tkv<sup>QD</sup>* (C', n=3). **C''.** *dpp<sup>p10638</sup>lacZ* expression was decreased with *tkv<sup>QD</sup>* over-expression. **D.** Expression of *dpp<sup>BS3.0</sup>lacZ* was examined in *ap-Gal4* (D) and *ap>tkv<sup>QD</sup>* (D') wing pouches, revealing an almost complete loss of *dpp<sup>BS3.0</sup>lacZ* in response to *tkv<sup>QD</sup>* expression. **E.** Expression of *dpp<sup>BS3.0</sup>lacZ* was observed in *MS1096-Gal4* (E) and *MS1096>tkv<sup>QD</sup>* (E') wing pouches. *tkv<sup>QD</sup>* expression leads to strong repression of *dpp<sup>BS3.0</sup>lacZ* throughout most of the wing pouch of *MS1096>tkv<sup>QD</sup>* animals.

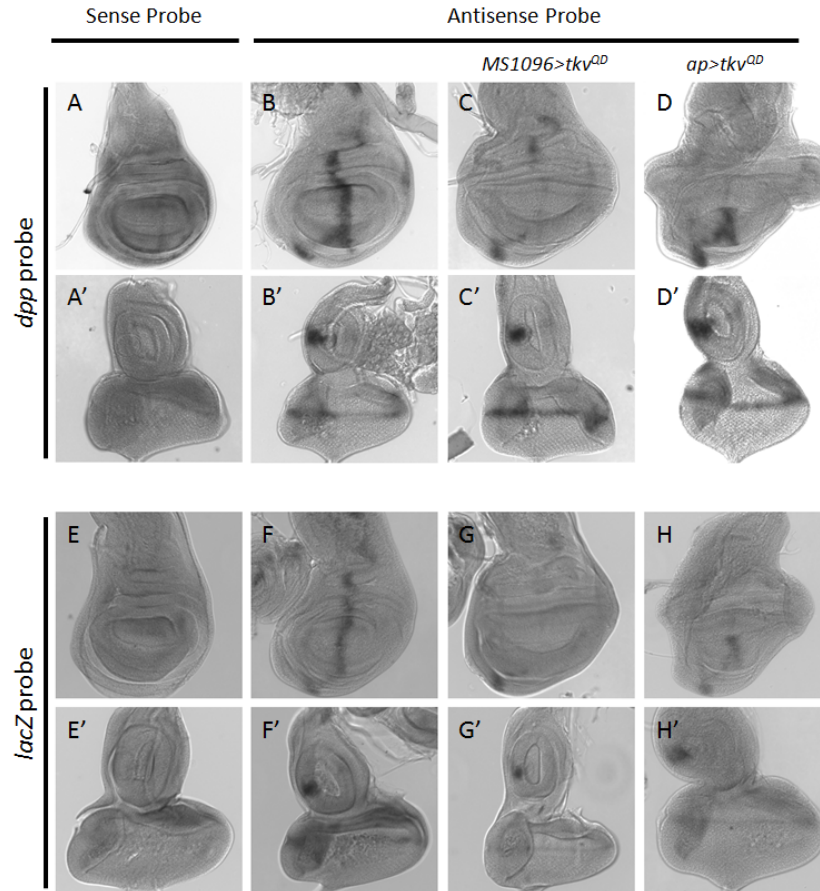
Consistent with repression of *dpp* in response to increased BMP signaling, over-expression of

*tkv<sup>QD</sup>* resulted in reduction in expression of both *dpp<sup>p10638</sup>lacZ* and *dpp<sup>BS3.0</sup>lacZ* reporters. In all genotypes

tested, expression of  $dpp^{p10638}lacZ$  is reduced in all or some of its expression domain in response to  $tkv^{QD}$ , but is not entirely lost. When  $UAS-tkv^{QD}$  was expressed specifically in the dorsal compartment of the wing disc by  $ap-Gal4$ , expression of  $dpp^{p10638}lacZ$  was strongly and specifically reduced in the dorsal wing pouch (Fig 2.2A).  $MS1096-Gal4$  and  $omb-Gal4$  driving  $UAS-tkv^{QD}$  gave similar results, with  $dpp^{p10638}lacZ$  levels reduced throughout its expression domain (Fig 2.2B,C). These results are not reporter-specific, as a similar effect is seen on expression of the reporter  $dpp^{BS3.0}lacZ$  in  $ap>tkv^{QD}$  and  $MS1096>tkv^{QD}$  wing discs (Fig 2.2D,E). In the case of  $dpp^{BS3.0}lacZ$ , however, expression of the reporter is almost completely lost in response to increased BMP signaling.

To confirm that the transcriptional responses of  $dpp^{p10638}lacZ$  and  $dpp^{BS3.0}lacZ$  to increased BMP signaling recapitulate the behavior of  $dpp$  RNA, we performed in situ hybridizations to directly observe the transcriptional response of  $dpp$  to increased BMP signaling. Over-expression of  $tkv^{QD}$  by  $MS1096-Gal4$  and  $ap-Gal4$  was used to increase BMP signaling levels. As a positive control for repression of  $dpp-lacZ$ , we performed  $lacZ$  in situ hybridizations on tissue from animals containing the  $dpp^{p10638}lacZ$  enhancer trap. As a positive control for hybridization efficiency, we performed  $dpp$  and  $lacZ$  in situ hybridizations on eye-antennae imaginal discs, as this tissue does not express either  $Gal4$  drivers used in these experiments and should exhibit normal expression of  $dpp$  and  $dpp^{p10638}lacZ$ . Both the  $dpp$  and  $lacZ$  anti-sense probes were highly specific and hybridized to control discs in the expected domains: along the anterior-posterior boundary of the wing disc, within the morphogenetic furrow of the eye disc, and in a ventral wedge of the antennal disc (Fig 2.3B, F). The  $dpp$  probe consistently led to higher signal than the  $lacZ$  probe, reflecting differences in RNA levels or probe efficiencies. Hybridization of  $dpp$  and  $lacZ$  sense probes, a negative control, display very little background hybridization and activity of the detection reagents (Fig 2.3A,E).

Over-expression of  $tkv^{QD}$  by  $MS1096-Gal4$  strongly reduced both  $dpp$  and  $lacZ$  RNA levels throughout the wing pouch (Fig 2.3C,G). Weak staining is still visible in the hinge region where the  $MS1096-Gal4$  driver is not expressed. When  $ap-Gal4$  is used to express  $tkv^{QD}$ ,  $dpp$  and  $lacZ$  RNA levels are decreased specifically in the dorsal compartment of the wing disc, the region where  $ap-Gal4$  is expressed (Fig 2.3D,H). With either  $Gal4$  driver used, levels of  $dpp$  and  $lacZ$  RNA are unaffected in eye-antennal discs, indicating that hybridization was efficient (Fig 2.3C',D',G',H').



**Figure 2.3. Expression of  $tkv^{QD}$  causes a reduction in  $dpp$  mRNA levels in the wing imaginal disc.** RNA in situ hybridization was performed against  $dpp$  RNA to detect endogenous  $dpp$  RNA (A-D) and  $lacZ$  RNA to detect  $dpp^{p10638}lacZ$  RNA (E-H) in animals with endogenous BMP signaling and with expression of  $UAS-tkv^{QD}$  driven by  $MS1096-Gal4$  (C, G) and  $ap-Gal4$  (D, H). Hybridization of probes to eye-antennal discs was performed as a positive control as  $dpp$  and  $dpp^{p10638}lacZ$  are expressed in this tissue but  $MS1096-Gal4$  and  $ap-Gal4$  are not. Levels of  $dpp$  and  $lacZ$  RNA are reduced in wing discs with over-expression of  $UAS-tkv^{QD}$  but not in eye-antennal discs. **A.** Hybridization of  $dpp$  sense probe in  $UAS-tkv^{QD}/+$  animals as a negative control. **B-D.** Hybridization of anti-sense  $dpp$  RNA probe in  $UAS-tkv^{QD}/+$  (B),  $MS1096-Gal4/+; UAS-tkv^{QD}/+$  (C), and  $ap-Gal4/+; UAS-tkv^{QD}/+$  (D) animals. **B.** Wildtype expression of  $dpp$  along the anterior-posterior boundary of the wing disc, in the morphogenetic furrow of the eye disc, and in a ventral wedge of the antennal disc. **C.** In the wing disc, but not the eye-antennal disc, of  $MS1096>tkv^{QD}$  animals,  $dpp$  RNA levels are strongly reduced in the wing pouch but remain at normal levels in the hinge, consistent with the expression pattern of  $MS1096-Gal4$ . **D.**  $dpp$  RNA levels are reduced in the dorsal compartment of  $ap>tkv^{QD}$  wing discs but not eye-antennal discs, as expected based on the expression pattern of  $ap-Gal4$ . **E.** Hybridization with sense  $lacZ$  probe in  $dpp^{p10638}lacZ/+; UAS-tkv^{QD}/+$  animals as a negative control. **F-H** Hybridization with antisense  $lacZ$  probe in  $dpp^{p10638}lacZ/+; UAS-tkv^{QD}/+$  (F),  $MS1096-Gal4/+; dpp^{p10638}lacZ/+; UAS-tkv^{QD}/+$  (G), and  $dpp^{p10638}lacZ/ap-Gal4; UAS-tkv^{QD}/+$  (H) animals. **F.**  $lacZ$  RNA from the  $dpp^{p10638}lacZ$  reporter construct is expressed in a pattern consistent with endogenous  $dpp$ . **G.**  $MS1096>tkv^{QD}$  animals with  $dpp^{p10638}lacZ$  display a reduction of  $lacZ$  RNA in the wing pouch but not the eye-antennal disc. **H.**  $ap>tkv^{QD}$  animals with  $dpp^{p10638}lacZ$  have reduced  $lacZ$  RNA in the dorsal compartment of the wing disc.

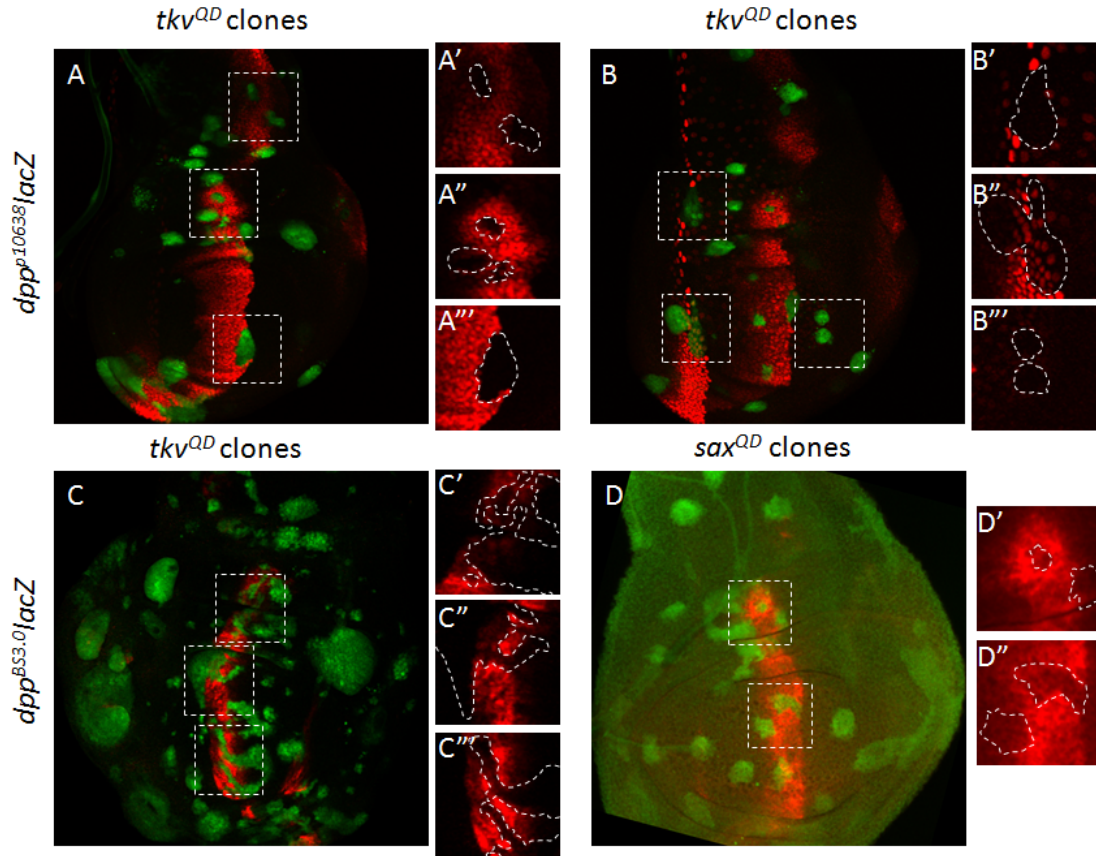
These results are consistent with previous results on expression of  $dpp^{p10638}lacZ$  and  $dpp^{BS3.0}lacZ$  in  $MS1096>tkv^{QD}$  and  $ap>tkv^{QD}$  disc tissue (Fig 2.2), indicating that  $dpp^{BS3.0}lacZ$  and  $dpp^{p10638}lacZ$  expression faithfully recapitulate not only the expression pattern of  $dpp$  in the wing imaginal disc, but also

the transcriptional regulation of *dpp* that occurs in response to altered BMP signaling levels. A potential concern of using *dpp-lacZ* reporters as a proxy to examining the RNA itself is that results we observe may be an artifact of the reporter system, such as a change in stability of *lacZ* RNA or  $\beta$ Gal protein instead of a bona fide change in transcription of *dpp*. However, the results of our in situ experiments reveal that the *dpp-lacZ* reporter constructs do, indeed, recapitulate the endogenous behavior of *dpp* RNA and demonstrate that transcription of *dpp* is negatively regulated by BMP signaling.

As the experiments thus far have over-expressed *tkv<sup>QD</sup>* throughout a large region of the wing disc, they did not allow us to ask whether the effect of increased BMP signaling on *dpp* expression is cell-autonomous. A non cell-autonomous effect may indicate that BMP signaling can influence *dpp* expression through the action of a secreted factor. To address the cell-autonomy of negative regulation, we generated “FLP-out” clones (Struhl and Basler, 1993) expressing either *UAS-tkv<sup>QD</sup>* or *UAS-sax<sup>QD</sup>*. Using this method to over-express a factor has the advantage of allowing over-expression to occur in only a subset of cells within the endogenous *dpp* expression domain, leaving the surrounding tissue as an internal control. Clones were induced in second instar larval stage and observed in late third instar larvae.

Within all clones examined, expression of both *dpp<sup>p10638</sup>-lacZ* and *dpp<sup>BS3.0</sup>-lacZ* reporters was strongly repressed in a cell-autonomous fashion (Fig 2.4A-C). Expression of *dpp<sup>p10638</sup>-lacZ* is reduced in clones that fall anywhere within the wing disc, including the wing pouch (Fig 2.4A’”), hinge (Fig 2.4A”), notum (Fig 2.4A’), and peripodium (Fig 2.4B). Interestingly, in contrast to broader over-expression of *UAS-tkv<sup>QD</sup>* driven by *ap-Gal4*, *MS1096-Gal4*, or *omb-Gal4*, over-expression of *tkv<sup>QD</sup>* in FLP-out clones results in near elimination of *dpp<sup>p10638</sup>-lacZ* expression. A similar response was observed for *dpp<sup>BS3.0</sup>-lacZ* expression in *tkv<sup>QD</sup>* clones (Fig 2.4C). We also tested whether over-expression of the activated form of the type I receptor *sax* leads to repression of *dpp<sup>BS3.0</sup>-lacZ* expression (Fig 2.4D). Indeed, clones of cells expressing *sax<sup>QD</sup>* display a cell-autonomous repression of *dpp<sup>BS3.0</sup>-lacZ* expression in both the wing pouch (Fig 2.4D’”) and the hinge (Fig 2.4D’). In every situation tested, expression of activated type I receptor led to strong and cell-autonomous repression of *dpp-lacZ* transcription.





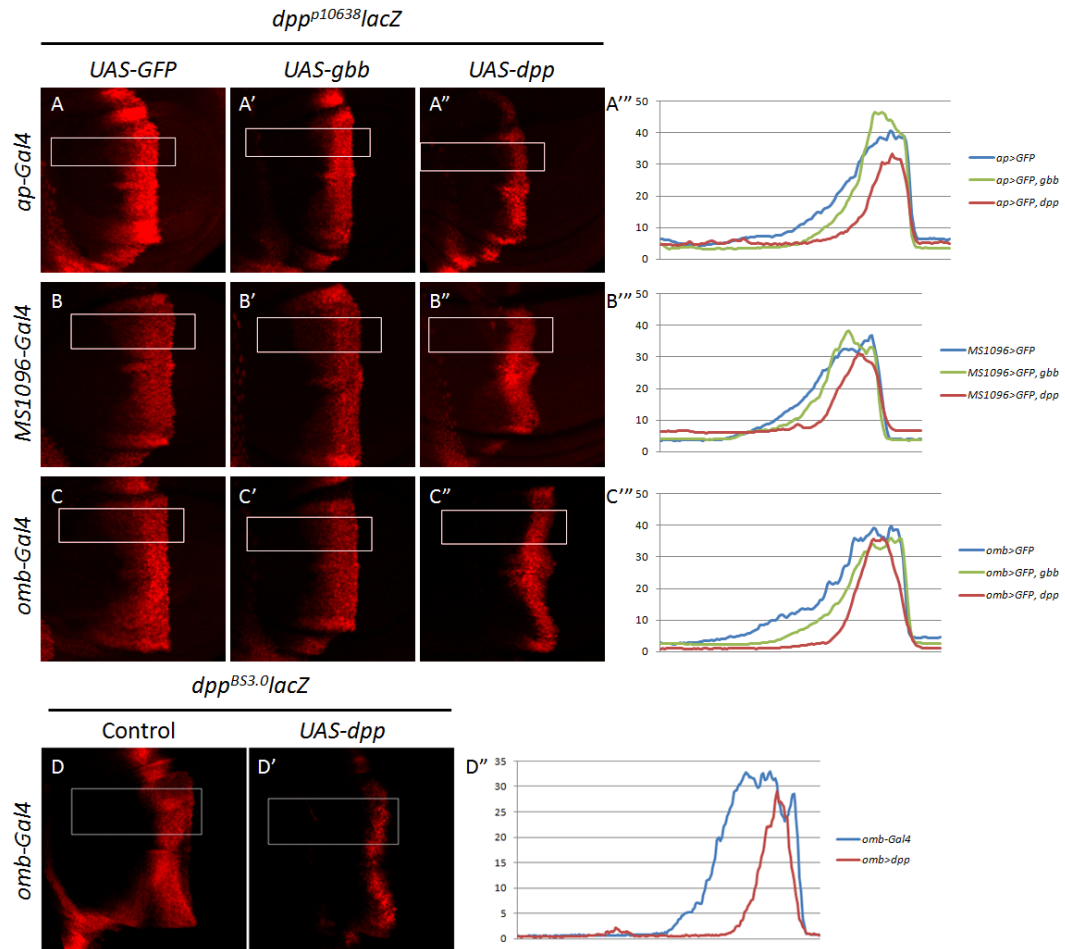
**Figure 2.4. FLP-out clones over-expressing activated Type I Receptors, *tkv<sup>QD</sup>* and *sax<sup>QD</sup>*, lead to cell autonomous repression of *dpp-lacZ* expression.** FLP-out clones were generated during first larval instar to over-express either *tkv<sup>QD</sup>* (A-C) or *sax<sup>QD</sup>* (D) and *dpp-lacZ* expression was examined in third larval instar. GFP in green marks clone location and *dpp-lacZ* is shown in red. We examined the effect of activated receptor on expression of both *dpp<sup>p10638</sup>lacZ* (A-B) and *dpp<sup>BS3.0</sup>lacZ* (C-D). For each, boxed areas in the left image are shown magnified in the right images. **A.** Within the disc proper, *tkv<sup>QD</sup>* over-expression leads to strong cell-autonomous repression of *dpp<sup>p10638</sup>lacZ* in the notum (A'), the hinge (A''), and the wing pouch (A'''). **B.** In the peripodium, all cells show low levels of *dpp<sup>p10638</sup>lacZ* expression except for within a stripe of cells that strongly expresses *dpp<sup>p10638</sup>lacZ*. *tkv<sup>QD</sup>* FLP-out clones throughout the peripodium display a reduction in *dpp<sup>p10638</sup>lacZ*, with the degree of *dpp<sup>p10638</sup>lacZ* expression reduction varying from strong (B' and B''') to moderate (B''), depending on location within the disc. **C.** *tkv<sup>QD</sup>* over-expression leads to strong repression of *dpp<sup>BS3.0</sup>lacZ* expression in the hinge (C') and wing pouch (C'' and C'''). **D.** Over-expression of *sax<sup>QD</sup>* leads to repression of *dpp<sup>BS3.0</sup>lacZ* in both the hinge (D') and wing pouch (D'') in the wing imaginal disc.

#### Changes in BMP ligand levels alters *dpp* transcription in the wing imaginal disc

Given the transcriptional response of *dpp* to expression of activated receptor, we asked if we could observe a similar effect with manipulation of other components of the BMP pathway. We tested whether increased ligand level would influence *dpp* expression, as many of our original observations of *dpp* feedback regulation were recognized in experiments altering *gbb* expression. Using the same methods employed for our *tkv<sup>QD</sup>* experiments, we over-expressed either *gbb* or *dpp* in the wing disc using

the *ap-Gal4*, *MS1096-Gal4*, and *omb-Gal4* drivers and examined the resulting expression of *dpp<sup>p10638</sup>lacZ* and *dpp<sup>BS3.0</sup>lacZ*.

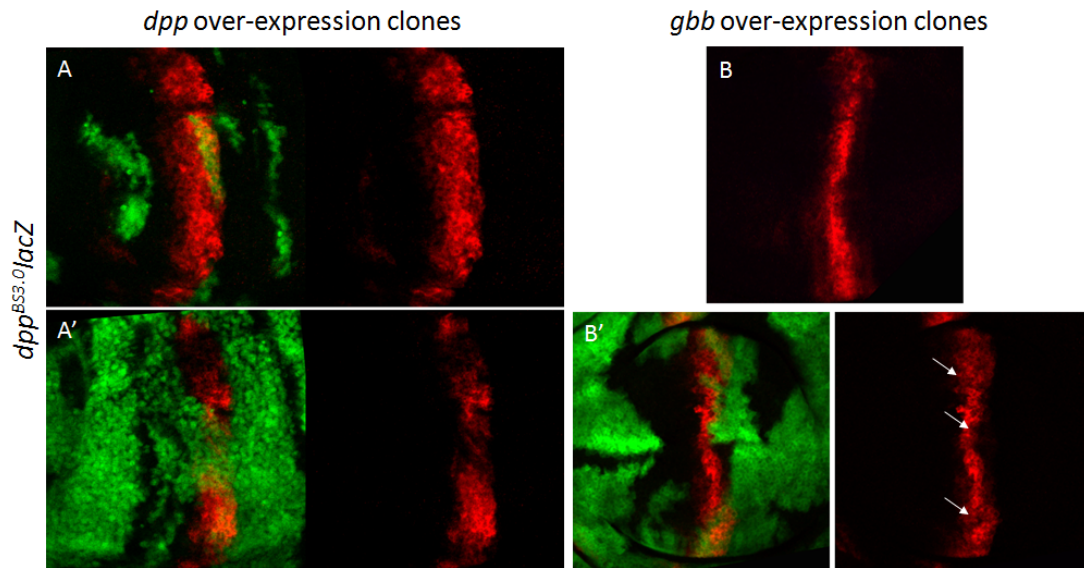
Over-expression of *gbb* caused only a mild reduction, if any, in the expression of *dpp<sup>p10638</sup>lacZ* with all of the *Gal4* drivers used (Fig 2.5A'-C'). Wing discs over-expressing *UAS-gbb* driven by *ap-Gal4* displayed a small reduction of *dpp<sup>p10638</sup>lacZ* levels in a subset of the *dpp<sup>p10638</sup>lacZ* expressing cells farthest



**Figure 2.5. Over-expression of the BMP ligands, *dpp* and *gbb*, have a variable effect on expression of *dpp-lacZ* reporters.** Ligands were over-expressed in the wing disc using various *Gal4* drivers and the resulting expression patterns of *dpp<sup>p10638</sup>lacZ* (A-C) and *dpp<sup>BS3.0</sup>lacZ* (D) were examined. **A.** *ap-Gal4* was used to drive *UAS-GFP* alone (A, n=3) and in combination with *UAS-gbb* (A', n=4) or *UAS-dpp* (A'', n=2). **A'''**. Expression of *dpp<sup>p10638</sup>lacZ* was reduced in a subset of its expression domain in response to *gbb* over-expression but was reduced throughout its expression domain in response to *dpp* over-expression. Despite dorsal-specific expression of *ap-Gal4* (see figure 2.1-A), *dpp* is able to elicit a change in expression of *dpp<sup>p10638</sup>lacZ* in both dorsal and ventral compartments. **B.** *MS1096-Gal4* was used to drive *UAS-GFP* alone (B, n=3) and in combination with *UAS-gbb* (B', n=3) or *UAS-dpp* (B'', n=3). **B'''**. Expression of *dpp<sup>p10638</sup>lacZ* was decreased in some cells in response to *gbb* over-expression and reduced in all cells in response to *dpp* over-expression. **C.** *omb-Gal4* was used to drive *UAS-GFP* alone (C, n=1) and in combination with *UAS-gbb* (C', n=4) or *UAS-dpp* (C'', n=4). **C'''**. *dpp<sup>p10638</sup>lacZ* expression was slightly decreased overall with *gbb* over-expression and repressed in some of its expression domain in response to *dpp* over-expression. **D.** Expression of *dpp<sup>BS3.0</sup>lacZ* was examined in *omb-Gal4* pouches alone (D, n=1) or with *UAS-dpp* (D', n=1). **D'''**. Expression of *dpp* leads to a reduction in expression of *dpp<sup>BS3.0</sup>lacZ*.

from the anterior-posterior boundary. The resulting  $dpp^{p10638}lacZ$  stripe, therefore, appears slightly narrowed compared to  $lacZ$  expression in a wildtype background (Fig 2.5A'). Over-expression of  $UAS-gbb$  controlled by  $omb-Gal4$  also caused a slight reduction in  $dpp^{p10638}lacZ$  expression throughout its expression domain (Fig 2.5B').  $MS1096>gbb$  wing pouches, on the other hand, displayed a  $dpp^{p10638}lacZ$  pattern that was mostly unchanged from control (Fig 2.5C').

Increased  $dpp$  levels had a more significant impact on expression of  $dpp^{p10638}lacZ$  than  $gbb$  did. We observed reduced expression of  $dpp^{p10638}lacZ$  in response to  $dpp$  expression directed by  $ap-Gal4$  (Fig 2.5A''),  $MS1096-Gal4$  (Fig 2.5B''), and  $omb-Gal4$  (Fig 2.5C''). While all  $dpp^{p10638}lacZ$ -expressing cells had reduced levels, some cells showed a stronger repression than others, resulting in a  $lacZ$  expression pattern that appeared much narrower than in control discs. This effect was non cell-autonomous in  $ap>tkv^{OD}$  wing pouches, as we observed a repression in both the dorsal and ventral compartment despite dorsal-specific expression of  $tkv^{OD}$ , likely reflecting movement of Dpp ligand from the dorsal to the ventral compartment (Fig 2.5A''). We also observed repression of  $dpp^{BS3.0}lacZ$  expression in  $omb>dpp$  wing



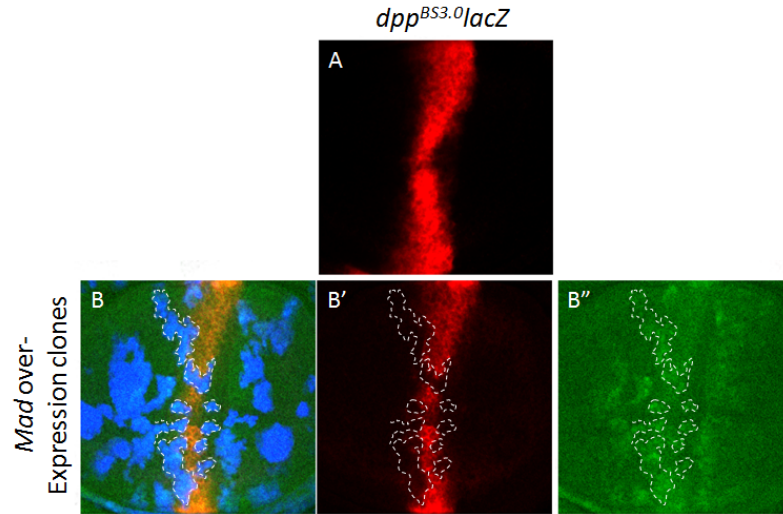
**Figure 2.6. Over-expression of  $dpp$  or  $gbb$  within FLP-out clones has a variable effect on expression of  $dpp^{BS3.0}lacZ$ .** FLP-out clones to over-expressing either  $UAS-dpp$  (A) or  $UAS-gbb$  (B) were generated during first larval instar and  $dpp^{BS3.0}lacZ$  expression was examined in late third larval instar. GFP expression in green marks clone location and  $dpp^{BS3.0}lacZ$  is shown in red. A.  $dpp$  expressing clones have a variable effect on expression of  $dpp^{BS3.0}lacZ$  depending on clone size and location. Smaller clones falling within the  $dpp^{BS3.0}lacZ$  expression domain (A) have no discernable effect on transcription of  $dpp^{BS3.0}lacZ$  while larger clones that cover the width of the  $dpp^{BS3.0}lacZ$  expression domain (A') lead to a cell autonomous decrease in expression of  $dpp^{BS3.0}lacZ$ . B.  $gbb$  over-expression has a more subtle effect on expression of  $dpp^{BS3.0}lacZ$  (expression of which without ligand over-expression is displayed in B). A clone that partially overlaps the  $dpp^{BS3.0}lacZ$  expression domain has a slight effect on  $dpp^{BS3.0}lacZ$  expression (B'). Regions displaying a slight change in  $dpp^{BS3.0}lacZ$  expression are marked by arrows in the second panel in B'.

pouches that resulted in a *dpp*<sup>BS3.0</sup>*lacZ* pattern that appeared narrowed compared to the wildtype control (Fig 2.5D). Of note, *dpp* expression in all three genotypes led to massive over-proliferation along the anterior-posterior axis, resulting in a wide and short wing disc.

We also generated FLP-out clones expressing either *UAS-gbb* or *UAS-dpp* and examined expression of *dpp*<sup>BS3.0</sup>*lacZ*. Clones expressing *dpp* did lead to a reduction in *dpp*<sup>BS3.0</sup>*lacZ* expression, but the response was dependent on clone size. Of the clones that fall within the expression domain of *dpp*<sup>BS3.0</sup>*lacZ*, small clones were not able to elicit a change in expression of *dpp*<sup>BS3.0</sup>*lacZ* (Fig 2.6A), but large clones did (Fig 2.6A'). Reduced *dpp*<sup>BS3.0</sup>*lacZ* expression occurred in a cell-autonomous fashion, curious considering that *dpp* is secreted can influence the surrounding cells (Nellen et al., 1996). *Gbb* FLP-out clones, on the other hand, had a more subtle effect on *dpp*<sup>BS3.0</sup>*lacZ* expression, with only a slight reduction occurring in the cells expressing *gbb* (Fig 2.6B', arrows mark areas of reduced *lacZ* staining).

#### **Changes in levels of the BMP transducer, Mad, alter *dpp* transcription in the wing imaginal disc**

Expression of *dpp* is modulated in response to both BMP ligand and receptor, suggesting that regulation of *dpp* is occurring through canonical BMP signaling. To rule out potential non-canonical signaling events through these factors, we asked whether regulation of *dpp* was Mad-specific by examining expression of *dpp*<sup>BS3.0</sup>*lacZ* in the presence of increased *Mad* levels. To test this, *Mad* was over-expressed by generating FLP-out clones during second instar and expression of *dpp*<sup>BS3.0</sup>*lacZ* was examined. Within these clones, there is a cell-autonomous decrease in expression of *dpp*<sup>BS3.0</sup>*lacZ*, indicating that an increase in *Mad* levels can repress expression of *dpp* (Fig 2.7B'). As *dpp* responds to *Mad* levels, this result supports regulation of *dpp* through canonical BMP signaling. In addition, since this process is *Mad*-mediated, these results highly suggest that the observed negative regulation of *dpp* is a component of a negative feedback loop through which *dpp* expression is regulated. Interestingly, over-expression of *Mad* leads to an increase in the amount of p*Mad* in these clones, suggesting either that *Mad* is limiting in endogenous BMP signaling in these cells or that increased *Mad* expression can drive ligand-independent signal transduction (Fig 2.7B'').



**Figure 2.7. Increased *Mad* levels leads to a cell-autonomous reduction in *dpp<sup>BS3.0</sup>lacZ* expression.** FLP-out clones over-expressing *Mad* were generated during first larval instar and the effect on pMad levels and *dpp<sup>BS3.0</sup>lacZ* expression was examined in late third larval instar. Clones expressing *GFP* are shown in blue, *dpp<sup>BS3.0</sup>lacZ* is shown in red, and pMad is shown in green. **A.** Expression of *dpp<sup>BS3.0</sup>lacZ* in a wildtype control wing pouch without clones. **B.** *Mad* over-expression clones overlapping the *dpp<sup>BS3.0</sup>lacZ* expression domain, outlined in white, display a cell-autonomous decrease in expression of *dpp<sup>BS3.0</sup>lacZ* (**B'**). These clones also display a cell-autonomous increase in pMad levels (**B''**).

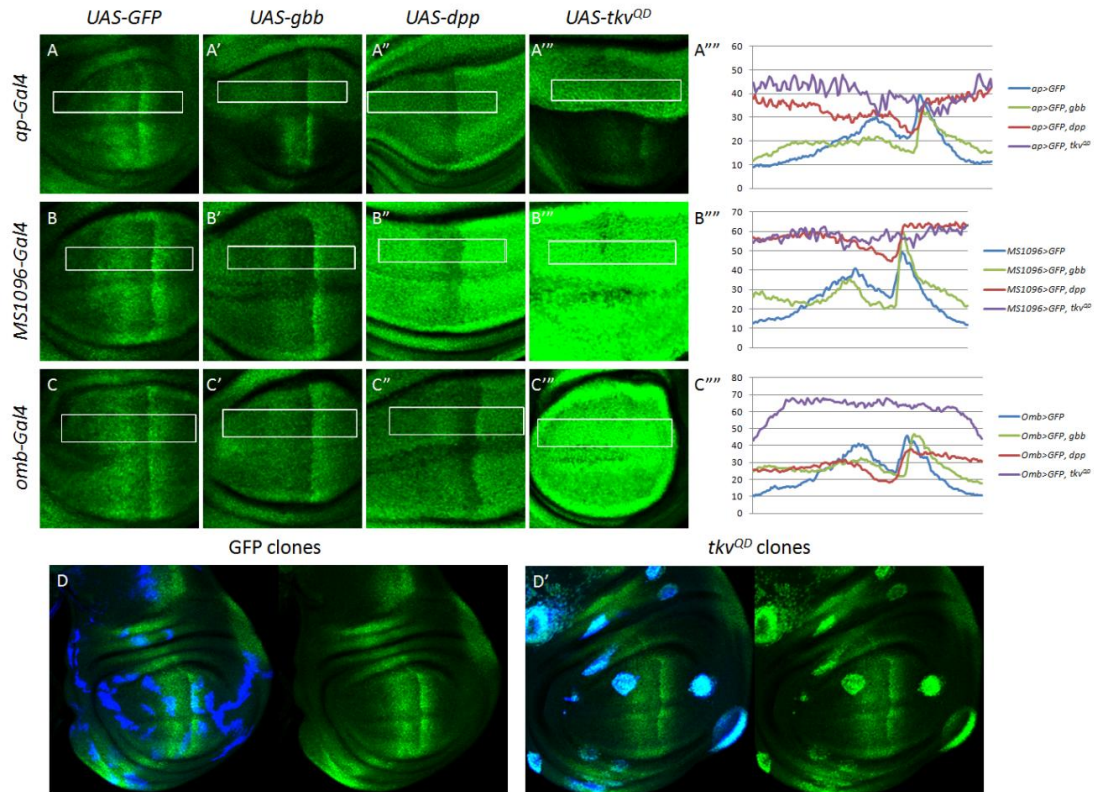
#### The degree of *dpp-lacZ* repression correlates with elevated pMad levels.

Independent of the *Gal4* driver used to drive expression, we consistently observed that over-expression of *tkv<sup>QD</sup>* resulted in a strong reduction in *dpp-lacZ* expression, over-expression of *dpp* gave a moderate reduction in *dpp-lacZ* expression, and over-expression of *gbb* led to little or no change in *dpp-lacZ* expression. We considered why there may be such a variable response depending on which component of the BMP signaling pathway was manipulated. Our lab has previously observed through S2 cell culture BMP signaling assays that *tkv<sup>QD</sup>* is a powerful activator of BMP signaling activity and that Dpp is far more potent in eliciting a signaling response than Gbb (data not shown, Takuya Akiyama, personal communication). Based on this, we hypothesized that the different responses in *dpp-lacZ* expression are due to the ability of the different BMP pathway components to influence the pMad gradient. We expected that the observed strengths of *dpp-lacZ* negative regulation would correlate with the level of pMad that each component of the pathway is able to generate.

To test this, we examined pMad levels in wing pouches with *UAS-GFP* and either *UAS-gbb*, *UAS-dpp*, or *UAS-tkv<sup>QD</sup>* expressed under the control of *ap-Gal4*, *MS1096-Gal4*, and *omb-Gal4*. We expected



that, independent of the *Gal4* driver used, over-expression of *tkv<sup>QD</sup>* would cause a strong increase in pMad levels, *dpp* a moderate increase in pMad levels, and *gbb* a weak increase in pMad levels. These experiments were performed in parallel with the *dpp<sup>p10638</sup>lacZ* experiments described in Fig 2.2 and Fig 2.5, so we can make a direct comparison between *dpp<sup>p10638</sup>lacZ* expression and pMad levels. For each *Gal4* driver, expression of *UAS-GFP* alone was used as a control and all images were taken at the same confocal settings to allow direct comparison between samples (Fig 2.8).



**Figure 2.8. Over-expression of *gbb*, *dpp*, or *tkv<sup>QD</sup>* has a variable effect on BMP signaling levels (pMad) in the wing pouch.** A-C. The BMP pathway components *gbb*, *dpp*, and *tkv<sup>QD</sup>* were over-expressed in the wing disc using various *Gal4* drivers and pMad levels were examined. Shown in green is pMad within wing pouches. **A.** *ap-Gal4* was used to drive *UAS-GFP* alone (A, n=3) and in combination with *UAS-gbb* (A', n=4), *UAS-dpp* (A'', n=2), or *UAS-tkv<sup>QD</sup>* (A''', n=1). **B.** *MS1096-Gal4* was used to drive *UAS-GFP* alone (B, n=3) and in combination with *UAS-gbb* (B', n=3), *UAS-dpp* (B'', n=3), or *UAS-tkv<sup>QD</sup>* (B''', n=2). **C.** *omb-Gal4* was used to drive *UAS-GFP* alone (C, n=2) and in combination with *UAS-gbb* (C', n=4), *UAS-dpp* (C'', n=4), or *UAS-tkv<sup>QD</sup>* (C''', n=3). **A'''-C'''**. Intensity profiles reveal that, in general, over-expression of *gbb* has only a mild effect on pMad levels. The posterior peak remains at the same level as control or slightly higher (in the case of *MS1096>gbb*, B') while the anterior peak is slightly decreased. pMad is increased in the lateral regions of the wing pouch. Over-expression of *dpp* results in fairly uniform levels of pMad in the anterior and posterior compartment with a trough along the anterior-posterior axis of the wing disc. Levels are elevated above the highest wildtype intensities seen in *ap>dpp* (A'') and *MS1096>dpp* (B'') animals, but not in *omb>dpp* (C'') animals, where pMad does not exceed the intensity seen in wildtype peaks. Over-expression of *tkv<sup>QD</sup>* results in elevated pMad in all genotypes examined. **D.** FLP-out clones were generated during first larval instar to over-express *tkv<sup>QD</sup>* and pMad was examined in late third larval instar. GFP in blue marks clone location and pMad is shown in green. Control clones expressing *UAS-GFP* alone display a wildtype pMad gradient (D). Clones over-expressing both *UAS-GFP* and *UAS-tkv<sup>QD</sup>* display a cell autonomous increase in pMad staining higher than endogenous levels

In our control wing discs pMad is observed in its characteristic wildtype distribution with a high posterior peak and a moderate anterior peak, between which is the trough of lower pMad levels. On either side of the peaks, pMad is graded towards the lateral regions of the wing disc (Fig 2.8A,B,C). As *dpp* expression is highest along the anterior-posterior boundary, we were particularly interested in the effect that over-expression of each component would have on pMad levels in the trough.

As expected, over-expression of *tkv<sup>QD</sup>* by all three *Gal4* drivers consistently led to a strong increase in pMad, causing an increase in pMad at or above the highest wildtype peaks (Fig 2.8A'''-C'''). The increase in pMad was cell-autonomous and occurred throughout the expression domain of each *Gal4* driver, including within the trough. A cell-autonomous increase in pMad is observed in *tkv<sup>QD</sup>* FLP-out clones as well (Fig 2.8D). This correlates with the strong repression of *dpp<sup>p10638</sup>lacZ* expression that was observed for these genotypes (Fig 2.2, 2.4).

In general, *dpp* over-expression had the second largest impact on pMad levels, leading to a uniform distribution of pMad in both anterior and posterior compartments while still maintaining the trough along the anterior-posterior boundary of the wing pouch (Fig 2.8A''-C''). In *ap>dpp* wing pouches, levels of pMad in the trough are slightly elevated compared to wildtype and the intensity of staining in the anterior and posterior compartments reaches the wildtype anterior and posterior peaks, respectively (Fig 2.8A''). *dpp* has a non cell-autonomous effect on pMad in this genotype; pMad is increased in both the dorsal and the ventral compartments despite being expressed exclusively in the dorsal compartment, consistent with the non cell-autonomous repression of *dpp<sup>p10638</sup>lacZ* that was observed for this genotype (Fig 2.5A''). Over-expression of *dpp* by *MS1096-Gal4* also results in a uniform distribution of pMad in the anterior and posterior compartments, but the overall increase in pMad is more dramatic than with *ap>dpp* (Fig 2.8B''). This is consistent with the reduction in expression of *dpp<sup>p10638</sup>lacZ* throughout its expression domain that we had observed (Fig 2.5A'',B''). The pMad gradient in *omb>dpp* wing discs is, again, uniform in the anterior and posterior compartments but the level of pMad is lower than in *ap>dpp* or *MS1096>dpp* wing discs. Furthermore, pMad in the trough is actually decreased compared to control (Fig 2.8C''), consistent with the *dpp<sup>p10638</sup>lacZ* expression observed in this genotype, where it was only repressed in regions farther from the anterior-posterior boundary (Fig 2.5C'').

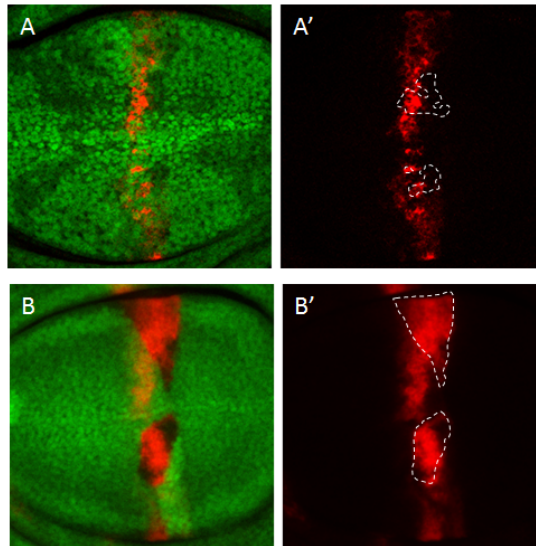
Finally, as we expected, over-expression of *gbb* has the mildest effect on pMad levels. In all three genotypes, pMad was increased in the lateral regions of the wing pouch in response to increased *gbb* but remained close to wildtype levels in the peaks and in the trough (Fig 2.8A'-C'). The anterior pMad peak and the trough in *ap>gbb* discs was slightly reduced but the posterior peak was unchanged compared to wildtype. Interestingly, unlike over-expression of *dpp* by *ap-Gal4*, only a cell-autonomous increase in pMad was observed in *ap>gbb* discs (Fig 2.8A'). In *MS1096>gbb* wing pouches, levels of pMad in the posterior peak were slightly elevated and levels in the anterior peak and trough were slightly decreased compared to wildtype (Fig 2.8B'). Expression of *gbb* controlled by *omb-Gal4* created a pMad profile that was similar to wildtype in all regions except for the lateral anterior portion where pMad was increased (Fig 2.8C'). These results correlate with our *dpp<sup>p10638</sup>lacZ* observations, where levels remained close to wildtype levels (Fig 2.5).

Based on these results, we conclude that over-expression of either *gbb*, *dpp*, or *tkv<sup>QD</sup>* leads to distinct changes in pMad. The degree of pMad change correlates with the degree to which *dpp* is repressed in each genotype, with *gbb* over-expression leading to the weakest effect on both pMad and *dpp-lacZ*, *dpp* over-expression causing moderate increase in pMad and a moderate repression of *dpp-lacZ* expression, and *tkv<sup>QD</sup>* expression causing a striking increase in pMad and a strong reduction in *dpp-lacZ* expression. The inverse relationship between pMad levels and *dpp<sup>p10638</sup>lacZ* expression suggests that regulation of *dpp* may respond in a dose-dependent manner to pMad activity.

#### ***dpp-lacZ* expression is up-regulated in response to decreased BMP signaling levels**

Our observations thus far indicate that increased BMP signaling leads to a repression of *dpp* expression and suggest that this repression may be a component of a negative feedback loop. A feedback loop should be able to sense increased signaling levels and compensate by reducing signaling capacity. The other hallmark of an effective negative feedback loop is the ability to respond to decreased activity levels and accommodate by increasing signaling output. As such, we hypothesized that decreased BMP signaling levels would lead to an increase in expression of *dpp-lacZ*.





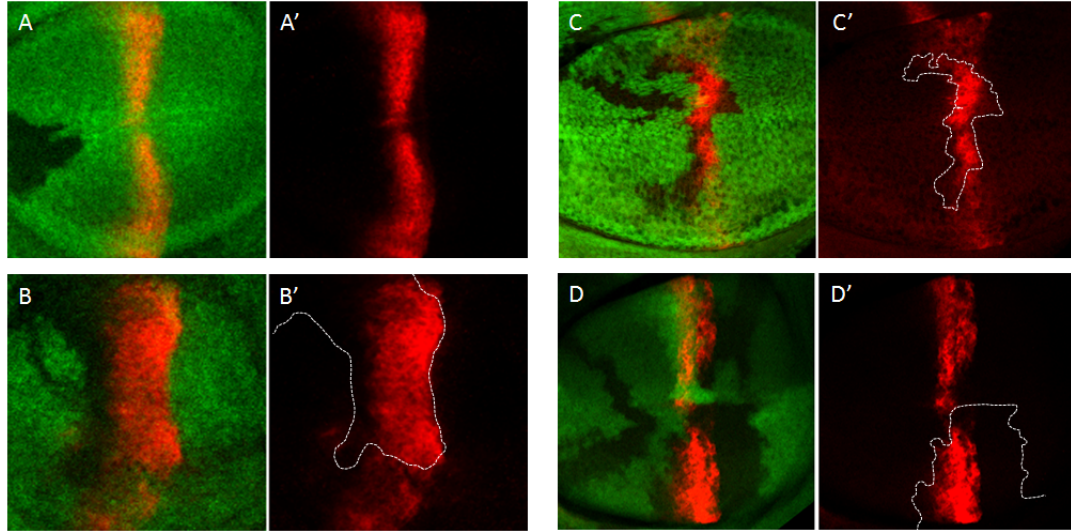
**Figure 2.9. Loss of BMP Type I receptor function leads to cell autonomous increase in  $dpp^{BS3.0}lacZ$  expression.** Clones were generated during first instar and examined in wandering third instar larvae. Clones are marked by the absence of GFP (in green) and are outlined in white. Shown in red is the expression of  $dpp^{BS3.0}lacZ$ . **A.** Clones of cells homozygous for a hypomorphic allele of *tkv*, *tkv<sup>a12</sup>*, are marked by the absence of GFP.  $dpp^{BS3.0}lacZ$  displays a cell-autonomous increase in expression with loss of *tkv*. **B.** Clones of cells homozygous for *sax<sup>4</sup>*, a null allele of *sax*, display a cell-autonomous increase in  $dpp^{BS3.0}lacZ$  expression.

increase in expression of  $dpp^{BS3.0}lacZ$  compared to the surrounding tissue (Fig 2.9A). Similar clones of cells containing two copies of a null allele of *sax*, *sax<sup>4</sup>*, also display an increase in  $dpp^{BS3.0}lacZ$  expression (Fig 2.9B). Importantly, up-regulation of  $dpp^{BS3.0}lacZ$  is only observed in clones that fall within the endogenous expression domain of the reporter. Ectopic expression of *dpp* outside of its stripe of expression was never seen in these experiments, indicating that a loss of BMP signaling is not sufficient to induce ectopic expression of *dpp*.

We next asked whether reducing ligand levels can influence the expression of  $dpp^{BS3.0}lacZ$ . As *dpp* is absolutely required for cell survival (Moreno et al., 2002; Posakony et al., 1991; Ziv et al., 2009), we, instead, generated *gbb* null clones. We generated *gbb<sup>2</sup>* loss-of-function clones during the first and second larval instars and measured the resulting expression of  $dpp^{BS3.0}lacZ$  (Fig 2.10). Indeed, cells that are null for *gbb* display an up-regulation in the expression of  $dpp^{BS3.0}lacZ$  regardless of clone size, with both small (Fig 2.10B) and large (Fig 2.10C,D) clones displaying increased  $dpp^{BS3.0}lacZ$  expression. In addition, some large clones that extend into the anterior compartment appeared to display a slight expansion of the

To test this, we generated clones of cells null for components of the BMP signaling pathway and compared the expression pattern of  $dpp^{BS3.0}lacZ$  in the tissue within and surrounding the null clone. We first reduced levels of the type I receptors, *tkv* and *sax*. *Tkv* is required for survival and cells null for *tkv* are difficult to recover as they extrude from the membrane (Burke and Basler, 1996; Gibson, 2005; Shen, 2005), but we were able to create small clones of cells homozygous for the *tkv* hypomorphic allele, *tkv<sup>a12</sup>*. Within these clones, we observed a cell-autonomous

expression domain of *dpp*<sup>BS3.0</sup>*lacZ* into the clone (Fig 2.10B,D). As with *tkv* and *sax* loss-of-function clones, ectopic *dpp*<sup>BS3.0</sup>*lacZ* up-regulation was never seen in clones that are non-overlapping with the endogenous expression domain of *dpp*.



**Figure 2.10. *gbb* loss-of-function clones show an increase in expression of *dpp*<sup>BS3.0</sup>*lacZ*.** *gbb*<sup>2</sup> loss-of-function clones were generated during first instar and observed in late third instar larvae. Clones are marked by the absence of GFP (in green) and are outlined in white. Shown in red is the expression of *dpp*<sup>BS3.0</sup>*lacZ*. **A.** Control pouch with no clones crossing the domain of endogenous *dpp*<sup>BS3.0</sup>*lacZ* expression. Both large clones (**B, D**) and small clones (**C**) overlapping the *dpp*<sup>BS3.0</sup>*lacZ* expression domain led to a cell autonomous increase in expression of *dpp*<sup>BS3.0</sup>*lacZ*. The clone in **B** that extends into the anterior compartment is associated with a slightly widened *dpp*<sup>BS3.0</sup>*lacZ* expression.

### Feedback regulation of *dpp* occurs only within its endogenous expression domain

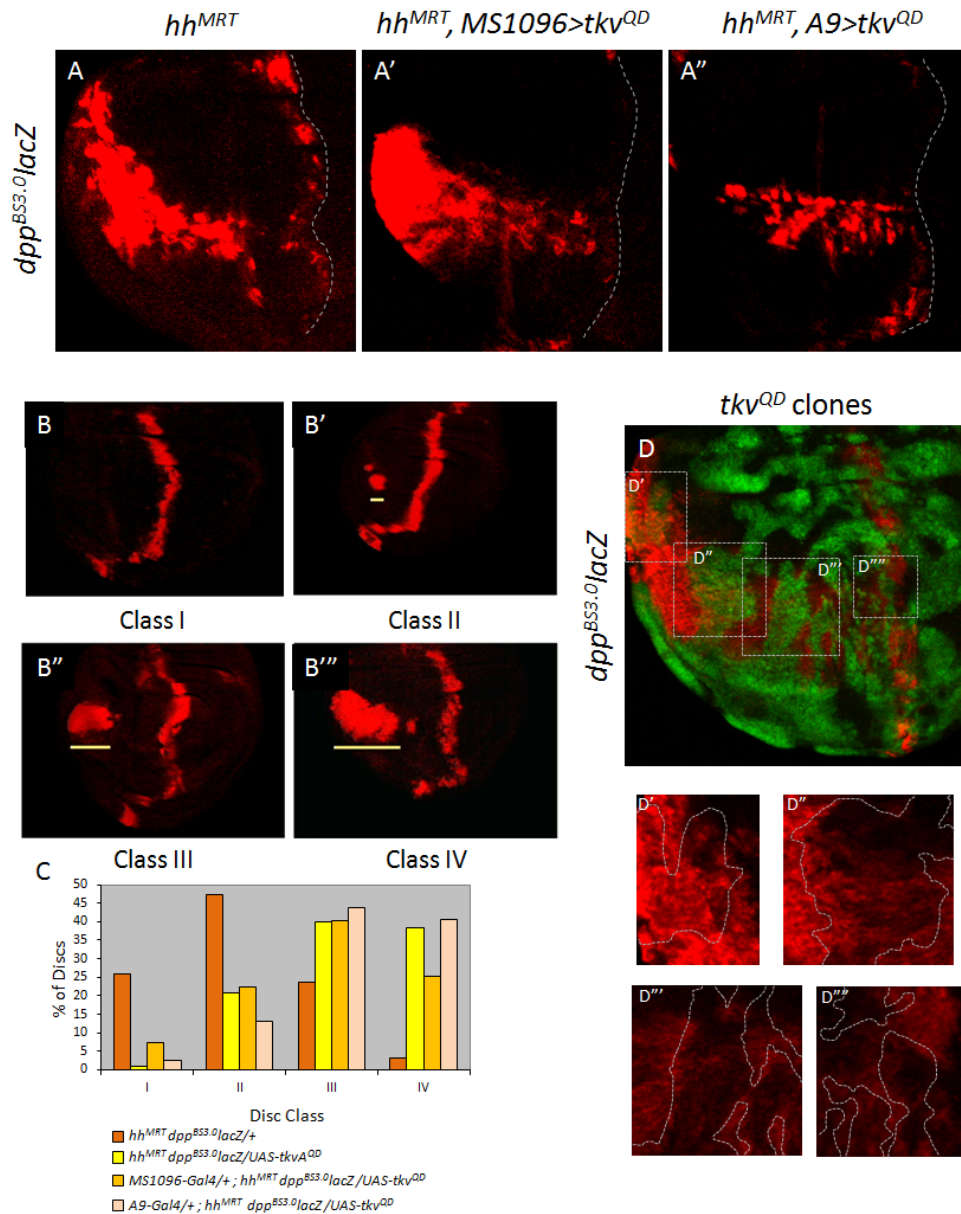
Having demonstrated that altered BMP signaling can influence the expression of endogenous *dpp* in the wing disc, we next used a sensitized background to ask if other domains of the wing disc are competent to negatively regulate *dpp*. To test this, we made use of the dominant gain-of-function *hh* allele, *hh*<sup>MRT</sup>, that leads to ectopic Hh signaling and *dpp* expression in the anterior compartment of the wing disc (Felsenfeld, 1995; Johnson et al., 1995). *hh*<sup>MRT</sup> adult wings display a variety of phenotypes in response to the increased anterior Hh and BMP signaling, including an enlarged anterior wing compartment, loss of central vein pattern, and ectopic vein tissue in the anterior-most region of the wing (see Fig 7.10A).

To test whether increased BMP signaling could repress ectopic *dpp*<sup>BS3.0</sup>*lacZ* throughout the anterior compartment, we over-expressed *tkv*<sup>QD</sup> in the wing disc using *MS1096-Gal4* and *A9-Gal4* and

examined the effect on  $dpp^{BS3.0}lacZ$  expression. *A9-Gal4*, not used in our experiments thus far, is expressed throughout much of the wing pouch, with higher levels in the dorsal than the ventral compartment (for expression pattern, see fig 7.1C). Consistent with previous observations (figure 2.2, 7.2), over-expression of  $tkv^{QD}$  by either *MS1096-Gal4* or *A9-Gal4* resulted in repression of  $dpp^{BS3.0}lacZ$  expression in the endogenous expression domain of *dpp* along the anterior-posterior compartment boundary (Fig 2.11A,A',A"). However, repression of ectopic  $dpp^{BS3.0}lacZ$  expression in the anterior compartment was never observed.

Wing discs derived from  $hh^{MRT}$  animals display variable levels of ectopic anterior *lacZ* expression, designed classes I to IV from least to most ectopic expression of *lacZ* (Fig 2.11B). To better understand the effect of increased BMP signaling specifically on the ectopic expression of  $dpp^{BS3.0}lacZ$ , we determined the distribution of ectopic expression phenotypes based on our classification system. As controls, we analyzed  $dpp^{BS3.0}lacZ$  expression in both  $hh^{MRT} dpp^{BS3.0}lacZ/+$  and  $hh^{MRT} dpp^{BS3.0}lacZ/UAS-tkv^{QD}$  backgrounds (Fig 2.11C). Surprisingly, the addition of the *UAS-tkv^{QD}*-containing chromosome alone created a significant shift from fairly mild phenotypes to more severe phenotypes ( $p < 0.001$ ). The reason for this shift is unclear, but it may reflect a consequence of "leaky" expression of the transgene, the particular insertion site of *UAS-tkv^{QD}*, or of the particular recombination event that occurred to generate the  $hh^{MRT} dpp^{BS3.0}lacZ$  chromosome. Several observations in our lab have led us to speculate that this construct may express at low levels in a *Gal4*-independent manner (data not shown). If, in fact, this construct is leaky then this implies that increased BMP signaling may actually increase  $dpp^{BS3.0}lacZ$  expression in the anterior compartment. Examining pMad staining between *UAS-tkv^{QD}* and wildtype backgrounds would address this concern. We could test the possibility that the observed effect is a consequence of *UAS-tkv^{QD}* insertion or of the recombination event that created the  $hh^{MRT} dpp^{BS3.0}lacZ$  chromosome, by examining the effect of a *UAS-tkv^{QD}* transgene inserted at different chromosomal location or of a different  $hh^{MRT} dpp^{BS3.0}lacZ$  recombinant.

We next determined the phenotypic distributions for both *MS1096>tkv^{QD}* and *A9>tkv^{QD}* animals in a  $hh^{MRT} dpp^{BS3.0}lacZ/+$  background (Fig 2.11C). Considering the discrepancy observed for our two control experiments, we chose to use the  $hh^{MRT} dpp^{BS3.0}lacZ/UAS-tkv^{QD}$  data as a control for our over-expression



trials. While the effect of  $tkv^{QD}$  expression is not drastic, there is a statistically significant change in comparison to the distribution of  $hh^{MRT} dpp^{BS3.0} lacZ/UAS-tkv^{QD}$  animals. The distribution of  $MS1096>tkv^{QD}$  animals is highly significantly different from the control ( $p<.001$ ), with a shift to less severe phenotypes and the distribution of  $A9>tkv^{QD}$  animals is significantly different from control ( $p<.05$ ), with a slight shift to more severe phenotypes. The different responses between  $MS1096>tkv^{QD}$  and  $A9>tkv^{QD}$  animals may be due to the different expression patterns of these two *Gal4* drivers, with *A9-Gal4* expressed in a more restricted domain than *MS1096-Gal4* (compare Figs 2.1B and 7.1C).

Our results thus far led us to hypothesize that the ability of BMP signaling to influence expression of *dpp* may be spatially restricted and that increased BMP signaling can only lead to a strong repression of  $dpp^{BS3.0} lacZ$  expression within the endogenous *dpp* expression domain. To better characterize the spatial restrictions of *dpp*'s negative feedback regulation, we generated FLP-out clones over-expressing  $tkv^{QD}$  in the  $hh^{MRT}$  background and examined the resulting expression of  $dpp^{BS3.0} lacZ$ . As each clone is independent, we can compare within one disc the response in a clone within the endogenous  $dpp^{BS3.0} lacZ$  expression domain to a clone in the lateral ectopic expression of  $dpp^{BS3.0} lacZ$  to determine how different regions of the wing disc can influence *dpp* expression. Consistent with our hypothesis, the ability of increased BMP signaling to repress expression of  $dpp^{BS3.0} lacZ$  varied depending on its location within the wing disc (Fig 2.11D). Clones located within or just anterior to the endogenous expression domain of  $dpp^{BS3.0} lacZ$  always displayed an almost complete reduction in expression of  $dpp^{BS3.0} lacZ$  (Fig 2.11D', D''') and clones that fell in the lateral region of the pouch could only weakly repress expression of  $dpp^{BS3.0} lacZ$  or could not repress expression of  $dpp^{BS3.0} lacZ$  at all (Fig 2.11D'', D).

These results suggest that increased levels of BMP signaling are not able to repress transcription of *dpp* in all cells of the wing disc. Instead, the ability of BMP signaling to repress expression of *dpp* is limited to the central regions of the wing pouch within or close to the endogenous expression domain of *dpp*. This spatial restriction on negative feedback regulation of *dpp* may be explained by the presence or activity of factors required to mediate transcriptional regulation of *dpp*.

### Lethality associated with compromised *dpp* levels is sensitive to *gbb* copy number

While negative feedback regulation of *dpp* transcription is confined to only certain regions of the wing imaginal disc, we believe that *dpp* is negatively regulated in other tissues as well. We have observed that lethality associated with *dpp* alleles is sensitive to *gbb* copy number in a manner consistent with BMP signaling negatively regulating transcription of *dpp*. An increase in *gbb* copy number exacerbates lethality in *dpp* mutant animals while a reduction in *gbb* copy number partially rescues lethality. These results were initially counterintuitive, but given our understanding of *dpp* regulation in the wing disc, they can now be explained through negative feedback regulation of *dpp* transcription.

Having recently elucidated a dual requirement for both Gbb and Dpp in patterning the wing disc (Bangi and Wharton, 2006; Khalsa et al., 1998; Ray and Wharton, 2001), we had expected that if Gbb and Dpp share other functions in development, an increase in *gbb* copy number may be able to compensate for reduced *dpp* function in other tissues. We tested the ability of increased *gbb* copy number to rescue a reduced viability phenotype seen in combinations of *dpp<sup>hr</sup>* and *dpp<sup>d</sup>* alleles. *dpp<sup>hr</sup>* alleles result from point mutations in the *dpp* protein coding region (Irish and Gelbart, 1987; Spencer et al., 1982; Wharton et al., 1996; Wharton et al., 1993), and *dpp<sup>d</sup>* alleles result from chromosomal rearrangements in *dpp* regulatory

| Genotype                                                                                                   | Viability | N   | Genotype                                                                                                    | Viability | N   |
|------------------------------------------------------------------------------------------------------------|-----------|-----|-------------------------------------------------------------------------------------------------------------|-----------|-----|
| Sp/Sm6a                                                                                                    | 100%      | 397 | Sp/Sm6a                                                                                                     | 100%      | 631 |
| <i>dpp<sup>hr4</sup></i> /Sp                                                                               | 91%       | 361 | <i>dpp<sup>hr56</sup></i> /Sp                                                                               | 67%       | 421 |
| <i>dpp<sup>d5</sup></i> /CyO                                                                               | 84%       | 334 | <i>dpp<sup>d5</sup></i> /CyO                                                                                | 94%       | 593 |
| <i>dpp<sup>hr4</sup></i> / <i>dpp<sup>d5</sup></i>                                                         | 84%       | 297 | <i>dpp<sup>hr56</sup></i> / <i>dpp<sup>d5</sup></i>                                                         | 58%       | 369 |
| <i>dpp<sup>hr4</sup></i> / <i>dpp<sup>d5</sup></i> ; <i>p[gbb<sup>+</sup>]</i> /+                          | 79%       | 112 | <i>dpp<sup>hr56</sup></i> / <i>dpp<sup>d5</sup></i> ; <i>p[gbb<sup>+</sup>]</i> /+                          | 66%       | 215 |
| <i>dpp<sup>hr4</sup></i> / <i>dpp<sup>d5</sup></i> ; <i>p[gbb<sup>+</sup>]</i> / <i>p[gbb<sup>+</sup>]</i> | 0%        | 0   | <i>dpp<sup>hr56</sup></i> / <i>dpp<sup>d5</sup></i> ; <i>p[gbb<sup>+</sup>]</i> / <i>p[gbb<sup>+</sup>]</i> | 19%       | 63  |

**Table 2.1. An increase in *gbb* copy number decreases the viability of *dpp* compromised animals.** Viability of *dpp<sup>hr</sup>* and *dpp<sup>d5</sup>* animals was determined in the presence or absence of one or two exogenous copies of *gbb*. In either a *dpp<sup>hr4</sup>*/*dpp<sup>d5</sup>* or a *dpp<sup>hr56</sup>*/*dpp<sup>d5</sup>* background, introduction of one and two copies of *p[gbb<sup>+</sup>]* leads to a decrease in viability. **Left column:** The effects of increased *gbb* copy number on the viability of *dpp<sup>hr4</sup>*/*dpp<sup>d5</sup>* animals reveals that introduction of one copy of *dpp<sup>hr4</sup>*, *dpp<sup>d5</sup>*, or both leads to a reduction in viability to 91%, 84%, and 84% respectively compared to *Sp/SM6a*. In a separate test cross, *dpp<sup>hr4</sup>*/*dpp<sup>d5</sup>*; *p[gbb<sup>+</sup>]*/+ animals display 79% viability compared to *dpp<sup>d5</sup>*/CyO; *p[gbb<sup>+</sup>]*/+. Viability is reduced to 0% (100% lethality) with the introduction of a second copy of *p[gbb<sup>+</sup>]* in the genotype *dpp<sup>hr4</sup>*/*dpp<sup>d5</sup>*; *p[gbb<sup>+</sup>]*/*p[gbb<sup>+</sup>]*. **Right column:** The effects of increased *gbb* copy number on the viability of *dpp<sup>hr56</sup>*/*dpp<sup>d5</sup>* animals reveals a reduction in viability to 94% in *dpp<sup>d5</sup>*/CyO animals and 67% in *dpp<sup>hr56</sup>*/Sp animals, compared to *Sp/SM6a*. Animals with both alleles, *dpp<sup>hr56</sup>*/*dpp<sup>d5</sup>*, display a further reduction in viability to 58%. Introduction of one copy of *p[gbb<sup>+</sup>]* gives 66% viability in *dpp<sup>hr56</sup>*/*dpp<sup>d5</sup>*; *p[gbb<sup>+</sup>]*/+ animals, relative to *dpp<sup>d5</sup>*/CyO; *p[gbb<sup>+</sup>]*/+ animals. Two copies of *p[gbb<sup>+</sup>]* in *dpp<sup>hr56</sup>*/*dpp<sup>d5</sup>*; *p[gbb<sup>+</sup>]*/*p[gbb<sup>+</sup>]* animals reduces viability to 19%. Due to the difference in genotypes used as an expected value, direct comparisons of viability values between control and test crosses cannot be made, but the general trend can be compared.

sequences that influence imaginal disc expression of *dpp* (Spencer et al., 1982; St Johnston et al., 1990). Surprisingly, we found that addition of one or two additional exogenous copies of *gbb* resulted in a dose-dependent reduction in the viability of both *dpp<sup>hr56</sup>/dpp<sup>d5</sup>* and *dpp<sup>hr4</sup>/dpp<sup>d5</sup>* animals (Table 2.1). These results suggested that an increase in *gbb* levels decreased the ability of *dpp* to function, presumably by decreasing transcription of *dpp*.

Given that an increase in *gbb* copy number enhanced *dpp* lethality, we next asked if a decrease in *gbb* copy number would instead rescue *dpp* mutant lethality. To test this hypothesis, we examined the effect on viability of introducing one null allele of *gbb* to animals with reduced *dpp* function in the following *dpp* allelic combinations: *dpp<sup>hr27</sup>/dpp<sup>d5</sup>*, *dpp<sup>hr4</sup>/dpp<sup>d5</sup>*, *dpp<sup>hr27</sup>/dpp<sup>d6</sup>*, *dpp<sup>hr4</sup>/dpp<sup>d6</sup>*. Furthermore, due to potential paternal effects observed for some *dpp* alleles (Table 7.1, 7.2, and data not shown), we performed reciprocal crosses for all genotypes tested. Indeed, a decrease in *gbb* copy number partially rescued lethality observed in *dpp<sup>hr27</sup>/dpp<sup>d5</sup>* and *dpp<sup>hr4</sup>/dpp<sup>d5</sup>* allelic combinations (Table 2.2). In flies in which the *dpp<sup>d5</sup>* allele was paternal, introduction of one copy of *gbb<sup>1</sup>* increased viability of *dpp<sup>hr27</sup>/dpp<sup>d5</sup>* progeny from 72% to 79% and of *dpp<sup>hr4</sup>/dpp<sup>d5</sup>* progeny from 72% to 82%. When the father instead provided the *dpp<sup>hr</sup>* allele, fewer *dpp<sup>hr</sup>/dpp<sup>d</sup>* progeny survived and a more pronounced rescue was seen with the introduction of one copy of *gbb<sup>1</sup>*, indicating a potential paternal effect of *dpp<sup>hr</sup>* alleles. In these crosses, introduction of one copy of *gbb<sup>1</sup>* increased viability of *dpp<sup>hr27</sup>/dpp<sup>d5</sup>* progeny from 12% to 34% and of *dpp<sup>hr4</sup>/dpp<sup>d5</sup>* progeny from 48% to 69%. We also confirmed that introduction of *gbb<sup>1</sup>* partially rescues the reduced viability observed in *dpp<sup>hr92</sup>/dpp<sup>d5</sup>* and *dpp<sup>hr56</sup>/dpp<sup>d5</sup>* allelic combinations (Table 7.2). Additional trials performed by previous graduate students (Table 7.1) revealed similar trends in viability, however, the absolute numbers varied significantly between trials. Variation in the absolute values could be due to the temperature sensitive nature of some *dpp* alleles (Wharton et al., 1996, personal communication), such that small fluctuations in incubator temperature could lead to large changes in viability. In addition, scoring these animals requires careful analysis of eye color, a technique that is prone to investigator error. Despite these discrepancies, the introduction of one copy of *gbb<sup>1</sup>* consistently rescued viability of *dpp<sup>hr</sup>/dpp<sup>d5</sup>* progeny.



| Genotype                                                | Viability                  | N    | Viability                    | N    |
|---------------------------------------------------------|----------------------------|------|------------------------------|------|
|                                                         | dpp <sup>d</sup> from male |      | dpp <sup>d</sup> from female |      |
| dpp <sup>d5</sup> /dpp <sup>hr27</sup>                  | 72%                        | 363  | 12%                          | 829  |
| dpp <sup>d5</sup> gbb <sup>1</sup> /dpp <sup>hr27</sup> | 79%                        | 883  | 34%                          | 271  |
| dpp <sup>d5</sup> /dpp <sup>hr4</sup>                   | 72%                        | 1184 | 48%                          | 1048 |
| dpp <sup>d5</sup> gbb <sup>1</sup> /dpp <sup>hr4</sup>  | 87%                        | 683  | 69%                          | 821  |
| dpp <sup>d6</sup> /dpp <sup>hr27</sup>                  | 73%                        | 902  | 4%                           | 118  |
| dpp <sup>d6</sup> gbb <sup>1</sup> /dpp <sup>hr27</sup> | 44%                        | 691  | 10%                          | 531  |
| dpp <sup>d6</sup> /dpp <sup>hr4</sup>                   | 68%                        | 834  | 12%                          | 179  |
| dpp <sup>d6</sup> gbb <sup>1</sup> /dpp <sup>hr4</sup>  | 45%                        | 864  | 36%                          | 634  |

**Table 2.2: A decrease in *gbb* copy number partially rescues viability defects in *dpp* compromised animals.**

Shown is a series of viability experiments testing the effect of a reduction in *gbb* copy number, by introduction of one copy of *gbb*<sup>1</sup>, on the viability of *dpp*<sup>hr</sup>/*dpp*<sup>d5</sup> (**top two rows**) and *dpp*<sup>hr</sup>/*dpp*<sup>d6</sup> (**bottom two rows**) animals. For each genotype, the viability is calculated as a percentage of the expected, either *dpp*<sup>d</sup>/*SM6a* or *dpp*<sup>d</sup> *gbb*<sup>1</sup>/*SM6a*. N represents the total number of animals counted for each experiment in all genotypes. Reciprocal crosses were performed; the left column displays results from crosses in which the male supplied the *dpp*<sup>d</sup> allele and the right column results from crosses in which the female supplied the *dpp*<sup>d</sup> allele. **Top two rows:** In all cases tested, animals with one copy of *dpp*<sup>d5</sup> display a partial rescue in the percent viable progeny with the introduction of one copy of *gbb*<sup>1</sup>. While the absolute values varied between individual experiments, the trend was consistent. **Bottom two rows:** The effect on viability of one copy of *gbb*<sup>1</sup> has different effects on *dpp*<sup>hr</sup>/*dpp*<sup>d6</sup> animals depending on the genotype of the parents. In crosses in which the *dpp*<sup>d6</sup> allele came from the father, introduction of one copy of *gbb*<sup>1</sup> led to an exacerbation of the viability defects, leading to increased lethality. If, instead, the *dpp*<sup>d6</sup> allele came from the mother, introduction of one copy of *gbb*<sup>1</sup> partially rescues the viability defects. In addition, maternal *dpp*<sup>d6</sup> results in overall lower viabilities observed.

Surprisingly, the same results were not observed in *dpp*<sup>hr</sup>/*dpp*<sup>d6</sup> allelic combinations. In crosses from which the father provided the *dpp*<sup>d6</sup> allele, introduction of one copy of *gbb*<sup>1</sup> actually exacerbated lethality, rather than rescued reduced viability, of *dpp*<sup>hr</sup>/*dpp*<sup>d6</sup> progeny (Table 2.1). Introduction of one copy of *gbb*<sup>1</sup> decreased viability of *dpp*<sup>hr27</sup>/*dpp*<sup>d6</sup> progeny from 73% to 44% and of *dpp*<sup>hr4</sup>/*dpp*<sup>d6</sup> progeny from 68% to 45%. We observed this trend in multiple trials of these experiments (Table 7.2, data not shown) and in *dpp*<sup>hr56</sup>/*dpp*<sup>d6</sup> progeny (Table 7.2). Consistent with a paternal effect in *dpp*<sup>hr</sup> alleles, when the father provided the *dpp*<sup>hr</sup> allele the opposite result was seen and reduced *gbb* function again partially rescued viability (Table 2.2). Introduction of one copy of *gbb*<sup>1</sup> rescued viability of *dpp*<sup>hr27</sup>/*dpp*<sup>d6</sup> progeny from 4% to 10% and of *dpp*<sup>hr4</sup>/*dpp*<sup>d6</sup> progeny from 12% to 36%. Our observation of a *dpp* paternal effect, while incredibly interesting, was not further pursued at the time but a more thorough characterization of this effect would be of interest for the future.

With a reduction in *gbb* copy number, we reproducibly observed a partial rescue in viability of *dpp*<sup>hr</sup>/*dpp*<sup>d5</sup> animals and observed an exacerbation of viability in *dpp*<sup>hr</sup>/*dpp*<sup>d6</sup> animals with paternal *dpp*<sup>d6</sup>

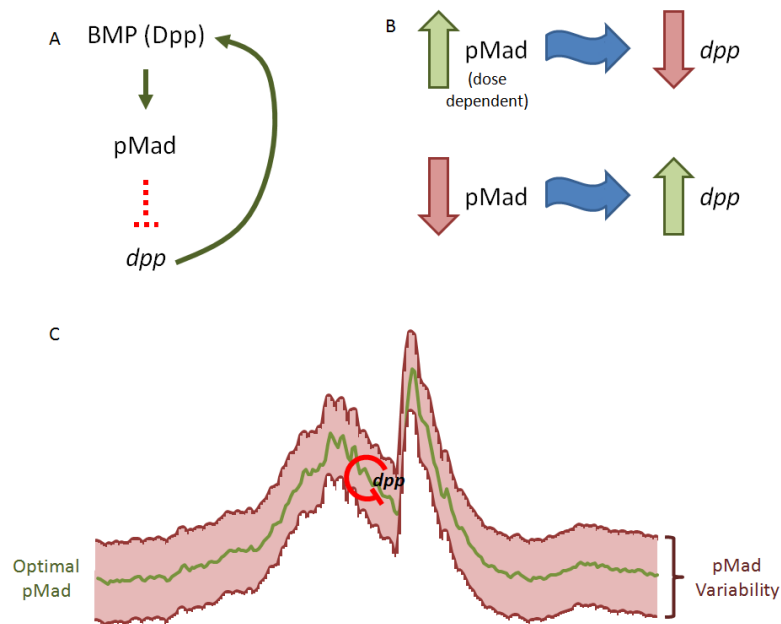


contribution. This difference in response to *gbb* reduction may be explained by the nature of the *dpp*<sup>d5</sup> and *dpp*<sup>d6</sup> alleles (Johnston et al., 1990). Although both of these alleles are the result of chromosomal rearrangements whose 5' breakpoints fall within the *dpp*<sup>disk</sup> regulatory region, there is considerable difference in the location of the two breakpoints. *dpp*<sup>d6</sup> breaks within the class III region of the *dpp*<sup>disk</sup> region, removing much of the *cis*-regulatory sequences required for expression of *dpp* in the wing imaginal discs. On the other hand, the breakpoint of *dpp*<sup>d5</sup> is at the junction of the class III and class II regions, leaving *dpp*<sup>d5</sup> with about five kb more *dpp*<sup>disk</sup> regulatory sequence than *dpp*<sup>d6</sup>. We hypothesize that the observed rescue in lethality of *dpp*<sup>hr</sup>/*dpp*<sup>d5</sup> progeny with one copy of *gbb*<sup>1</sup> is due to up-regulation of *dpp* expression that is possible because *dpp*<sup>d5</sup> retains sequences required to mediate regulation of *dpp* transcription. In *dpp*<sup>hr</sup>/*dpp*<sup>d6</sup> allelic combinations, reduced *gbb* copy number cannot up-regulate *dpp* expression and rescue lethality because *dpp*<sup>d6</sup> lacks sufficient regulatory sequences to mediate negative feedback of *dpp* transcription. Instead there is an overall reduction in the amount of BMP ligand available, leading to increased lethality of progeny.

In light of our previous observations demonstrating that levels of both *dpp-lacZ* reporter expression and *dpp* RNA itself are negatively regulated in response to BMP signaling, we propose that the viability changes of *dpp*<sup>hr</sup>/*dpp*<sup>d</sup> allelic combinations with altered *gbb* copy number is due to negative feedback regulation of *dpp* expression. Importantly, these results suggest that expression of *dpp* is regulated by this mechanism in multiple points during development, as the observed changes in viability are unlikely to be due to changes in wing imaginal disc expression of *dpp* alone. In addition, the different responses observed in animals containing *dpp*<sup>d5</sup> or *dpp*<sup>d6</sup> alleles of *dpp* suggest an important role for *dpp*<sup>disk</sup> *cis*-regulatory sequences in mediating feedback regulation of *dpp*.

## Discussion

In the wing imaginal disc, expression of *dpp* is altered in response to increased or decreased BMP signaling levels as part of a negative feedback loop. This effect is BMP specific since the response is observed when levels or activity of many of the core signaling components of the pathway is modulated, including the ligands Dpp and Gbb, the type I receptors Tkv and Sax, and the transducer, Mad. We monitored the behavior of *dpp* expression in the wing disc through examination of the *dpp* reporter constructs, *dpp<sup>BS3.0</sup>lacZ* and *dpp<sup>p10638</sup>lacZ*, with either increased or decreased levels or activity of BMP pathway components. Consistently, the expression of these *dpp* reporter constructs was reduced in cells over-expressing *dpp*, *gbb*, *tkv<sup>QD</sup>*, *sax<sup>QD</sup>*, or *Mad* and increased in cells null for *gbb*, *tkv*, or *sax*. We propose a model in which transcription of *dpp* can be modulated by BMP signaling levels (Fig 2.12A,B). In this model, the expression of *dpp* is modulated in response to changes in BMP signaling and pMad levels. As of now, it is unclear whether this is through direct regulation by the pMad/Med complex, or if this can be mediated indirectly through a second factor.



**Figure 2.12. A BMP-signaling-dependent negative feedback mechanism regulates expression of *dpp*.** Expression of *dpp* is negatively regulated by Mad-mediated BMP signaling. **B.** Increased BMP activity levels leads to a repression of *dpp*. This response is Mad dose-dependent. Decreased BMP activity levels leads to an up-regulation of *dpp*. **C.** Transcriptional regulation of *dpp* may serve to maintain robustness in pMad activity. Actual pMad activity may vary (in red) from the optimal value (shown in green). Negative feedback regulation fine-tunes expression of *dpp* to maintain pMad within a functional window of activity levels.

### Regulation of *dpp* correlates with pMad levels

We consistently observed a transcriptional response in *dpp-lacZ* expression with increased or decreased BMP signaling, but the strength of the effect varied between experiments. In experiments in which BMP signaling was increased by expressing a constitutively active form of Tkv, *tkv<sup>QD</sup>*, expression of both *dpp<sup>p10638</sup>-lacZ* and *dpp<sup>BS3.0</sup>-lacZ* was significantly or completely reduced in a cell-autonomous manner. We observed this effect when *tkv<sup>QD</sup>* was expressed in a large portion of the wing pouch, controlled by *ap-Gal4*, *MS1096-Gal4*, or *A9-Gal4*, or when *tkv<sup>QD</sup>* was expressed in small FLP-out clones in the wing pouch. Over-expressing of *dpp* had a more modest effect on expression of *dpp<sup>BS3.0</sup>-lacZ* and *dpp<sup>p10638</sup>-lacZ* in comparison to activated Tkv. In wing discs with *dpp* broadly over-expressed throughout the wing pouch or specifically in the dorsal compartment, expression of *dpp<sup>BS3.0</sup>-lacZ* and *dpp<sup>p10638</sup>-lacZ* was reduced within the *lacZ*-expressing cells of the wing pouch. In addition, large *dpp* FLP-out clones that fell within the *dpp<sup>BS3.0</sup>-lacZ* –expression domain of the wing pouch displayed reduced *dpp<sup>BS3.0</sup>-lacZ* expression levels. *gbb* over-expression, however, only had a mild effect on *dpp<sup>BS3.0</sup>-lacZ* and *dpp<sup>p10638</sup>-lacZ* expression in all genetics schemes examined.

To explain the variable response on expression of *dpp<sup>BS3.0</sup>-lacZ* and *dpp<sup>p10638</sup>-lacZ* with increased expression or activity of various BMP pathway components, we considered the possibility that these different factors are able to elicit different BMP signaling responses. We hypothesized that a stronger reduction in *dpp-lacZ* expression would correlate to a higher increase in BMP signaling activity. Indeed, when we stained for the phosphorylated form of Mad, pMad, we observed that these factors displayed different signaling capabilities that correlated to the strength of observed *dpp-lacZ* expression reduction. *tkv<sup>QD</sup>* expression always led to strong cell-autonomous BMP signaling independent of the method used to over-express it. Over-expression of *dpp* lead to a modest increase in pMad levels when expressed under the control of *ap-gal4*, *MS1096-Gal4*, or *omb-Gal4*. Conversely, expression of *gbb* led to only slight changes in the pMad gradient in the wing disc and, unlike Dpp, did not cause a significant increase in wing pouch size. These results indicate that expression of *dpp* and pMad levels are inversely correlated, and suggests that *dpp* expression responds to increased pMad levels in a dose-dependent manner (Fig 2.12B). It will be useful in for future experiments to specifically address the requirement for Mad in this process

and test the behavior of *dpp* expression in the absence of *Mad*. This will be achieved by observing *dpp-lacZ* expression in clones of cells homozygous for *Mad* mutants or in cells expressing RNAi directed against *Mad*. Furthermore, we would like to test whether expression of *dpp* responds to a reduction in pMad in a dose-dependent manner as well.

It makes sense that over-expression of activated receptor compared to ligand should have a differential effect on pMad. A constitutively active receptor will lead to phosphorylation of Mad independent of up-stream components, such as ligand or extracellular components. Activity of ligand, on the other hand, is dependent on the presence or activity of extracellular regulators and all downstream components. BMP ligands could also have different signaling capabilities as homodimers or heterodimers and over-expression of one particular ligand would most likely shift the ratio of dimer type produced (Israel et al., 1996; Kusumoto et al., 1997; Little and Mullins, 2009; Shimmi et al., 2005; Suzuki et al., 1997). We also observed a difference in signaling ability between Dpp and Gbb that may reflect intrinsic differences in signaling strength or range, or specific dimer or receptor requirements. Consistent with our *in vivo* observations, our lab has previously observed through S2 cell culture BMP signaling assays that Dpp is far more potent in eliciting a signaling response than Gbb (data not shown, Takuya Akiyama and Viet Le, personal communication).

### **BMP signaling in the wing imaginal disc is a highly robust system**

We must also consider robustness of the BMP signaling pathway, the ability of the pathway to accommodate for fluctuations in signaling levels, in determining the final pMad output of over-expressing *tkv<sup>QD</sup>*, *dpp*, or *gbb*. Several autoregulatory mechanisms have already been identified within BMP signaling in *Drosophila* wing development that may play a role in ensuring robust signaling. The inhibitory Smad, *dad*, is both a target of BMP signaling and an intracellular negative regulator of signaling (Ogiso et al., 2011; Tsuneizumi et al., 1997; Weiss et al., 2009). The activity of over-expressed *tkv<sup>QD</sup>*, *dpp*, and *gbb* could all be subject to regulation by Dad, as Dad is believed to function at or below the step of receptor-Mad interaction (Inoue et al., 1998). In mammalian cell culture assays, Dad has been shown to bind *tkv<sup>QD</sup>* and inhibit *tkv<sup>QD</sup>*-induced phosphorylation of Mad (Kamiya et al., 2008). Ligands, however, may be subject to

negative regulatory mechanisms that *tkv<sup>QD</sup>* is not, such as the secreted extracellular regulators *pent*, which may regulate ligand distribution, and *Itl*, which may regulate ligand activity (Hamaratoglu et al., 2011; Szuperak et al., 2011; Vuilleumier et al., 2010). It is possible that these factors influence the activity of Gbb more so than Dpp, explaining the different pMad distributions observed in response to expression of either ligand. With more regulatory mechanisms in place to regulate ligand activity, it is not surprising that increased *dpp* and *gbb* expression does not impact pMad at the same level as *tkv<sup>QD</sup>*.

Negative feedback regulation of *dpp* transcription may represent an additional mechanism through which BMP signaling maintains robustness (Fig 2.12C). Throughout development, pMad may fluctuate from an optimal level (Fig 2.12C, in green) in response to environmental or genetic perturbations or as a result of tissue growth and proliferation. We propose that the pathway is able to sense the variability in pMad activity levels (Fig 2.12C, in red) and accommodate by fine-tuning expression of the ligand *dpp*. That the degree of *dpp-lacZ* repression correlates to the degree of pMad increase suggests that this mechanism can not only sense a change in signaling activity but also sense the degree of activity change and react accordingly. Repression of *dpp-lacZ* expression may not be visible or obvious by our reporter assays when only a subtle change in expression is occurring.

### Spatial restrictions on feedback

Our experiments testing the effect of raising or lowering BMP signaling levels on *hh<sup>MRT</sup>* phenotypes reveal that there are spatial restrictions within the wing imaginal disc for where feedback regulation of *dpp* transcription can occur. The *hh<sup>MRT</sup>* allele displays a variable amount of ectopic *dpp* expressed in the anterior compartment, which can be exploited to address the ability of BMP signaling to repress *dpp* transcription outside of its endogenous expression domain. Understanding the spatial limitations of regulation may help identify specific factors involved in this mechanism of *dpp* regulation.

Analysis of *tkv<sup>QD</sup>* clones reveals that *dpp<sup>BS3.0</sup>lacZ* expression is only strongly repressed by increased BMP signaling within or close to the endogenous *dpp* expression domain and ectopically expressed *dpp<sup>BS3.0</sup>lacZ* is not reduced. This spatial restriction to feedback regulation of *dpp* suggests that the factor or factors responsible are active in the medial region of the wing disc but are either not

expressed or not active in the lateral regions of the wing disc. In addition, that negative feedback regulation of *dpp* is confined to the endogenous expression domain of *dpp* suggests that it is not a mechanism for in regulating *dpp* expression globally. Instead, this supports our model that negative feedback regulation of *dpp* functions in fine-tuning endogenous expression of *dpp* to regulate pMad activity during development.

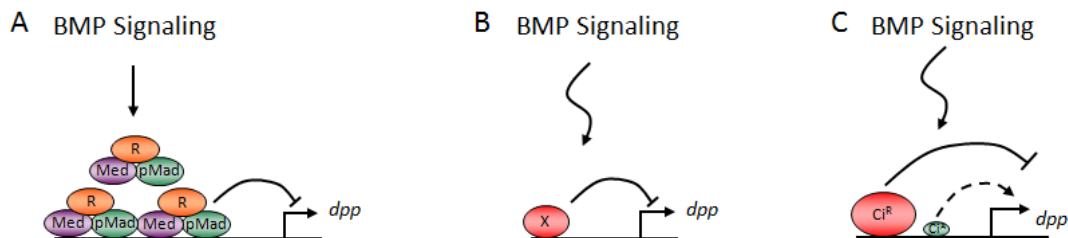
Negative feedback regulation of *dpp* is most likely not, however, restricted to the wing imaginal disc. Our analysis of *dpp<sup>hr</sup>/dpp<sup>d</sup>* lethality with altered *gbb* copy number suggests that negative regulation of *dpp* expression may occur in different contexts and tissues. We made the observation that an increase in *gbb* copy number can exacerbate lethality of *dpp<sup>hr</sup>/dpp<sup>d</sup>* allelic combinations while a decrease in *gbb* copy number can partially rescue lethality of *dpp<sup>hr</sup>/dpp<sup>d</sup>* allelic combinations. These results were unexpected, as we had hypothesized that Gbb would compensate for reduced *dpp* expression. However, they can be explained by negative feedback regulation of *dpp*: increases in *gbb* level further decrease expression of *dpp* which leads to higher lethality, and decreases in *gbb* level up-regulates *dpp* expression enough to rescue lethality. The observed change in viability of *dpp<sup>hr</sup>/dpp<sup>d</sup>* animals could be due to a number of reasons and these experiments have not tested the particular stage of development that is impacted, but it is unlikely to be due to wing development alone. Our results suggest that feedback regulation of *dpp* expression may occur at various times throughout development to influence viability, perhaps even quite early.

### **Proposed models for the molecular mechanism of dpp feedback regulation**

Our results suggest that negative feedback of *dpp* occurs in response to canonical BMP signaling, as expression of *dpp<sup>BS3.0</sup>lacZ* is sensitive to the level of Mad and pMad. We have demonstrated that an increase in Mad leads to a reduction in *dpp<sup>BS3.0</sup>lacZ* expression and that repression of *dpp-lacZ* correlates to pMad levels. As Mad is the transducer of BMP signaling and is itself a transcription factor, we expect that Mad plays an important role in facilitating the transcriptional regulation of *dpp*. This effect may be direct, in which pMad interacts with the *cis*-regulatory sequences of *dpp*, or indirect, through a secondary regulatory downstream of BMP signaling. In figure **2.13**, we propose three models through which Mad

## Direct regulation

## Indirect regulation



**Figure 2.13 Models for mechanisms of BMP-mediated regulation of *dpp*.** We consider several models of how BMP signaling may regulate expression of *dpp* directly (**A**) or indirectly (**B-C**). **A.** BMP signaling directly regulates *dpp* through repression by Mad and a repressive co-factor, R. Expression of *dpp* is sensitive to levels of this complex and responds accordingly. A potential factor that could behave as a co-factor in this model is Shn. **B.** BMP signaling indirectly regulates *dpp* by regulating expression of a repressive transcription factor, X. As BMP signaling increases, so does expression of the repressor, X. **C.** BMP indirectly regulates *dpp* expression by influencing the processing or activity of Ci. BMP signaling influences the ratio of  $Ci^A:Ci^R$ , resulting in a lower ratio at increased levels of BMP signaling. A higher relative concentration of  $Ci^R$  causes a reduction in *dpp* expression.

may influence the transcription of *dpp*, either directly in concert with a repressive co-factor or indirectly through regulation of a downstream repressor. In all models, this system requires that *dpp* be expressed in its endogenous expression domain under normal BMP signaling conditions and that the expression of *dpp* be sensitive to both increased and decreased BMP signaling levels.

If Mad can directly regulate the transcription of *dpp*, it would likely require a repressive cofactor since Mad is not known to behave as a repressor on its own (Fig 2.13A). Since repression of *dpp* occurs within its endogenous expression domain where pMad is present, this model would require that expression of *dpp* be sensitive to changes in the level or activity of the pMad/co-repressor complex that may occur with changes in level of BMP activity. Shn is a candidate to play the role as the repressive co-factor in the direct model (Fig. 2.13A), as it has been reported to form a complex with pMad and Med and confer a repressive function onto these factors (Gao et al., 2005; Pyrowolakis et al., 2004). This complex is essential in establishing the expression domain of many BMP target genes within the wing imaginal disc. In this tissue, the Shn/pMad/Med complex directly binds the regulatory region of the gene *brinker* and represses its expression within the medial region of the wing pouch (Marty et al., 2000; Muller et al., 2003). By extension, it is possible that the Shn/pMad/Med complex could directly bind *dpp* cis-regulatory sequences to regulate its transcription in the wing pouch. Since *dpp* is expressed in the region where this complex is active, our model would require that *dpp* be sensitive to changes in levels of the

Shn/pMad/Med complex. Furthermore, this model requires that *dpp* be less sensitive to the Shn/pMad/Med complex than *brinker* is, possibly as a result of affinity or number of binding sites for the Shn/pMad/Med complex.

We have also considered a second possibility by which BMP signaling regulates *dpp* indirectly. In this model, BMP signaling regulates the transcription of a factor that itself directly regulates *dpp* (Fig 2.13B). Due to its sensitivity to BMP signaling activity, expression of this downstream transcription factor could serve as a sensing mechanism for BMP signaling activity. With increased BMP signaling, the factor is highly expressed, leading to repression of *dpp* expression (Fig 2.13B). Conversely, decreased BMP signaling would lead to lower expression of this transcription factor, releasing *dpp* from negative regulation. As expression of this factor responds to pMad in a concentration-dependent manner, this model is consistent with the known role of pMad as a morphogen.

In our third model, we propose a second way in which pMad could indirectly regulate *dpp* through regulating the activity of Ci, the downstream effector of Hh signaling (Fig 2.13C). Ci is critical in generating the expression domain of *dpp* in the wing disc where its active form,  $Ci^A$ , is known to activate *dpp* and its truncated repressor form,  $Ci^R$ , is known to repress *dpp* (Méthot and Basler, 1999; Müller and Basler, 2000). In the wing pouch, *dpp* is expressed in cells with a moderately high  $Ci^A:Ci^R$  ratio, such as those in anterior cells closest to the anterior-posterior boundary where Hh signaling activity is highest. In anterior cells far from the boundary, where Hh signaling is inactive, these cells do not express *dpp* because  $Ci^R$  is the predominant form of Ci. This dual function of Ci on the expression of *dpp* makes it a particularly attractive candidate to mediate negative feedback regulation of *dpp* transcription. Negative feedback regulation of *dpp* in the wing imaginal disc might therefore be explained by a model in which BMP signaling alters the ratio of  $Ci^A:Ci^R$  (Fig 2.13C). In response to increased BMP signaling, the endogenous ratio of  $Ci^A:Ci^R$  is reduced along the anterior-posterior boundary of the wing disc, resulting in a reduction in the expression of *dpp* in this domain (Fig 2.13C). BMP signaling may be altering the  $Ci^A:Ci^R$  ratio through regulating the expression of a factor that is involved in processing or activating Ci. Alternatively, pMad may have a more direct effect on Ci and work in concert with Ci to facilitate its binding to the *dpp* cis-regulatory sequences and ultimately influence its activity. Although an interaction



between Ci and Mad has not been observed in *Drosophila*, an interaction between Smads and Gli3, the repressive form of the Gli protein in vertebrates, have been demonstrated in mammalian cell culture (Liu et al., 1998).

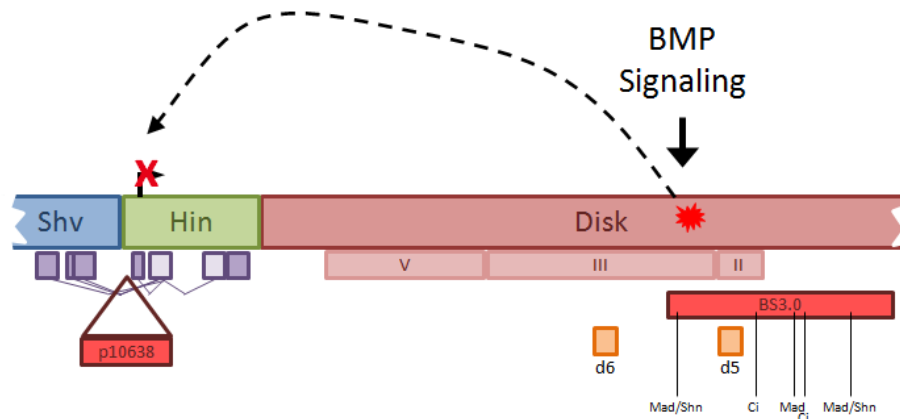
Given the complex regulation of *dpp* currently appreciated and the large region of the *dpp* locus devoted to transcriptional regulation, it is possible that feedback regulation of *dpp* transcription could occur through multiple mechanisms involving a variety of factors. Therefore, we also consider the possibility that *dpp* is subject to negative feedback by more than one of the models proposed, as they are not mutually exclusive. At this time, we are most interested in addressing the individual impact of Mad, Shn, and Ci in negative feedback regulation of *dpp*. To address the role of these potential factors, future experiments will explore how *dpp* transcription responds to perturbations in BMP signaling in their absence or with reduced function. If, indeed, a particular component is required for the negative feedback of *dpp*, then we expect to see no change in the expression of *dpp* in the absence of that factor.

#### **Cis-regulatory sequences required for negative feedback of *dpp***

Our work testing the effect of *gbb*<sup>1</sup> on viability of *dpp*<sup>d</sup>/*dpp*<sup>hr</sup> animals points to particular *dpp* cis-regulatory regions that might be required for negative feedback of *dpp* transcription. We observed that a reduction in *gbb* copy number could partially rescue viability of *dpp*<sup>d5</sup>/*dpp*<sup>hr</sup> animals but not *dpp*<sup>d6</sup>/*dpp*<sup>hr</sup> animals. Differential rescue of *dpp*<sup>d5</sup>/*dpp*<sup>hr</sup> and *dpp*<sup>d6</sup>/*dpp*<sup>hr</sup> viability with one copy of *gbb*<sup>1</sup> could be explained by the difference in the cis-regulatory regions still present in these alleles. While both of these *dpp*<sup>d</sup> alleles arise from chromosomal inversions whose breakpoints fall within the *dpp* cis-regulatory sequence, the class II *dpp*<sup>d6</sup> allele contains 3kb less sequence than the class II *dpp*<sup>d5</sup> allele does (St Johnston et al., 1990). The region of *dpp* regulatory sequences located between these two breakpoints might contain cis-regulatory sequences required to mediate negative feedback of *dpp* transcription. If this is the case, *dpp*<sup>d5</sup> may contain a binding site for a transcription factor that can regulate *dpp* transcription while *dpp*<sup>d6</sup> may have this site deleted. As a result, *dpp*<sup>d5</sup> animals could sense altered BMP signaling and modulate *dpp* transcription accordingly while *dpp*<sup>d6</sup> animals could not. The model in figure 2.14 displays this hypothesis.

To the best of our knowledge, the precise breakpoints for  $dpp^{d5}$  and  $dpp^{d6}$  are unknown but we do have an approximation of where they break (St Johnston et al., 1990). A closer examination of the sequences between the approximate breakpoints for  $dpp^{d5}$  and  $dpp^{d6}$  reveals the presence of consensus binding sites for several factors important to BMP signaling and to expression of  $dpp$  in the wing disc. These sites and factors may play an important role in feedback regulation of  $dpp$  transcription as well. Consensus binding sites for Mad and for the Shn/pMad/Med complex are located in this region, such that they are present in  $dpp^{d5}$  but not in  $dpp^{d6}$  (Gao et al., 2005; Hepker et al., 1999; Kim et al., 1997; Kirkpatrick et al., 2001; Newfeld and Takaesu, 2002; Pyrowolakis et al., 2004; Szuts et al., 1998). Furthermore, due to the imprecise location of the breakpoints,  $dpp^{d5}$  may retain consensus binding sites for the transcription factor Ci that would be absent in  $dpp^{d6}$  (Fig 2.14) (Müller and Basler, 2000). The presence of these binding sites within the  $dpp$  regulatory sequence supports a possible function for these factors in negative feedback regulation of  $dpp$  transcription.

Our results thus far suggest an importance for the *cis*-regulatory regions near the breakpoints of both  $dpp^{d5}$  and  $dpp^{d6}$  in determining the transcriptional response of  $dpp$ . Future experiments will seek to map the negative feedback regulation of  $dpp$  to specific  $dpp$  control regions.  $dpp$  reporter constructs are



**Figure 2.14.  $dpp^{disk}$  cis-regulatory sequences mediate negative feedback regulation of  $dpp$ .** Map depicting the *cis*-regulatory regions of *dpp*: *shortvein*, *Hin*, and *disk*. Purple denotes the sequences of *dpp* found within cDNA. In red, are the approximate locations of the two *dpp* reporter constructs,  $dpp^{p1038}lacZ$  which is an enhancer trap inserted 5' to the start site, and  $dpp^{BS3.0}lacZ$  which is a transgenic reporter construct with 10kb of  $dpp^{disk}$  regulatory sequence. The regions of the  $dpp^{disk}$  sequence corresponding to allelic classes are marked as V, III, and II. The approximate breakpoints for  $dpp^{d6}$ , within the class III region, and  $dpp^{d5}$ , within the class II region, are denoted in orange. Our model assumes that sequences required for negative regulation of *dpp*, marked in bright red, are located between the  $dpp^{d6}$  and  $dpp^{d5}$  breakpoints. Binding of a factor to these sequences will then influence expression of *dpp*. Binding sites for potential regulators of *dpp*, Shn/pMad/Med, Ci, and Mad, are denoted.

available that are constructed from small fragments of the *dpp<sup>disk</sup>* *cis*-regulatory sequences. We will exploit these different *dpp* reporter constructs and examine their transcriptional response to altered BMP signaling levels. We will map the sequences necessary for negative feedback regulation of *dpp* by determining which reporter constructs are competent to respond to negative feedback cues. Once we have determined a small region of the *dpp cis*-regulatory sequence that is competent to mediate feedback regulation of *dpp* transcription, we can examine the sequences to further investigate the binding of candidate mediators to this region.

### Chapter 3:

Feedback regulation of *dpp* in the wing pouch is achieved through functional redundancy of the *cis*-regulatory sequences and through the activity of both direct and indirect mechanisms

I performed all of the experiments presented in this chapter.

## Abstract

Patterning in the wing imaginal disc occurs in response to a graded distribution of the BMP transducer, pMad, which is generated in response to signaling through the ligands Dpp and Gbb. As pMad functions as a morphogen in this tissue and generates responses in a concentration-dependent manner, it is important that BMP signaling be robust and capable of accommodating for fluctuations in signaling levels. We have previously identified and characterized a system of negative feedback regulation of *dpp* transcription mediated by BMP signaling that might serve to compensate for perturbations in signaling activity and contribute to robustness of the pMad gradient. We have observed that expression of *dpp* within the wing pouch of the wing imaginal disc is negatively regulated in response to both increases and decreases in BMP signaling in a pMad dose-dependent manner. In the experiments described here, we have dissected the *cis*-regulatory regions of *dpp* to uncover sequences that mediate feedback regulation as a way to identify the molecular mechanisms through which negative feedback regulation of *dpp* is achieved. Through these experiments we have revealed redundancy within *cis*-regulatory regions for the ability to mediate negative transcriptional regulation of *dpp* and have realized a dual function for BMP signaling in regulating wing pouch expression of *dpp*: BMP signaling negatively regulates expression of *dpp* within its endogenous expression domain and has the ability to positively regulate expression of *dpp* in the lateral wing disc. In our analysis of potential factors that may be required for feedback regulation, we have revealed that Ci, which is well-known as both a positive and negative regulator of *dpp* transcription, is not required for feedback regulation of *dpp*. Instead, our experiments have identified both a potential direct and indirect role for Mad in regulating *dpp*: directly through a Mad consensus binding site, and indirectly through action of a factor downstream of Shn.

## Introduction

In the *Drosophila* wing imaginal disc, the BMP signaling pathway behaves as a morphogen and influences cell fate specification in a concentration dependent manner along the anterior-posterior axis (Khalsa et al., 1998; Lecuit et al., 1996; Nellen et al., 1996; Singer et al., 1997). This is accomplished through a graded distribution of phosphorylated BMP transducer, pMad, which plays a critical role in directing the expression of BMP target genes important in directing cell fate specification. In the wing disc, the pMad gradient is established in response to BMP signaling by the ligands Dpp and Gbb through a heterotetrameric receptor complex consisting of two type II, Put or Wit, and two type I, Sax or Tkv, serine/threonine-protein kinase receptors (Bangi and Wharton, 2006; Khalsa et al., 1998; Raftery and Sutherland, 1999; Ray and Wharton, 2001; Sivasankaran et al., 2000). Upon complex formation, the constitutively active type II receptors phosphorylate the type I receptors which, in turn, phosphorylate the intracellular transducer of BMP signaling, Mad, generating pMad.

In the wing pouch, BMP signaling through pMad has both a direct and indirect effect on the expression of BMP target genes. It directly activates expression of the target genes *vestigial (vg)*, *spalt (sal)*, *larval translucida (ltl)*, and *daughters-against-dpp (dad)* (Barrio and de Celis, 2004; Kim et al., 1997; Szuperák et al., 2011; Weiss et al., 2009). pMad can also directly repress expression of the target genes *brinker (brk)* and *pentagone (pent)* in cooperation with Schnurri (Shn) (Jazwinska et al., 1999; Kopp et al., 1999; Marty et al., 2000; Moser and Campbell, 2005; Müller et al., 2003; Vuilleumier et al., 2010).

Association with the co-repressor Shn converts the pMad/Med complex, which is normally a transcriptional activator, into a repressor capable of directly repressing target genes. Expression of *brk* and *pent* is repressed in the medial wing pouch, where BMP activity is high, and active in the lateral wing pouch, where BMP activity is low. *Brk* is expressed in an inverse gradient in relation to pMad and is of particular importance because it directly represses the BMP target genes *optomotor-blind (omb)*, *sal*, *ltl*, and *dad* (Barrio and de Celis, 2004; Campbell and Tomlinson, 1999; Grimm and Pflugfelder, 1996; Sivasankaran et al., 2000; Szuperák et al., 2011; Weiss et al., 2009). Through the repressive action of Brk, pMad indirectly up-regulates expression of target genes. The balance of direct positive input from pMad

and negative input from Brk determines the expression domains of the target genes *sal*, *ltl*, and *dad*. Together, these genes are responsible for a variety of cellular processes important for proper wing development, such as establishment of the vein primordia (Cook et al., 2004; de Celis and Barrio, 2000; Kissinger et al., 1990), control of cell growth (Martin et al., 2004), and maintenance of epithelial architecture (Gibson, 2005; Shen, 2005; Shen and Dahmann, 2005; Shen et al., 2010; Shen et al., 2008; Umemori et al., 2007)

Given that the expression domains of these target genes are established, directly and indirectly, in response to pMad thresholds, modulation of the pMad gradient could lead to improper target gene expression and, consequently, improper fate specification. Therefore, precise regulation of the pMad output from BMP signaling is essential and several mechanisms exist as safeguards against inappropriate changes in BMP signaling activity. Negative feedback is a recurrent theme in BMP signaling regulation as two transcriptional targets, the intracellular inhibitory Smad, Dad, and the extracellular antagonist, Ltl, function to negatively regulate output of the pathway (Ogiso et al., 2011; Szuperák et al., 2011). An additional target which is repressed by BMP signaling, *pentagone (pent)*, facilitates signaling in the wing disc (Ben-Zvi et al., 2011; Hamaratoglu et al., 2009; Hamaratoglu et al., 2011; Vuilleumier et al., 2010). We have identified and characterized an additional negative feedback mechanism that regulates transcription levels of the BMP ligand *dpp*. In previous studies, we have demonstrated that increasing BMP signaling levels represses the expression of *dpp* while reducing BMP signaling levels leads to increased expression of *dpp*. Furthermore, transcriptional regulation of *dpp* occurs in response to modulation of components at every step of the pathway and is pMad dose-dependent, indicating that regulation of *dpp* is occurring in response to canonical BMP signaling. Having verified that *dpp* transcription is negatively regulated by BMP signaling in the wing pouch, we set out to identify *cis*-regulatory sequences and transcriptional regulators responsible for this regulation.

During development, spatial and temporal regulation of gene expression is achieved through the coordinated action of regulatory factors upon control regions known as *cis*-regulatory modules (CRMs) (Bonn and Furlong, 2008). These modular units incorporate regulatory information from many *trans*-acting factors to determine the expression behavior of a gene. The *dpp* locus contains over 50kb of *cis*-

regulatory sequences, half of which are largely responsible for directing expression of *dpp* in the larval imaginal discs (St Johnston et al., 1990). The region responsible for imaginal disc expression, the *dpp*<sup>disk</sup> region, has long been known to direct expression of *dpp* in a modular fashion, such that particular sequences direct expression of *dpp* in different imaginal discs or regions of discs (Blackman et al., 1991; Masucci et al., 1990). If CRMs exist in the *dpp*<sup>disk</sup> region to direct *dpp* expression in each disc, CRMs may also exist for feedback regulation of *dpp* expression. Identifying the particular sequences that mediate negative feedback of *dpp* would, in turn, help us determine the factors that are responsible for this transcriptional regulation of *dpp*.

One factor well-known to direct expression of *dpp* in the wing imaginal disc through action on distinct CRMs is Ci (Hepker et al., 1999; Méthot and Basler, 1999; Müller and Basler, 2000; Parker et al., 2011). Ci is critical in establishing the expression domain of *dpp* in the wing pouch and plays a dual role in determining the expression pattern of *dpp*: Ci<sup>A</sup> activates *dpp* along the anterior-posterior compartment boundary and Ci<sup>R</sup> represses *dpp* throughout the remainder of the anterior compartment. In the absence of Ci protein, expression levels of *dpp* are reduced along the anterior-posterior boundary of the wing pouch, where expression of *dpp* is normally at its highest, and ectopically expressed throughout the remainder of the anterior compartment (Méthot and Basler, 1999; Müller and Basler, 2000). Furthermore, reporter constructs that cannot bind Ci either do not express in the wing disc or display a reduced but expanded expression domain in comparison to wildtype reporter lines (Hepker et al., 1999; Müller and Basler, 2000; Parker et al., 2011). Within the *dpp*<sup>disk</sup> region are two clusters of binding sites for the transcription factor Ci found within a proximal and a distal CRM. The first cluster, within the proximal CRM, consists of three Ci binding sites as well as sequences for yet unidentified activating factors that can activate expression of *dpp* in the absence of Ci (Müller and Basler, 2000; Parker et al., 2011). The second cluster, within the distal CRM, consists of two Ci binding sites and is also near binding sites for a variety of other factors, including Mad and dTCF, the Wg effector protein (Hepker et al., 1999).

To determine the particular *cis*-regulatory sequences that allow negative feedback of *dpp* and the transcription factors that mediate this response, we took multiple experimental approaches, both unbiased and biased. To identify *cis*-regulatory sequences, we took a two-pronged unbiased approach to



determine functionally relevant CRMs within the *dpp cis*-regulatory sequence that are required for negative feedback. Given that we have observed negative feedback of *dpp<sup>BS3.0</sup>lacZ* expression in response to altered BMP signaling, we hypothesized that *trans*-acting factors must bind to the *cis*-regulatory sequences contained in *dpp<sup>BS3.0</sup>lacZ* and mediate negative feedback regulation of *dpp* expression. To narrow down regions potentially important in negative feedback, we performed sequence comparisons of the *dpp<sup>BS3.0</sup>lacZ* regulatory sequences to identify regions of high conservation and tested *dpp* reporter constructs containing smaller *cis*-regulatory regions for their response to altered BMP signaling. These results indicate that CRMs that mediate negative feedback regulation are spread across large regions of *cis*-regulatory sequence. Unexpectedly, these experiments also revealed positive regulation of *dpp-lacZ* in the lateral wing pouch and along the dorsal-ventral boundary.

We also took a biased approach to test several candidate transcription factors that may mediate feedback regulation of *dpp*. Given its established function in both activating and repressing *dpp*, we hypothesized that Ci could mediate BMP-induced repression of *dpp* transcription. However, negative regulation still occurred when the binding sites for Ci were mutated in *dpp* CRMs and in the absence of Ci protein, indicating that Ci likely is not required in mediating the negative feedback regulation of *dpp*. Another candidate factor that could regulate expression of *dpp* is the downstream effector of BMP signaling itself, pMad. We have identified that Mad plays both a direct role in regulating *dpp*, specifically in repressing *dpp* in the lateral region of the wing pouch, and an indirect role through the action of a factor downstream of the co-repressor Shn. Our results indicate that feedback regulation of *dpp* is complex, requiring the input from many CRMs and several transcription factors.

## Materials and Methods

### ***Drosophila melanogaster* strains, crosses, and husbandry**

Flies were raised on standard cornmeal/yeast medium at 25°C in plastic bottles or glass vials. Flies used for virgin collection were raised at 18°C or 22°C in plastic bottles. Crosses were performed using virgin females. Unless otherwise state, all alleles used are described in flybase (<http://flybase.org/>)

### **Dissections and immunostaining**

Unless otherwise stated, larvae were dissected as third instar crawlers. All steps were carried out in glass wells without rocking. Briefly, larvae were collected and the anterior two thirds of the larvae was everted in cold PBS. They were stored on ice for no longer than 30 minutes. Tissue was fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature (RT). After fixation, discs were washed 3X 20 minutes in PBST (0.1% Triton-X in PBS) at RT. Discs were blocked for one hour in PBST + 1% NGS (normal goat serum) at RT and then incubated overnight at 4°C with the appropriate primary antibody in PBST + 1% NGS + 0.02% sodium azide. Following incubation, discs were washed 3X 20 minutes in PBST at RT, followed by a one hour block in PBST + 1% NGS at RT. Discs were incubated for 2 hours at RT in the appropriate secondary antibody in PBST + 1% NGS + 0.02% sodium azide. When used, Hoechst-33432 was added to the secondary antibody solution. Tissue was next blocked for one hour in PBST + 1% NGS at RT and then stored in mounting solution (80% glycerol, 0.1% N-propyl-gallate in water) at 4°C until mounting. Discs were dissected away from cuticles and mounted on glass slides in 8µL mounting solution and covered with a #1.5 cover slip.

The following antibodies were used: anti-pMad (rabbit monoclonal Smad3 phospho (pS423/425), 1:1000, Epitomics), anti-βGal (mouse monoclonal, 1:3000, Promega; rabbit polyclonal, 1:3000, Cappel), and anti-Ci (monoclonal 2A1, rat, 1:2, a generous gift from Robert Holmgren). Secondary antibodies used were Alexa Fluor 633 goat anti-rat IgG, Alexa Fluor 633 goat anti-mouse IgG, Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 555 goat anti-rabbit IgG, Alexa Fluor 555 donkey anti-mouse IgG, Alexa Fluor 488 goat anti-

mouse IgG, Alexa Fluor 405 goat anti-mouse IgG, Molecular Probes. All were used at a 1:3000 concentration. Nuclei were labeled with Hoechst-33432 at a 1:1000 dilution.

### **Confocal microscopy and image analysis**

Images were taken with a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope. Confocal images were taken as z-stacks with optimal slice size (usually .95µM) at either 512X512 or 1024X1024 pixels. All images were taken sequentially at a scan speed of 6 was used. Within one experiment, confocal laser settings and slice size was kept consistent.

Unless noted in the figure legend, images shown represent a collapsed z-projection of the sum of slice intensities, generated in Fiji.

Intensity profiles were created using Fiji software using the “plot profile” function. For each experiment, profiles were plotted from a rectangular selection, which gives a vertically averaged pixel intensity at each x-position. The rectangular selection size varied between experiments, but was kept constant within an experiment. Rectangular selections were placed at approximately the same location in all disc images within an experiment. Location was determined by relative position to the anterior-posterior border or to location of intensity peaks. In experiments in which average profiles were created, Microsoft Excel was used to average the intensity value at each position of all profiles for each genotype. In all intensity plots, the X axis represents each pixel and the Y axis represents pixel intensity.

### **Construction of *dpp* reporters with mutant transcription factor binding sites**

We created the following series of *dpp-lacZ* reporter lines to test Shn/pMad/Med and Ci consensus binding sites for their role in the regulation of *dpp* in response to increased BMP signaling: *dpp*<sup>MA3.0</sup>*lacZ*, *dpp*<sup>MA3.0-MS2</sup>*lacZ*, *dpp*<sup>APRD</sup>*lacZ*, *dpp*<sup>APRD-Ci5</sup>*lacZ*, *dpp*<sup>APRD-Ci5-M</sup>*lacZ*, *dpp*<sup>APRD-Ci4-M</sup>*lacZ*, *dpp*<sup>APR</sup>*lacZ*, and *dpp*<sup>APR-Ci3</sup>*lacZ*. For each, *dpp* cis-regulatory sequences with wildtype and mutant sites were cloned upstream of an *hsp70* minimal promoter and *lacZ* coding sequence with *sv40* 3'UTR within the *pAttB* transformation vector. *pAttB* contains attB recombination sites for ΦC31 site specific integration and a selectable *w*<sup>+</sup> marker.

Specific details of cloning are described below:

All locations within the *dpp* cis-regulatory sequences are based on a sequence file called dppdisc.seq that contains 30,060bp of *dpp<sup>disk</sup>* regulatory sequence. This genomic sequence was obtained from the NCBI database, entry AE003583, in 2002. For reference, the first 20bp of this sequence are ATGTTGGATAAATTAATAGA and the last 20bp are AAAGATGGGCTTTCAGGGAT. Location numbers given in the work presented in this thesis refer to the specific base pair location within this 30,060 base pair sequence.

#### Construction of *dpp<sup>MA3.0</sup>lacZ* series:

We obtained a *pCaSpeR4-dpp<sup>BS3.0</sup>-hsp70-Gal4* clone, a generous gift from Dr. Jessica Treisman. The *dpp<sup>BS3.0</sup>* region spans 10.2kb of sequence from a KpnI site at location 18460 to site 28683. From thus point on, this region will be referred to as MA3.0 instead of BS3.0. We first created *pHSG398-dpp<sup>MA3.0</sup>-hsp70-lacZ*. *pCaSpeR4-dpp<sup>MA3.0</sup>-hsp70-Gal4* was digested with KpnI to obtain a 10.3kb *dpp<sup>MA3.0</sup>-hsp70* fragment. This piece was identified on an agarose gel, extracted with a Qiagen gel extraction kit, and ligated into KpnI-digested *pHSG398.lacZ* sequences containing an *SV40 3'UTR* were amplified by PCR from a *pCaSpeR-hs43-lacZ* clone using primers that introduced a KpnI restriction enzyme recognition site (underlined) (forward primer: GGCGGC GGTACC ACCATGTCGTTTACTTTGACCAACAAG, reverse primer: GGCGGC GGTACC GGGCTGCAGGTCGACCAATTCGAGC). This PCR product was then digested with KpnI and cloned into a 13kb *pHSG398-dpp<sup>MA3.0</sup>-hsp70* fragment that was partially digested with KpnI and had been extracted from an agarose gel. Plasmids were selected by cracking. For cracking: spin 100μL of *e. coli* culture at 13k for 5min and discard supernatant. Add 20μL of cracking buffer (0.1N NaOH, 10mM EDTA, 10% glycerol, 1% SDS), vortex for 10sec, incubate at RT for 10min, vortex for 10sec, and spin at 13k for 5min at RT. Run 10μL of supernatant and 5μL loading dye in a 0.5% agarose gel to identify slowing travelling samples. Insertion was confirmed by test digestion and sequencing, giving *pHSG398-dpp<sup>MA3.0</sup>-hsp70-lacZ*. Shn/pMad/Med binding sites at locations 19403 (site 1) and 25384 (site 2) were mutated within the context of *pHSG398-dpp<sup>MA3.0</sup>-hsp70-lacZ* based on the Stratagene QuickChange Site Directed Mutagenesis kit using PFU high-fidelity DNA polymerase. Binding sites were altered by mutating the last

four base pairs of each site from GTCT to AGAC. The following primers were used for site directed mutagenesis with altered base pairs in bold:

site 1 forward primer CGTTGCAGTGGCGACTCGTT**AGACTT**GAAAAAATTTTCCATCGTCG,

site 1 reverse primer CGACGATGGAAAATTTTTCA**GTCT**AACGAGTCGCCACTGCAACG,

site 2 forward primer GCTCGACGGCGTCCGTT**AGAC**ATGCCAGCTTCATTATTTTTTCTCC, and

site 2 reverse primer GGAGAAAAATAATGAAGCTGGCAT**GTCT**GAACGGACGCCGTCGAGC. To avoid the

inclusion of point mutations generated during mutagenesis, we excised the mutated sites and some

surrounding sequence and cloned them into *pHSG398-dpp<sup>MA3.0</sup>-hsp70-lacZ* that had not been

mutagenized. The first site was excised as a 3.6kb *Apal* (19243) and *Stul* (22847) fragment, purified from

an agarose gel, and cloned into a gel-purified 13.5kb fragment of *pHSG398-dpp<sup>MA3.0</sup>-hsp70-lacZ* cut with

*Apal* and *Stul* to generate *pHSG398-dpp<sup>MA3.0-MS1</sup>-hsp70-lacZ*. The second site was excised as a 1.8kb *SpeI*

(24034) and *XmnI* (25798) fragment, extracted from agarose, and cloned into a 15.3kb gel-purified *SpeI*/

*XmnI* fragment of *pHSG398-dpp<sup>MA3.0-MS1</sup>-hsp70-lacZ*. Proper insertion was checked by PCR and confirmed

by sequencing. *dpp<sup>MA3.0</sup>-hsp70-lacZ* and *dpp<sup>MA3.0-MS2</sup>-hsp70-lacZ* were then cloned into *pAttB* by partial

digestion with *KpnI*, extraction of the 14.5kb fragment, and ligation into *KpnI*-digested *pAttB*. Proper

ligation was tested by cracking, PCR, test digestion, and sequencing. Correct construction of final

constructs was confirmed by sequencing *Shn/pMad/Med* binding sites and cloning junctions.

Construction of *dpp<sup>APRD</sup>-lacZ* series:

A 2.9kb *HindIII* (21045)-*NheI* (23900) fragment of *dpp cis*-regulatory sequence, digested from *pHSG398-*

*dpp<sup>MA3.0</sup>-hsp70-lacZ*, was isolated and cloned into *HindIII/XbaI* digested *pHSG398* to generate *pHSG398-*

*dpp<sup>APRD</sup>*. *Ci* binding sites were mutated within the context of a smaller clone of *dpp* sequence, *pDonr221-*

*dpp<sup>BS3.2</sup>*, containing a 2.9kb *PvuII* (21860)/*HindIII* (24754) fragment of control region. *dpp* sequence was

amplified by PCR using the forward primer,

*GGGGACAAGTTTGTACAAAAAAGCAGGCTAAGCTT*GCTGCAGGTGTGTGTTTATGG, which introduces an

*attB1* site (italicized) and a *HindIII* recognition site (underlined) and the reverse primer,

*GGGGACCACTTTGTACAAGA AAGCTGGGTCCCGGGT*GCGAACTATAAAAAATACACCC, which introduces an

*attB2* site (italicized) and a *SmaI* recognition site (underlined). This sequence was then recombined into

the pDonr221 Gateway Cloning vector to create *pDonr221-dpp<sup>BS3.2</sup>*. Within this vector, Ci binding sites located at positions 22200 (site 1), 22250 (site 2), 22290 (site 3), 23680 (site 4), and 23870 (site 5) of the *dpp* cis-regulatory sequence were mutated based on the Stratagene QuickChange Site Directed Mutagenesis kit using Pfu high-fidelity DNA polymerase. The first four sites were mutated at base six of the Ci consensus sequence, TGGGt/aGGTC, from a G to an A. At site five, which is overlapping with a potential Mad binding site, three versions were created: A, which is the same mutation as in sites one to four and which alters both the Ci and Mad binding sites, B, which alters only the Mad binding site, and Ci, which alters only the Ci binding site. The following primers were used for site directed mutagenesis with altered bases bolded:

site 1 forward primer GTATATAAATGAGATGCGG**C**AACCTAATGAGCCTGATTAAC,

site 1 reverse primer GTTAATCAGGCTCATTAGG**T**GCCGCATCTCATTATATAC,

site 2 forward primer CGGTCCTCACGGG**C**TGTCTCTACACCCAGC,

site 2 reverse primer GCTGGGTGTAGAGAC**A**GCCCGTGAGGAACCG,

site 3 forward primer GCCGCTCCCTTGTA**C**TCCCCCATCATCAAC,

site 3 reverse primer GTTGATGATGGGGGGG**A**TGTACAAGGGAGCGGC,

site 4 forward primer GACTTCATTGCTG**A**CTACCAGGCGAGCGGAG,

site 4 reverse primer CTCCGCTCGCCTGG**T**AGTCAGCAAATGAAGTC,

site 5 forward primer version "A" GCTTTCCGATTGGG**T**AGCGGCAGCCCCTCAG,

site 5 reverse primer version "A" CTGAGGGGCTGCCG**C**TACCCAATCGGAAAGC,

site 5 forward primer version "B" GCTTTCCGATTGGG**T**GCG**A**TAGCCCCTCAGC,

site 5 reverse primer version "B" GCTGAGGGGCT**A**TCGCCACCCAATCGGAAAGC,

site 5 forward primer version "C" GATCAGCTTCCG**A**T**G**AAGTGGCGGCAGCCCCTCAG, and

site 5 reverse primer version "C" CTGAGGGGCTGCCGCC**A**CT**T**CATCGGAAAGCTGATC.

Once mutated, regions containing the mutant Ci binding sites were removed from the *pDonr221-dpp<sup>BS3.2-Ci</sup>* plasmids and introduced into *pHSG398-dpp<sup>APRD</sup>*. The first three Ci sites were removed from *pDonr221-dpp<sup>BS3.2-Ci123</sup>* as a 611 PstI digestion product that was purified from an agarose gel. This product was then ligated to a 4.5kb gel-purified PstI digestion product of *pHSG398-dpp<sup>APRD</sup>* and orientation of insert was

checked by PCR. The last two mutant Ci sites were amplified from *pDonr221-dpp<sup>BS3.2-Ci1-5</sup>* by PCR using a forward primer (CCTTTTGACCTCGAGCCGGG) and a reverse primer (GGCGGCGGATCCGCTAGCCAATTCCGGTGGTG), the latter of which introduces a BamHI recognition site (underlined) that allows for insertion within the *pHSG398-dpp<sup>APRD-Ci123</sup>* multiple cloning site. The PCR product was purified, digested with BamHI, and cloned into a 3.7kb band of *pHSG398-dpp<sup>APRD-Ci3</sup>* that had been digested with BamHI and purified. The orientation of inserts was determined by PCR amplification. The following combinations of Ci binding site mutants were created: *dpp<sup>APRD-Ci5</sup>* containing mutations in all Ci sites with site five mutated by primer A, *dpp<sup>APRD-Ci4-Mad</sup>* containing mutations in four Ci sites and one Mad site with site five mutated by primer B, and *dpp<sup>APRD-Ci4-M</sup>* containing mutations in all Ci sites and one Mad site with site five mutated by primer C. To create the *lacZ* reporter construct, we first created a *pHSG398-hsp70-lacZ* vector by digesting *pHSG398-dpp<sup>MA3.0</sup>-hsp70-lacZ* with SmaI, gel-purifying the 6.8kb fragment containing vector and *hsp70-lacZ*, and self-ligating the vector. Wildtype and mutant *pHSG398-dpp<sup>APRD</sup>* was digested with HindIII and SmaI and the gel-purified 2.2kb product was ligated to HindIII/ SmaI digested *pHSG398-hsp70-lacZ* vector to insert regulatory sequences upstream of the *hsp70* promoter. Wildtype and mutant versions of *dpp<sup>APRD</sup>-hsp70-lacZ* sequence were then isolated from *pHSG398* by digestion with HindIII, klenow treatment to generate a blunt end, digestion with EcoRI, and gel extraction. This fragment was then cloned into *pAttB* by ligation to EcoRI/HpaI digested *pAttB*. Correct construction was confirmed by sequencing of Ci binding sites and cloning junctions.

Construction of *dpp<sup>APR</sup>lacZ* series:

To create the *dpp<sup>APR</sup>lacZ* and *dpp<sup>APR-Ci3</sup>lacZ* reporters, a 3' region of *dpp* sequence was removed from the *pHSG398-dpp<sup>APRD</sup>* and *pHSG398-dpp<sup>APRD-Ci3</sup>* constructs by BsaBI/SmaI digestion, purification of the 4.5kb fragment, and self-ligation. These truncated *dpp* sequences were then digested with KpnI, treated with Klenow to generate a blunt end, digested with HindIII, purified, and then ligated to HindIII/SmaI digested *pHSG398-hsp70-lacZ* to insert the *dpp* sequences upstream of *hsp70*. *pHSG398-dpp<sup>APR</sup>-hsp70-lacZ* and *pHSG398-dpp<sup>APR-Ci3</sup>-hsp70-lacZ* were then digested with HindIII, treated with Klenow to generate blunt ends, digested with EcoRI, purified, and then inserted into EcoRI/HpaI digested *pAttB* to generate *pAttB-*

*dpp<sup>APR</sup>-hsp70-lacZ* and *pAttB-dpp<sup>APR-Ci3</sup>-hsp70-lacZ*. Correct construction was confirmed by sequencing of Ci binding sites and cloning junctions.

Construction of transgenic lines:

The ΦC31 system was chosen to obtain efficient site-specific transgene incorporation into the *Drosophila* genome and allow for comparison of expression levels and patterns between reporter lines. An attP landing platform on the third chromosome, ZH-86Fb, was chosen based on reported high survival rates and transformation efficiency and based on high expression observed for *UAS* lines inserted into this location by a member of our lab (Takuya Akiyama, personal communication). The genotype of the stock used for injection, Bloomington stock 24749, was *y[1] M{vas-int.Dm}ZH-2A w[\*]; M{3xP3-RFP.attP}ZH-86Fb*. Injections were performed by Rainbow Transgenic Flies, Inc. Males recovered from injected embryos were crossed to *yw* females, progeny were screened for transgene insertion by *w<sup>+</sup>* selection, and males with successful incorporation were used to balance the transgene over *TM3 Sb*.

#### **Analysis of *dpp* cis-regulatory sequences for transcription factor binding sites and conservation**

The *dpp<sup>disk</sup>* cis-regulatory sequence was manually scanned by eye for consensus binding sites for several factors within the BMP signaling pathway or known to directly regulate *dpp* or associate with Mad. The factors and their consensus sequences are: Engrailed (En) with consensus sequence TAAATCA (Desplan et al., 1988; Kissinger et al., 1990), Brinker (Brk) with consensus sequence TGGCG c/t c/t (Zhang et al., 2001), Mad with consensus sequence GCCGNCGC (Kim et al., 1997), Ci with consensus sequence TGGGt/aGGTC (Kinzler and Vogelstein, 1990), Shn/pMad/Med with consensus sequence GNCGNCCCCNNNGTCT (Gao et al., 2005; Pyrowolakis et al., 2004), and dTCF with consensus sequence a/t a/t CAAAG (Wetering and Clevers, 1992). Sequences were examined in both the forward (*cis*) and reverse (*trans*) orientations.

The UCSC genome browser was used to determine the sequence conservation along the sequences found within the *dpp<sup>B53.0</sup>lacZ* reporter construct. Conservation was determined between twelve *Drosophila* species, the mosquito (*A. gambiae*), the honeybee (*A. mellifera*), and the beetle (*A. castaneum*). In the conservation display, peaks represent conservation score, with high peaks representing areas of high conservation. (<http://genome.ucsc.edu/>, BDGP Release 5 (Kent et al., 2002)).



### Analysis of *dpp-lacZ* reporter expression in *tkv<sup>QD</sup>* over-expression wing discs

The following crosses were performed to examine expression of the *dpp-lacZ* reporter lines noted:

*dpp<sup>p10638</sup>lacZ*: *w/Y* ; *dpp<sup>p10638</sup>lacZ* /+ ; *UAS-tkv<sup>QD</sup>* /+ males to *w* ; *ap-Gal4*, *UAS-GFP* ; +/*T(2;3) SM5-TM6b* females to generate *w* ; *dpp<sup>p10638</sup>lacZ* / *ap-Gal4 UAS-GFP* ; *UAS-tkv<sup>QD</sup>* /+ and *w* ; *dpp<sup>p10638</sup>lacZ* / *ap-Gal4* larvae. This experiment was performed to observe both *dpp<sup>p10638</sup>lacZ* expression and *Ci<sup>A</sup>* levels.

*dpp<sup>BS3.23</sup>lacZ*: *w/Y* ; *ap-Gal4*, *UAS-GFP* /+ ; *dpp<sup>BS3.23</sup>lacZ* /+ males to *UAS-tkv<sup>QD</sup>* females to generate *ap-Gal4*, *UAS-GFP* /+ ; *dpp<sup>BS3.23</sup>lacZ* / *UAS-tkv<sup>QD</sup>* and *dpp<sup>APR</sup>lacZ* / *UAS-tkv<sup>QD</sup>* larvae. *A9-Gal4/Y* ; *dpp<sup>BS3.23</sup>lacZ* /+ males to *UAS-tkv<sup>QD</sup>* and *Or* females to generate *A9-Gal4* /+ ; *dpp<sup>BS3.23</sup>lacZ* / *UAS-tkv<sup>QD</sup>* and *A9-Gal4* /+ ; *dpp<sup>BS3.23</sup>lacZ* /+ , respectively

*dpp<sup>Exel-1</sup>lacZ*: *w/Y* ; *dpp<sup>Exel-1</sup>lacZ* /+ ; *UAS-tkv<sup>QD</sup>* /+ males to *ap-Gal4*, *UAS-GFP* / *T(2;3) SM5-TM6b* females to generate *ap-Gal4*, *UAS-GFP* / *dpp<sup>Exel-1</sup>lacZ* and *ap-GFP*, *UAS-GFP* / *dpp<sup>Exel-1</sup>lacZ* ; *UAS-tkv<sup>QD</sup>* /+ larvae.

*dpp<sup>D</sup>GFP*: *w/Y* ; *ap-Gal4* /+ ; *dpp<sup>WT30</sup>GFP* /+ males to *UAS-tkv<sup>QD</sup>* females to generate *ap-Gal4* /+ ; *dpp<sup>WT30</sup>GFP* / *UAS-tkv<sup>QD</sup>* and *dpp<sup>WT30</sup>GFP* / *UAS-tkv<sup>QD</sup>* larvae

*dpp<sup>D-Ci3</sup>GFP*: *w/Y* ; *dpp<sup>KO19</sup>GFP* /+ ; *UAS-tkv<sup>QD</sup>* /+ males to *ap-Gal4* / *Sm6a* females to generate *ap-Gal4* / *dpp<sup>KO19</sup>GFP* and *ap-GFP* / *dpp<sup>KO19</sup>GFP* ; *UAS-tkv<sup>QD</sup>* /+ larvae.

*dpp<sup>APRD</sup>lacZ*, *dpp<sup>APRD-Ci5</sup>lacZ*, *dpp<sup>APRD-Ci5-M</sup>lacZ*, *dpp<sup>APRD-Ci4-M</sup>lacZ*, *dpp<sup>APR</sup>lacZ*, and *dpp<sup>APR-Ci3</sup>lacZ*:

*w/Y* ; *ap-Gal4*, *UAS-GFP* /+ ; *dpp-lacZ* /+ males to *UAS-tkv<sup>QD</sup>* and to *w* females to generate *ap-Gal4*, *UAS-GFP* /+ ; *dpp-lacZ* / *UAS-tkv<sup>QD</sup>* and *ap-Gal4*, *UAS-GFP* /+ ; *dpp-lacZ* /+ larvae, respectively.

*dpp<sup>MA3.0</sup>lacZ* and *dpp<sup>MA3.0-MS2</sup>lacZ*:

*w/Y* ; *ap-Gal4*, *UAS-GFP* /+ ; *dpp-lacZ* /+ males to *UAS-tkv<sup>QD</sup>* and to *w* females to generate *ap-Gal4*, *UAS-GFP* /+ ; *dpp-lacZ* / *UAS-tkv<sup>QD</sup>* and *ap-Gal4*, *UAS-GFP* /+ ; *dpp-lacZ* /+ larvae, respectively.

*dpp<sup>APRD</sup>lacZ*, *dpp<sup>APRD</sup>lacZ*, and *dpp<sup>APRD</sup>lacZ* (Theisen et al., 2007):

*dpp-lacZ/+ ; UAS- $tkv^{QD}$ /+ males to *w; ap-Gal4, UAS-GFP ; +/T(2;3) SM5-TM6b* females to generate *ap-Gal4, UAS-GFP/ dpp-lacZ ; UAS- $tkv^{QD}$ /+ and *ap-Gal4, UAS-GFP/ dpp-lacZ* larvae.**

*dpp<sup>APRd</sup>lacZ*, and *dpp<sup>APrd</sup>lacZ* (Theisen et al., 2007):

*ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+ males to *w ; UAS- $tkv^{QD}$  females to generate *ap-Gal4, UAS-GFP/+ ; UAS- $tkv^{QD}$ / dpp-lacZ and *UAS- $tkv^{QD}$ / dpp-lacZ* larvae.***

#### **Analysis of *dpp-lacZ* reporter expression in *tkv<sup>QD</sup>* over-expression FLP-out clones**

Clones of cells over-expressing *UAS- $tkv^{QD}$*  were generated by the FLP-out method (Struhl and Basler, 1997). Animals over-expressing *UAS- $tkv^{QD}$*  were generated by crossing:

*y w/Y ; AyGal4 UAS-GFP/+ ; dpp<sup>BS3.23</sup>lacZ* males to *y w f hsFLP122 ; UAS- $tkv^{QD}$  and *Ore-R* wildtype females to generate *y w f hs-FLP122/+ ; AyGal4 UAS-GFP /+ ; dpp<sup>BS3.23</sup>lacZ /UAS- $tkv^{QD}$  and *AyGal4 UAS-GFP /+ ; dpp<sup>BS3.23</sup>lacZ /+ larvae.***

Crosses were pre-mated in glass vials and then moved to apple juice plates with yeast paste and brooded every 24 hours. To induce the expression of FLP recombinase, apple juice plates containing larvae were sealed and heat shocked at 37°C by submersion in a water bath for 10-15 minutes at either 48-72 or 54-76 hours post lay to generate early or mid second instar clones. Clones were identified by the expression of GFP.

#### **Analysis of *ptc-lacZ* reporter expression in *tkv<sup>QD</sup>* over-expression wing discs**

The following crosses were performed to examine expression of *ptc-lacZ* with increased BMP signaling:

*MS1096-Gal4/Y ; ptc-lacZ/+ males were crossed to *Ore-R* wildtype and *UAS- $tkv^{QD}$  females to generate *MS1096-Gal4/Y ; ptc-lacZ/+ ; UAS- $tkv^{QD}$ /+ and *ptc-lacZ/+ ; UAS- $tkv^{QD}$ /+ larvae****

*A9-Gal4/Y ; ptc-lacZ/+ males were crossed to *Ore-R* wildtype and *UAS- $tkv^{QD}$  females to generate *A9-Gal4/Y ; ptc-lacZ/+ ; UAS- $tkv^{QD}$ /+ and *ptc-lacZ/+ ; UAS- $tkv^{QD}$ /+ larvae****

#### **Analysis of *dpp-lacZ* expression in the presence of *UAS-ci-RNAi* and *UAS- $tkv^{QD}$***

The *UAS-ci-RNAi* line used in these experiments is line #51479 from the VDRC.

The following crosses were performed to examine expression of *dpp<sup>p10638</sup>lacZ* :

*dpp<sup>p10638</sup>lacZ/+ ; UAS-tnv<sup>QD</sup>/+* males were crossed to *w ; ap-Gal4, UAS-GFP ; +/ T(2;3) SM5-TM6b* females to generate *ap-Gal4, UAS-GFP/ dpp<sup>p10638</sup>lacZ ; UAS-tnv<sup>QD</sup>/+* and *ap-Gal4, UAS-GFP/ dpp<sup>p10638</sup>lacZ* larvae.

*dpp<sup>p10638</sup>lacZ, UAS-ci-RNAi/Sm6a* males were crossed to *w ; ap-Gal4, UAS-GFP ; +/ T(2;3) SM5-TM6b* females to generate *ap-Gal4, UAS-GFP/ dpp<sup>p10638</sup>lacZ, UAS-ci-RNAi* larvae

*dpp<sup>p10638</sup>lacZ, UAS-ci-RNAi/CyO-GFP ; UAS-tnv<sup>QD</sup>/TM6b* males were crossed to *w ; ap-Gal4, UAS-GFP ; +/ T(2;3) SM5-TM6b* females to generate *ap-Gal4, UAS-GFP/ dpp<sup>p10638</sup>lacZ, UAS-ci-RNAi ; UAS-tnv<sup>QD</sup>/+* larvae

The following crosses were performed to examine expression of *dpp<sup>APRD</sup>lacZ*, *dpp<sup>APRD-Ci5</sup>lacZ*, and *dpp<sup>APRD-Ci5-M</sup>lacZ*:

*ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* males were crossed to *w* females to generate *ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* larvae

*ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* males were crossed to *w ; UAS-tnv<sup>QD</sup>* females to generate *ap-Gal4, UAS-GFP/+ ; dpp-lacZ/ UAS-tnv<sup>QD</sup>* larvae

*ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* males were crossed to *w ; UAS-ci-RNAi* females to generate *ap-Gal4, UAS-GFP/ UAS-ci-RNAi ; dpp-lacZ/ +* larvae

*ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* males were crossed to *w ; UAS-ci-RNAi/+ ; UAS-tnv<sup>QD</sup>/+* females to generate *ap-Gal4, UAS-GFP/ UAS-ci-RNAi ; dpp-lacZ/ UAS-tnv<sup>QD</sup>* larvae

#### **Analysis of *dpp<sup>p10638</sup>lacZ* expression in the presence of *UAS-shn-RNAi* and *UAS-tnv<sup>QD</sup>***

The *UAS-shn-RNAi* line used in these experiments is line #105643 from the VDRC. Clones of cells over-expressing *UAS-tnv<sup>QD</sup>* or *UAS-shn-RNAi* were generated by the FLP-out method (Struhl and Basler, 1997) through the following crosses:

*y w f hs-FLP122/Y ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/CyO-GFP* males were crossed to *y w ; dpp<sup>p10638</sup>lacZ/Sm6a* females to generate *y w f hs-FLP122/+ ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ* larvae

*y w f hs-FLP122/Y ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/CyO-GFP* males were crossed to were crossed to *y w ; dpp<sup>p10638</sup>lacZ, UAS-shn-RNAi/Sm6a* females to generate *y w f hs-FLP122/+ ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ, UAS-shn-RNAi* larvae

*y w f hs-FLP122/Y ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/CyO-GFP* males were crossed to were crossed to *w ; dpp<sup>p10638</sup>lacZ ; UAS-tkv<sup>QD</sup>/T(2;3) SM5-TM6b* females to generate *y w f hs-FLP122/+ ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ ; UAS-tkv<sup>QD</sup>* larvae

*y w f hs-FLP122/Y ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/CyO-GFP* males were crossed to were crossed to *w ; dpp<sup>p10638</sup>lacZ, UAS-shn-RNAi/CyO-GFP ; UAS-tkv<sup>QD</sup>/TM6b* females to generate *y w f hs-FLP122/+ ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ, UAS-shn-RNAi ; UAS-tkv<sup>QD</sup>/+* larvae

Crosses were pre-mated in glass vials and then moved to apple juice plates with yeast paste and brooded every 24 hours. To induce FLP recombinase expression, apple juice plates containing larvae were sealed and heat shocked at 37°C by submersion in a water bath for 10-15 minutes at either 48-72 or 54-76 hours post lay to generate early or mid second instar clones. Clones were identified by the expression of GFP.

#### **Analysis of *omb-lacZ* expression in the presence of *UAS-shn-RNAi* and *UAS-tkv<sup>QD</sup>***

Clones of cells over-expressing *UAS-tkv<sup>QD</sup>* or *UAS-shn-RNAi* were generated by the FLP-out method (Struhl and Basler, 1997) through the following crosses:

*y w f hs-FLP122/Y ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/CyO-GFP* males were crossed to were crossed to *omb-lacZ/+ ; UAS-shn-RNAi/+* females to generate *y w f hs-FLP122/omb-lacZ ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/+ ; UAS-shn-RNAi /+* larvae

*y w f hs-FLP122/Y ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/CyO-GFP* males were crossed to were crossed to *omb-lacZ/+ ; UAS-tkv<sup>QD</sup>/+* females to generate *y w f hs-FLP122/omb-lacZ ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/+ ; UAS-tkv<sup>QD</sup>/+* larvae

*y w f hs-FLP122/Y ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/CyO-GFP* males were crossed to were crossed to *omb-lacZ* females to generate *y w f hs-FLP122/omb-lacZ ; Actin-FRT-y<sup>+</sup>-FRT-Gal4 UAS-GFP/+* larvae

Crosses were pre-mated in glass vials and then moved to apple juice plates with yeast paste and brooded every 24 hours. To induce FLP recombinase expression, apple juice plates containing larvae were sealed

and heat shocked at 37°C by submersion in a water bath for 10-15 minutes at either 48-72 or 54-76 hours post lay to generate early or mid second instar clones. Clones were identified by the expression of GFP.

## Results

### **The *dpp*<sup>BS3.0</sup>*lacZ* cis-regulatory sequences are characterized by many highly conserved regions**

Our findings thus far have demonstrated that transcription of the *dpp* reporter *dpp*<sup>BS3.0</sup>*lacZ* is negatively regulated in response to increased BMP signaling, indicating that the *cis*-regulatory sequences mediating this negative response must map within the control sequences contained within this construct. To determine potential *trans*-acting factors that mediate this effect, we wished to identify the specific *cis*-regulatory sequences responsible for mediating the negative regulation of *dpp* transcription. A notable characteristic of developmentally relevant CRMs is that they are often highly conserved (Dickmeis and Müller, 2005), and given the important role that BMP signaling plays across many species, it is possible that negative regulation of *dpp* is a conserved mechanism.

The *dpp*<sup>BS3.0</sup>*lacZ* reporter contains approximately 10kb of *dpp*<sup>disk</sup> sequence which directs expression of *lacZ* in the wing imaginal disc, among other discs, and contains distinct CRMs that generate the wing disc expression domains (Fig 3.1A) (Blackman et al., 1991; Hepker et al., 1999; Masucci et al., 1990; Müller and Basler, 2000; Parker et al., 2011). Considering the large size of this fragment, we first took an unbiased bioinformatics approach to identify smaller, conserved regions of this sequence that might mediate transcriptional regulation of *dpp*. If BMP-mediated negative transcriptional regulation of *dpp* plays an important role in maintaining robust BMP signaling, then we might expect this behavior to be conserved and be reflected as highly conserved *cis*-regulatory regions to mediate regulation of *dpp*. We used the UCSC Genome Browser (<http://genome.ucsc.edu/>) to identify the conserved sequences in the 10kb of regulatory sequence of *dpp*<sup>BS3.0</sup>*lacZ* between twelve species of Drosophilids, the mosquito (*A. gambiae*), the honeybee (*A. mellifera*), and the beetle (*A. castaneum*) (Kent et al., 2002). We expected to see peaks of conservation in regions known to direct *dpp* expression as well as previously uncharacterized peaks that could potentially mediate feedback regulation of *dpp* expression. While we did observe high

**Figure 3.1. Expression of *dpp-lacZ* reporter constructs is reduced in response to increased BMP signaling. A:** Map depicting 10.2kb of *dpp<sup>disk</sup>* regulatory region within *dpp<sup>B53.0</sup>lacZ* (Blackman et al., 1991). The regions of regulatory sequence contained within various reporter constructs tested in **C-F** are denoted in red. For each, the regions highlighted direct expression of the reporter gene *lacZ*. These reporter constructs include: *dpp<sup>B53.23</sup>lacZ* (360bp) (Blackman et al., 1991; Hepker et al., 1999; Kopp et al., 1999), *dpp<sup>Exel1</sup>lacZ* (3.7kb) (Parks, 2004),

*dpp<sup>APRD</sup>lacZ* (2.9kb), and *dpp<sup>APR</sup>lacZ* (2.3kb) (Theisen et al., 2007)(materials and methods). Locations of consensus binding sites for several transcriptional factors known to be associated with BMP signaling are marked: B, Brinker in blue (Sivasankaran et al., 2000; Zhang et al., 2001); C, Cubitus Interruptus in green (Kinzler and Vogelstein, 1990); T, *Drosophila* TCF/Pangolin in pink (Tetsu and McCormick, 1999; van de Wetering et al., 1997); E, Engrailed in purple (Desplan et al., 1988; Kissinger et al., 1990); M, Mad in red (Kim et al., 1997); and S, Shn/pMad/Med complex in orange (Gao et al., 2005; Pyrowolakis et al., 2004). Asterisks mark highly conserved binding sites (for higher resolution see figures 7.12-7.16). The numbers at the bottom of the map denote the map locations based on a chromosomal walk performed on this region (St Johnston et al., 1990). **B:** Conservation across this region is seen in many small but highly conserved and potentially functionally relevant regions distributed throughout the sequence (<http://genome.ucsc.edu/>) (Kent et al., 2002). **C-F.** For this and all experiments, unless otherwise noted, expression of *dpp-lacZ* reporters were examined in control and *ap>tkv<sup>QD</sup>* wing discs. Late third instar larvae were dissected and expression of reporters was visualized by anti-βGal antibody (shown in red). For each individual experiment, images were taken at identical confocal settings to allow comparison. Intensity plots generated from the boxed regions display the expression pattern of each reporter. When noted, these profiles represent an average profile generated from multiple samples. **C.** Expression patterns of *dpp<sup>Exel1</sup>lacZ* in control (**C**, n=1) and *Ap>tkv<sup>QD</sup>* (**C'**, n=2) wing pouches. **C''.** Expression of *dpp<sup>Exel1</sup>lacZ* is reduced in a subset of its expression domain with increased BMP signaling. **D.** Expression patterns of *dpp<sup>BS3.23</sup>lacZ* in control (**D**, n=2) and *Ap>tkv<sup>QD</sup>* (**D'**, n=3) wing pouches. **D''.** Expression of *dpp<sup>BS3.23</sup>lacZ* is reduced throughout its expression domain with increased BMP signaling. **E.** Expression patterns of *dpp<sup>APRD</sup>lacZ* in control (**E**, n=8) and *Ap>tkv<sup>QD</sup>* (**E'**, n=2) wing pouches. **E''.** Expression of *dpp<sup>APRD</sup>lacZ* is reduced in a subset of its expression domain with increased BMP signaling. **F.** Expression patterns of *dpp<sup>APR</sup>lacZ* in control (**F**, n=9) and *Ap>tkv<sup>QD</sup>* (**F'**, n=6) wing pouches. **F''.** Expression of *dpp<sup>BS3.23</sup>lacZ* is reduced in the medial region of the wing pouch with increased BMP signaling. For **E** and **F**, see Materials and Methods for details of reporter construction.

conservation between Drosophilids in the expected regions, analysis of the entire 10kb sequence did not reveal any distinct regions that could mediate feedback regulation of *dpp* (Fig 3.1B). Instead, peaks of high conservation were observed throughout the region, making it difficult to focus on or exclude any particular regions. It is likely that regulation of *dpp*, both general regulation and feedback regulation, occurs in response to binding of several factors at many locations throughout its *cis*-regulatory sequence.

### Many regions of the *dpp cis*-regulatory sequence mediate negative feedback of *dpp*

In order to narrow down the region of potential *cis*-regulatory sequences responsible for the negative feedback regulation of *dpp*, a number of reporter constructs with smaller control regions than contained within *dpp<sup>BS3.0</sup>lacZ* were tested for their response to increases in BMP signaling. In all cases except where noted, BMP signaling was increased by directing the expression of *UAS-tkv<sup>QD</sup>* in the dorsal compartment of the wing imaginal disc using the *ap-Gal4* driver. For all over-expression experiments, a representative example of a control and an over-expression wing pouch are shown. We generated intensity profiles representing *lacZ* expression from the boxed regions in the displayed pouches. When noted, average profiles were generated from intensity profiles from two or more samples, which include the displayed sampled, to more accurately reflect the expression behavior between samples. The



expression of four *dpp* reporter lines were examined in this manner and the general conclusions from this analysis are that increased BMP signaling affects the expression of *dpp* in some or all of the *dpp* expression domain and that several regions of the control sequences can mediate this response.

We first tested whether the sequences within the reporter lines *dpp<sup>Exel1</sup>lacZ* and *dpp<sup>BS3.23</sup>lacZ* are competent to mediate negative feedback of *dpp* lines in response to altered BMP signaling. These two reporters contain 3.7kb and 360bp, respectively, of non-overlapping *dpp* control sequence (Hepker et al., 1999; Kopp et al., 1999; Masucci et al., 1990; Parks, 2004). In tissue with normal levels of BMP signaling, they display strong and distinct staining along the anterior-posterior compartment of the wing pouch, recapitulating expression of *dpp<sup>BS3.0</sup>lacZ*, *dpp<sup>p10638</sup>lacZ*, and *dpp* RNA itself (Fig 3.1C,D, Fig 2.3B). Elevated BMP signaling affects the expression of both *dpp<sup>Exel1</sup>lacZ* and *dpp<sup>BS3.23</sup>lacZ*. The expression of *dpp<sup>Exel1</sup>lacZ* is repressed in a subset of its expression domain, resulting in an expression pattern that appears narrowed compared to the wildtype control wing pouch (Fig 3.1C), whereas *dpp<sup>BS3.23</sup>lacZ* expression is reduced throughout its entire expression domain (Fig 3.1D).

We next tested two other reporter lines, *dpp<sup>APRD</sup>lacZ* and *dpp<sup>APR</sup>lacZ* (Theisen et al., 2007)(see materials and methods). The regulatory sequences in *dpp<sup>APRD</sup>lacZ* span the sequences between *dpp<sup>Exel1</sup>lacZ* and *dpp<sup>BS3.23</sup>lacZ*, while the regulatory sequences in *dpp<sup>APR</sup>lacZ* lack those of *dpp<sup>BS3.23</sup>lacZ*. Expression of both reporters is reduced with increased BMP signaling, with expression of *dpp<sup>APRD</sup>lacZ* repressed in a subset of its expression domain and *dpp<sup>APR</sup>lacZ* repressed throughout its expression domain (Fig 3.1E,F). Our analysis of these four *dpp* reporter constructs indicates that the control sequences present in all reporters tested are sufficient to mediate negative feedback regulation of *dpp* in response to increased BMP signaling. Furthermore, as the sequences present within *dpp<sup>BS3.23</sup>lacZ* does not overlap with those of *dpp<sup>Exel1</sup>lacZ* or *dpp<sup>APR</sup>lacZ*, these results indicate that the sequences that mediate feedback are duplicated or functionally redundant.

***dpp* control regions contain many potential binding sites for *cis* acting factors potentially mediating negative feedback of *dpp* transcription**

To identify potential factors responsible for mediating feedback regulation of *dpp* expression we searched the *dpp*<sup>BS3.0</sup>*lacZ* sequence for consensus binding sites of known transcription factors. A closer examination of the sequences present within the *dpp*<sup>BS3.0</sup>*lacZ* regulatory region revealed the presence of consensus binding sites for several factors known to be associated with BMP signaling or specifically with regulation of *dpp*: Brk, Ci, dTCF, En, Mad, and Shn/pMad/Med. Many of these had previously been identified, but we also manually searched for sequences matching published consensus binding sites for these factors. The locations of these sites are shown in figure 3.1A within the context of the *dpp*<sup>BS3.0</sup>*lacZ* regulatory region.

Four potential binding sites for Brk were identified that matched the consensus site, TGGCG C/T C/T (Sivasankaran et al., 2000; Zhang et al., 2001), two of which have previously been identified and tested. While a role for Brk in regulating *dpp* in the wing disc has not been established, binding of Brk to two of these sites represses *dpp* expression in the leg disc (Theisen et al., 2007). Five binding sites for Ci were identified that display considerable similarity to the consensus sequence, TGGG T/A GGTC (Kinzler and Vogelstein, 1990), all of which have been previously identified, tested, and found to regulate *dpp* expression in the wing imaginal disc (Hepker et al., 1999; Johnson et al., 2008; Müller and Basler, 2000; Parker et al., 2011). The Ci binding sites are found in two clusters, three in a proximal CRM (henceforth referred to as sites 1, 2, or 3) and two in a distal CRM (henceforth referred to as sites 4 or 5). Seventeen consensus binding sites for dTCF (*Drosophila* TCF or *pangolin*), the downstream effector of WNT/Wg signaling, were identified matching the consensus sequences, AACAAAG and TTCAAAG (Tetsu and McCormick, 1999; Van de Wetering and Clevers, 1992). dTCF is known to directly repress *dpp* in both the embryonic visceral mesoderm (Yang et al., 2000) and the leg imaginal disc (Theisen et al., 2007). Of the seventeen dTCF sites identified, five are important in leg imaginal disc expression of *dpp* (Theisen et al., 2007) and two are important in wing imaginal disc expression of *dpp*<sup>BS3.23</sup>*lacZ* (Hepker et al., 1999). Six Engrailed consensus binding sites, TAATCA (Desplan et al., 1988; Kissinger et al., 1990), were identified, one of which has previously been identified, directly tested, and shown to mediate repression of *dpp* in the posterior wing disc (Müller and Basler, 2000). Five consensus binding sites for Mad were identified showing similarity to the consensus sequence GCCGNCGC (Hepker et al., 1999; Kim et al., 1997;

Kirkpatrick et al., 2001; Newfeld and Takaesu, 2002). We also identified two consensus binding sites for the Shn/pMad/Med complex, GNCGNCNNNNGTCT (Gao et al., 2005; Pyrowolakis et al., 2004). While this complex is important in regulation of several BMP targets, no role has been established for it in the regulation of *dpp*. Sequence alignments of the *dpp<sup>BS3.0</sup>lacZ* sequence revealed numerous putative binding sites that were highly conserved (Fig 3.1B starred, Figs 7.12-7.16) (Kent et al., 2002). The high conservation of these binding sites suggests they may play a role in development.

Of the factors whose consensus binding sites were found within the *dpp<sup>BS3.0</sup>lacZ* cis-regulatory region, all but En were chosen for further study. Despite the role that En plays in repressing *dpp* in the posterior compartment, its limited expression in the anterior compartment makes it an unlikely candidate to mediate repression of *dpp* (Blair, 1992; Raftery et al., 1991; Sanicola et al., 1995; Zecca et al., 1995). To determine if these factors were responsible for mediating feedback, we tested how both mutation of a factor's consensus binding site, and presumably loss of binding to that site, or the loss of protein affected the expression of *dpp-lacZ* reporters in response to increased BMP signaling. To test the role of putative binding sites, we obtained or created *dpp-lacZ* reporters with mutated consensus binding sites for each factor and asked if expression is altered with increased BMP signaling. To test the requirement for the transcription factor itself, we asked if expression of *dpp<sup>p10638</sup>lacZ* is reduced in response to increased BMP signaling in the absence of that factor, achieved through the use of RNAi to knockdown the expression of the factor.

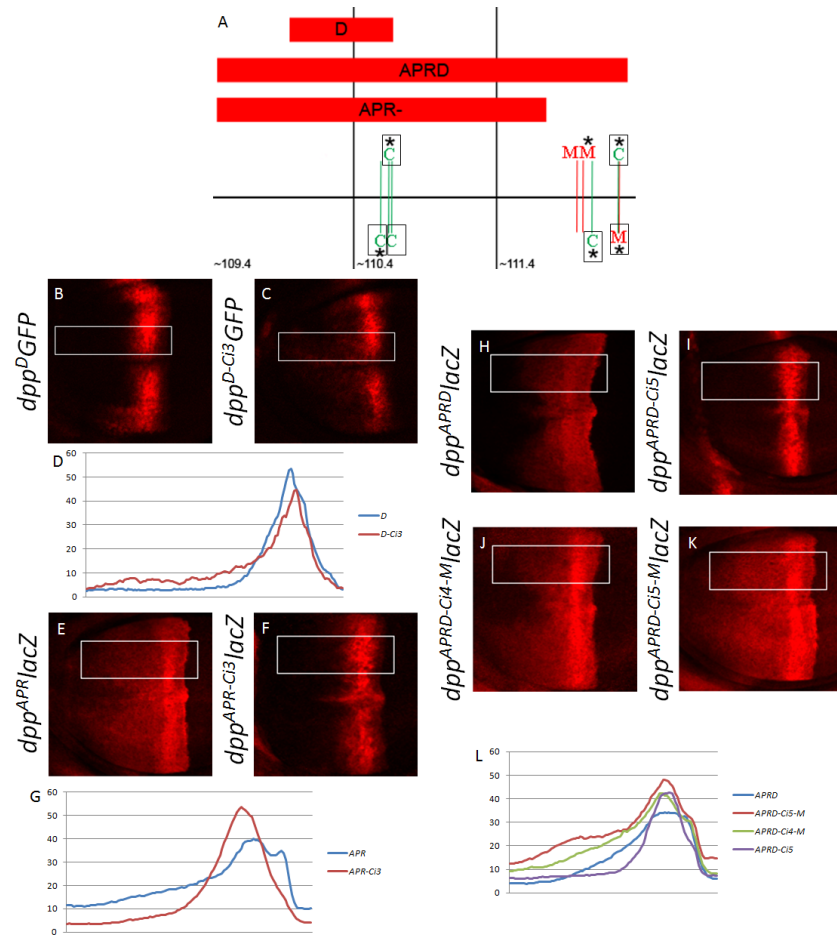
**Ci and Mad consensus binding sites are important in regulating expression of *dpp*, but not in mediating repression of *dpp*.**

Common to all of the cis-regulatory regions capable of mediating a *dpp* transcriptional response to increased BMP signaling is the presence of Ci binding sites. Ci is an attractive candidate factor to mediate negative regulation of *dpp* as it has been shown to both activate and repress *dpp* in the anterior wing pouch (Méthot and Basler, 1999; Müller and Basler, 2000). To test this we first asked whether binding of Ci to *dpp-lacZ* reporters is required for negative feedback regulation of *dpp* expression. If binding of Ci is required, we predict that reporter lines with mutated Ci binding sites would be

unresponsive to changes in BMP signaling. To disrupt binding of Ci, we obtained and created *dpp* reporter constructs with mutated and non-functional consensus Ci binding sites. It is of note that previous studies on the role of Ci in regulating *dpp* have indicated that, while Ci is important in generating the wildtype expression pattern of *dpp*, it is not absolutely required for basal *dpp* expression in the wing disc. This was a key consideration in the design of these experiments, as the remaining basal expression of *dpp* allows us to test whether any *dpp* expression can be repressed by increased BMP signaling in the absence of Ci.

*dpp<sup>D</sup>GFP* and *dpp<sup>D-Ci3</sup>GFP* are *dpp* reporter lines with wildtype and non-functional Ci binding sites, respectively (Johnson et al., 2008; Parker et al., 2011). These particular reporters are constructed from a 720bp region of the *dpp<sup>disk</sup>* cis-regulatory sequence that contains the proximal CRM with three Ci binding sites and has been identified as a minimal enhancer for the endogenous expression domain of *dpp* of the wing pouch (Fig 3.2A) (Müller and Basler, 2000). Unlike other reporters that we have used, this one directs expression of *GFP* instead of *lacZ*. Under endogenous signaling condition, *dpp<sup>D</sup>GFP* is expressed in a narrow stripe of cells along the anterior posterior compartment of the wing pouch (Fig 3.2B) while *dpp<sup>D-Ci3</sup>GFP* is expressed at slightly lower levels in an expanded expression domain far into the anterior compartment (Fig 3.2C,D). The expression pattern of the *dpp<sup>D-Ci3</sup>GFP* reporter can be explained by the loss of transcriptional activation by Ci<sup>A</sup> along the anterior-posterior boundary and the loss of repression by Ci<sup>R</sup> throughout the remainder of the anterior compartment. Expression in the absence of Ci is due to activation by the currently unknown “basal” activator of *dpp* expression (Müller and Basler, 2000).

In addition to the sequences present in *dpp<sup>D</sup>GFP*, we were particularly interested in the control sequences used in the construction of *dpp<sup>APRD</sup>lacZ* as this region contains five Ci binding sites and is competent to mediate a transcriptional response of *dpp-lacZ* expression (Fig 3.2A). These Ci sites have, independently, been found critical in regulation of *dpp* but they have not yet been tested together. Using site-directed mutagenesis, the first four Ci consensus binding sites were disrupted in the context of *dpp<sup>APRD</sup>lacZ* to interfere with binding of Ci to the DNA (Pavletich and Pabo, 1992). Sequence analysis of the fifth Ci binding site (henceforth referred to as “site 5”) revealed that it partially overlaps with a potential Mad binding site (Hepker et al., 1999) (Fig 3.2A) at the sequence, TGGGTGGCGGC, with the Ci site in italics



**Figure 3.2. Mad and Ci binding sites are important in generating the expression pattern of *dpp* reporters.** The expression patterns of reporter constructs with wildtype or mutant binding sites for Mad and Ci were examined under endogenous levels of BMP signaling. **A.** Map depicting the sequences present in the three reporter constructs examined and the locations of binding sites for Ci and Mad. Those that were mutated and tested are boxed. **B-L.** Third instar larvae were dissected and expression of reporters was visualized by anti-βGal antibody or direct immunofluorescence of GFP (in red). Confocal images of all GFP (**B, C**) and *lacZ* (**E-F, H-K**) reporters were taken at the same confocal settings to allow for comparison between samples. **B-D.** Expression of *dpp<sup>D</sup>GFP* (**B**, n=1) compared to *dpp<sup>D-Ci3</sup>GFP* (**C**, n=1). Expression is reduced and expanded into the anterior compartment when Ci binding sites are mutated (**D**). (Johnson et al., 2008; Parker et al., 2011) **E-G.** Expression of *dpp<sup>APR</sup>lacZ* (**E**, n=6) compared to *dpp<sup>APR-Ci3</sup>lacZ* (**F**, n=6). Expression is narrowed and increased when all Ci binding sites present are mutated (**G**). **H-L.** Comparison of the expression patterns of *dpp<sup>APRD</sup>lacZ* (**H**, n=5), *dpp<sup>APRD-Ci5-M</sup>lacZ* (**I**, n=5), *dpp<sup>APRD-Ci4-M</sup>lacZ* (**J**, n=6), and *dpp<sup>APRD-Ci5</sup>lacZ* (**K**, n=4). **L.** Expression of *dpp<sup>APRD</sup>lacZ* is influenced by binding of both Mad and Ci. The *dpp<sup>APRD-Ci5</sup>lacZ* expression domain is narrowed and decreased compared to *dpp<sup>APRD</sup>lacZ*, similar to *dpp<sup>APR-Ci3</sup>lacZ* (**F**). Expression of both *dpp<sup>APRD-Ci4-M</sup>lacZ* and *dpp<sup>APRD-Ci5-M</sup>lacZ* with a mutated Mad binding site is increased and in a wider domain than *dpp<sup>APRD</sup>lacZ*.

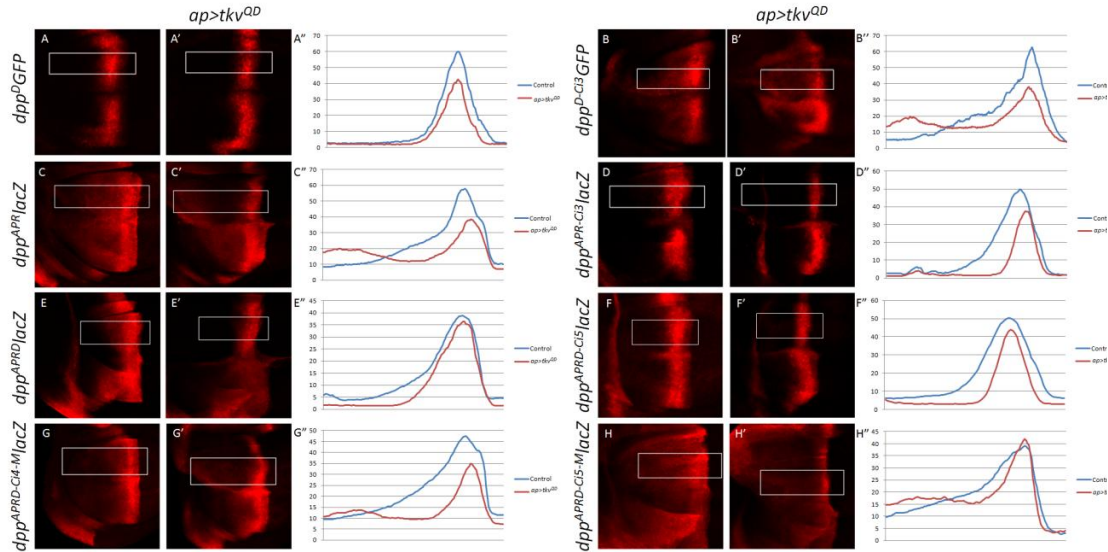
and the Mad site underlined. Creating the corresponding Ci mutation from the first four Ci sites would disrupt both the Ci and Mad binding sites; in the event that this Mad site was important, we chose to create three independent mutations at site five in *dpp<sup>APRD</sup>lacZ* such that only Ci, or only Mad, or both Ci and Mad binding would be disrupted (see material and methods for details of mutagenesis and transgenic creation). In all, the following constructs and transgenic lines were generated: *dpp<sup>APRD</sup>lacZ* wildtype

reporter,  $dpp^{APRD-Ci5}lacZ$  reporter with all Ci sites mutated,  $dpp^{APRD-Ci5-M}lacZ$  reporter with all Ci sites and one Mad site mutated, and  $dpp^{APRD-Ci4-M}lacZ$  reporter with four Ci sites and one Mad site mutated. Considering the strong repression of  $dpp^{APR}lacZ$  expression that we saw in response to increased BMP signaling, we also created  $dpp^{APR}lacZ$  with wildtype and mutant Ci binding sites, denoted as:  $dpp^{APR}lacZ$  with all wildtype sites, and  $dpp^{APR-Ci3}lacZ$  with three mutant Ci sites.

Mutation of the Ci and Mad binding sites leads to slightly different expression patterns in each reporter in a wildtype background (Fig 3.2-E-L). Surprisingly, loss of Ci binding in the context of the larger *cis*-regulatory sequences contained within  $dpp^{APR}lacZ$  and  $dpp^{APRD}lacZ$  did not recapitulate the expression pattern of  $dpp^{D-Ci3}GFP$ . Instead of the expanded expression domain seen for  $dpp^{D-Ci3}GFP$ , the expression patterns of  $dpp^{APR-Ci3}lacZ$  and  $dpp^{APRD-Ci5}lacZ$  were both narrower and stronger than their wildtype counterparts (compare Fig 3.2C,F,I to 3.2B,E,H).

Even more surprising was the significant impact on *lacZ* expression of mutating the Mad binding site that overlaps with Ci site five. Both  $dpp^{APRD-Ci5-M}lacZ$  and  $dpp^{APRD-Ci4-M}lacZ$  are broadly expressed in the anterior compartment at an overall higher level than wildtype  $dpp^{APRD}lacZ$  (Fig 3.2J-L), suggesting that this previously uncharacterized Mad site may play a functional role in regulation of *dpp*. Furthermore, the overall increase in expression of  $dpp^{APRD-Ci5-M}lacZ$  and  $dpp^{APRD-Ci4-M}lacZ$  throughout their entire expression domains compared to  $dpp^{APRD}lacZ$  suggests that Mad may directly repress *dpp* in the lateral anterior compartment of a wildtype pouch, a region thought to be largely absent of Mad activity.

We next tested the effect of loss of Ci binding on BMP-induced *dpp* transcriptional repression. Importantly, the wildtype versions of  $dpp^{APR}lacZ$ ,  $dpp^{APRD}lacZ$ , and  $dpp^D GFP$  display a change in their expression patterns in response to increased BMP signaling (Fig 3.3A,C,E), indicating that the sequences present are competent to respond to the repressive influence from BMP signaling. We predicted that if binding of Ci or Mad is absolutely required for transcriptional regulation of *dpp*, then the expression of reporters with mutated binding sites should be unchanged in response to increased BMP signaling. However, the expression patterns of all reporters with mutated Ci binding sites were variably altered in response to  $ap>tkv^{QD}$  expression in wing pouches (Fig 3.3). Expression of the reporters that cannot bind Ci,  $dpp^{D-Ci3}GFP$ ,  $dpp^{APR-Ci3}lacZ$ , and  $dpp^{APRD-Ci5}lacZ$  was reduced throughout their expression domains in



**Figure 3.3. Expression of reporters with mutated Ci and Mad binding sites is altered in response to increased BMP signaling.** The expression patterns of *dpp-lacZ* and *dpp-GFP* reporter constructs with wildtype or mutant binding sites for Mad and Ci were examined in control (left panel) and *ap>tkv<sup>QD</sup>* (center panel) wing pouches. All reporters show an altered expression pattern with increased BMP signaling. **A.** Expression of *dpp<sup>D</sup>GFP* in wildtype (**A**, n=1) and *ap>tkv<sup>QD</sup>* (**A'**, n=2) wing pouches. **A''.** Expression is slightly reduced throughout its expression domain with increased BMP signaling. **B.** Expression of *dpp<sup>D-C13</sup>GFP* in wildtype (**B**, n=1) and *ap>tkv<sup>QD</sup>* (**B'**, n=2) wing pouches. **B''.** Expression is reduced along the anterior-posterior compartment boundary and slightly increased in the lateral region of *ap>tkv<sup>QD</sup>* pouches. **C.** Expression of *dpp<sup>APR-C13</sup>lacZ* in wildtype (**C**, n=9) and *ap>tkv<sup>QD</sup>* (**C'**, n=6) wing pouches. **C''.** Expression is reduced in the medial region of the wing pouch with increased BMP signaling. **D.** Expression of *dpp<sup>APR-C13</sup>lacZ* in wildtype (**D**, n=10) and *ap>tkv<sup>QD</sup>* (**D'**, n=5) wing pouches. **D''.** Expression is reduced in response to increased BMP signaling. **E.** Expression of *dpp<sup>APRD</sup>lacZ* in wildtype (**E**, n=8) and *ap>tkv<sup>QD</sup>* (**E'**, n=2) wing pouches. **E''.** Expression is reduced in a subset of its expression domain in *ap>tkv<sup>QD</sup>* pouches. **F.** Expression of *dpp<sup>APRD-C15</sup>lacZ* in wildtype (**F**, n=8) and *ap>tkv<sup>QD</sup>* (**F'**, n=8) wing pouches. **F''.** Expression is reduced in response to increased BMP signaling. **G.** Expression of *dpp<sup>APRD-C14-M</sup>lacZ* in wildtype (**G**, n=9) and *ap>tkv<sup>QD</sup>* (**G'**, n=7) wing pouches. **G''.** Expression of this reporter is reduced throughout its expression domain with increased BMP signaling. **H.** Expression of *dpp<sup>APRD-C15-M</sup>lacZ* in wildtype (**H**, n=8) and *ap>tkv<sup>QD</sup>* (**H'**, n=5) wing pouches. **H''.** In response to increased BMP signaling, expression is reduced in cells near the anterior-posterior compartment boundary.

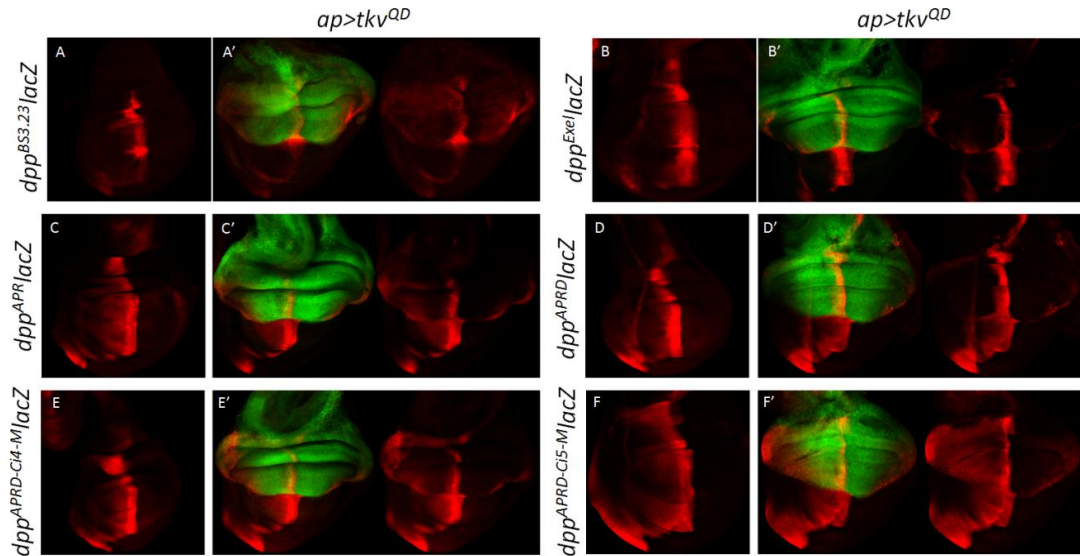
response to increased BMP signaling (Fig 3.3B,D,E), while expression of *dpp<sup>APRD-C14-M</sup>lacZ* was reduced throughout its endogenous expression domain in the medial region of *ap>tkv<sup>QD</sup>* wing pouches (Fig 3.3G). *dpp<sup>APRD-C15-M</sup>lacZ*, on the other hand, exhibited an altered expression pattern, but this effect was limited to a subset of its expression domain slightly anterior to cells displaying its highest level of expression (Fig 3.3H). These results indicate that neither the Ci-binding sites nor the Mad binding site (at Ci site 5) are required for BMP-induced negative regulation of *dpp* transcription and suggests that direct binding of either Ci or Mad is not necessary to repress *dpp* transcription.

#### Domain-specific effects of BMP signaling on *dpp-lacZ* expression

Unexpectedly, we noted that BMP signaling up-regulated the expression of some reporter lines in the lateral regions of *ap>tkv<sup>QD</sup>* wing imaginal pouches. This effect appeared to be most pronounced along the dorsal-ventral boundary of the dorsal compartment and was evident in *dpp<sup>BS3.23</sup>lacZ*, *dpp<sup>Exel</sup>lacZ*, *dpp<sup>APR</sup>lacZ*, and *dpp<sup>APRD</sup>lacZ* (Fig 3.4A-D), indicating that, as with negative feedback, this effect maps to several regions of the *dpp cis*-regulatory sequence. Surprisingly, loss of a Mad binding site in the context of reporters with compromised Ci binding, *dpp<sup>APRD-Ci5-M</sup>lacZ* and *dpp<sup>APRD-Ci4-M</sup>lacZ*, led to a significant up-regulation of expression in the lateral region of the wing pouch (Fig 3.4E-F). We had previously noted that lateral expression of these two reporters that cannot bind Mad is already higher than *dpp<sup>APRD</sup>lacZ* in wildtype tissue (Fig 3.2J-L), but with increased BMP signaling lateral expression is even further up-regulated. These results suggest that binding of Mad at that particular site may play a role in directly repressing *dpp*, particularly in the lateral wing pouch, and that BMP signaling may indirectly up-regulate the expression of *dpp* in the lateral region of the wing pouch. As this was observed in tissue with both endogenous and elevated BMP signaling levels, Mad binding at this site may contribute to restricting *dpp* expression from these cells. Future experiments will address the specific role of this and other, untested putative Mad binding sites present (Fig 3.2A) within the *dpp<sup>APRD</sup>lacZ* control sequences independent of mutations in the Ci binding sites.

While positive regulation of *dpp* by BMP signaling in the lateral regions of the wing pouch was unexpected, it is not entirely inconsistent with earlier experiments performed in our lab. We have previously observed that the *hedgehog* allele, *hh<sup>MRT</sup>* results in ectopic expression of *dpp* in the anterior compartment of the wing pouch along the dorsal-ventral axis (Felsenfeld, 1995; Johnson et al., 1995). When we tested the effect of increased BMP signaling on the ectopic expression of *dpp* in a *hh<sup>MRT</sup>* background, we noticed that clones of cells over-expressing *tkv<sup>QD</sup>* were unable to repress the lateral-most ectopic *dpp* expression (see Fig 2.12C). We concluded that *dpp* expression cannot be negatively regulated by BMP signaling in the lateral region of the wing pouch. Our recent observations suggest that, in addition to the inability of BMP signaling to repress *dpp* in the lateral wing pouch, *dpp* transcription may actually be up-regulated by BMP signaling in this region.





**Figure 3.4. Expression of *dpp-lacZ* reporters is increased in the lateral wing disc in response to BMP signaling.** Images of whole wing discs are shown to better display lateral up-regulation. Reporters displaying positive transcriptional regulation include: (A) *dpp*<sup>BS3.23</sup>*lacZ*, (B) *dpp*<sup>Exel1</sup>*lacZ*, (C) *dpp*<sup>APR</sup>*lacZ*, and (D) *dpp*<sup>APRD</sup>*lacZ*. Ectopic expression of *dpp*<sup>APRD</sup>*lacZ* in the anterior compartment is enhanced by a mutation in a Mad binding site, as observed for *dpp*<sup>APRD-Ci4-M</sup>*lacZ* (E) and *dpp*<sup>APRD-Ci5-M</sup>*lacZ* (F). The version of this reporter with all Ci sites mutated but not the Mad site mutated, *dpp*<sup>APRD-Ci5</sup>*lacZ*, does not display up-regulation of expression (see figure 7.28). For all reporters examined, up-regulation of *dpp-lacZ* expression is largely cell-autonomous.

These results suggest that BMP signaling plays multiple, domain-specific roles in regulating *dpp* expression in the wing pouch. Along the anterior-posterior boundary within the endogenous *dpp* expression domain, increased BMP signaling leads to a repression of *dpp* expression. In the lateral region of the wing pouch, where *dpp* is normally not expressed, BMP signaling can lead to ectopic expression of *dpp-lacZ*. Paradoxically, direct binding of Mad at one particular site seems to repress *dpp* expression in lateral cells both with and without altered BMP signaling. In the case of increased BMP signaling, binding of Mad may counteract the up-regulation of *dpp* occurring in response to increased BMP signaling.

#### Ci protein is not required for negative feedback of *dpp* transcriptional regulation

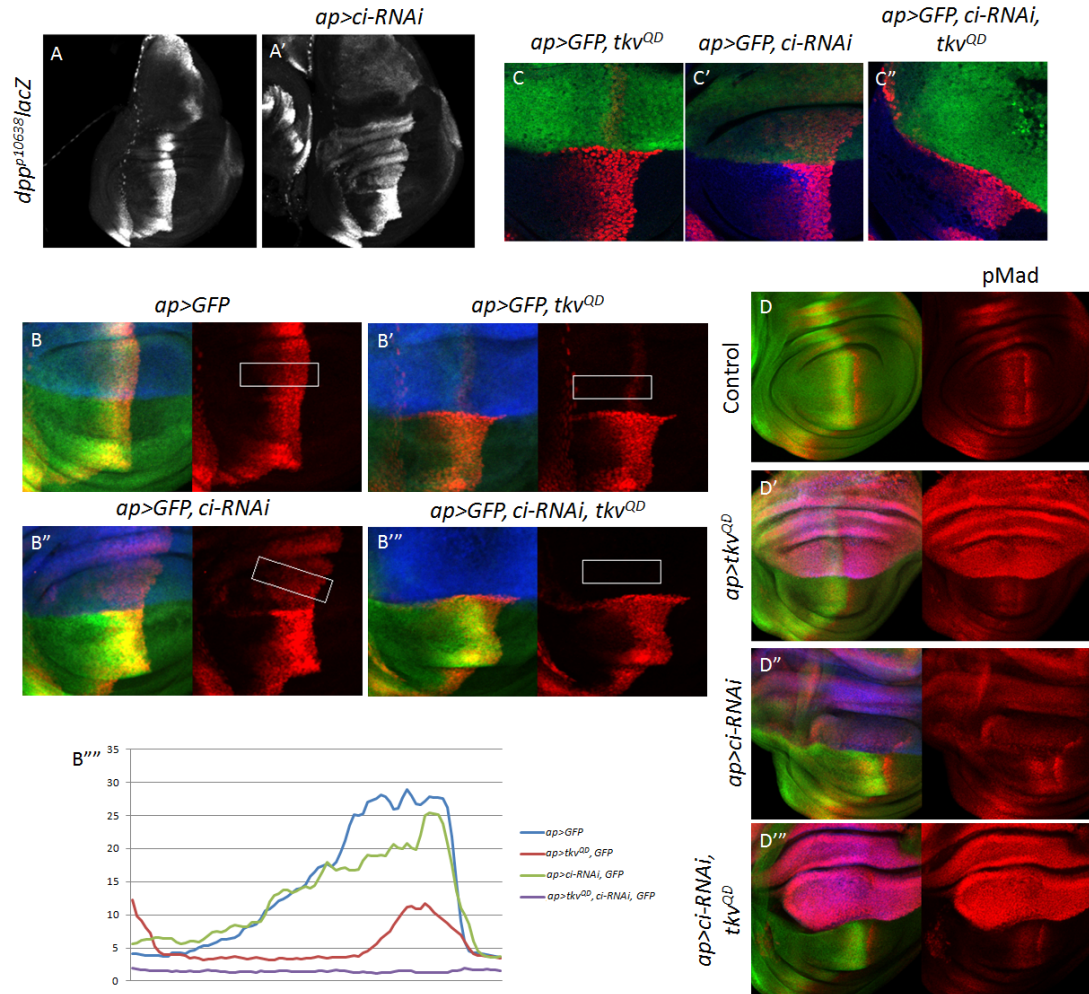
While our experiments thus far have demonstrated that direct binding of Ci to *dpp* control regions is not necessary to negatively regulate *dpp* expression, it is possible that Ci still plays a role in feedback regulation of *dpp*, perhaps indirectly. To determine whether Ci is absolutely required for negative feedback, we tested whether negative feedback regulation of *dpp* could occur in the absence of Ci. Cells in which Ci was knocked down by RNAi (without Ci) display ectopic expression of *dpp*<sup>p10638</sup>*lacZ*

throughout the dorsal anterior compartment of the wing pouch, hinge, and notum (Fig 3.5A). In addition, expression levels of *dpp*<sup>p10638</sup>*lacZ* in the anterior cells along the anterior-posterior compartment boundary were reduced compared to ventral expression, as expected based on previously published clonal data (Méthot and Basler, 1999).

Co-expression of *tkv*<sup>QD</sup> and *ci-RNAi* in the dorsal compartment resulted in a complete loss of *dpp*<sup>p10638</sup>*lacZ* expression in the medial wing pouch (Fig 3.5B'''). Intensity profiles (Fig 3.5B''') revealed that expression levels of *dpp*<sup>p10638</sup>*lacZ* are reduced in *ap>ci-RNAi*, *tkv*<sup>QD</sup> pouches compared to *ap>ci-RNAi* or *ap>tkv*<sup>QD</sup> wing pouches (Fig 3.5B'-3.5B''). We attribute the complete loss of *lacZ* expression in *ap>ci-RNAi*, *tkv*<sup>QD</sup> to a combination of two effects: reduced *dpp* transcription in the absence of the activator Ci<sup>A</sup> and BMP-signaling induced repression of *dpp*. This result indicates that Ci is not absolutely essential for negative feedback of *dpp* transcription in response to increased BMP signaling.

Interestingly, expression of *tkv*<sup>QD</sup> led to repression of *dpp* only within its endogenous expression domain in the center of the wing imaginal pouch. The ectopic *dpp*<sup>p10638</sup>*lacZ* expression in the lateral regions of the wing pouch, resulting from the loss of Ci, is resistant to BMP-mediated repression (Fig 3.5C), revealed by co-localization of GFP and *dpp*<sup>p10638</sup>*lacZ* in an *ap>tkv*<sup>QD</sup>, *ci-RNAi* pouch (Fig 3.5C''). This result is consistent with several of our previous observations on ectopic *dpp* expression in a *hh*<sup>MRT</sup> background (Fig 2.12) and on our *dpp*<sup>APRD-Ci4-M</sup>*lacZ* and *dpp*<sup>APRD-Ci5-M</sup>*lacZ* reporters (Fig. 3.4) that indicated *dpp* expression is not and cannot be negatively regulated in the lateral regions of the wing pouch.

To confirm that BMP signaling was still being highly activated in *ap>tkv*<sup>QD</sup>, *ci-RNAi* wing pouches without Ci protein, we examined pMad protein levels in pouches with endogenous and elevated BMP signaling both with and without Ci protein. pMad was increased to equally high levels in both *ap>tkv*<sup>QD</sup> (Fig 3.5D') and *ap>tkv*<sup>QD</sup>, *ci-RNAi* (Fig 3.5D''') wing pouches. Interestingly, *ap>ci-RNAi* pouches without Ci in the dorsal compartment displayed uniform pMad distribution throughout the anterior compartment with only part of the posterior peak remaining (Fig 3.5D''). This suggests that the ectopic *dpp* expressed in this tissue is competent to initiate BMP signaling uniformly across the anterior compartment.

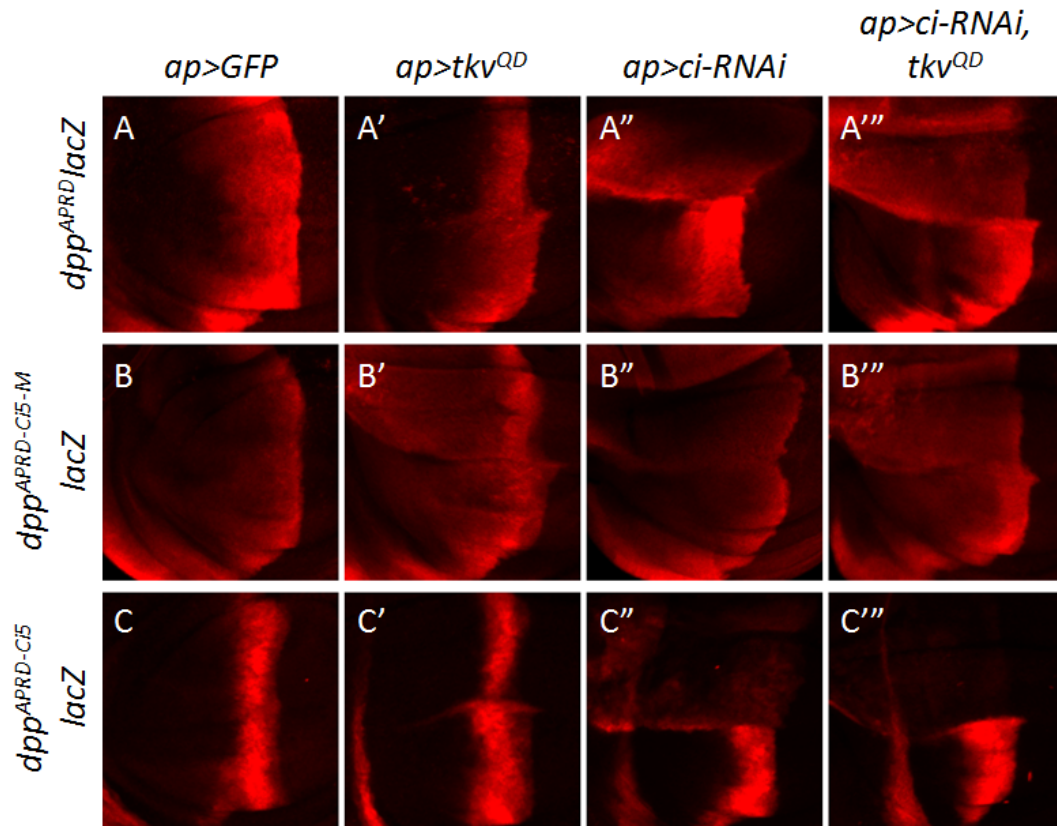


### Ci may indirectly regulate expression of *dpp-lacZ*

Our experiments thus far have revealed that Ci is not absolutely required for negative feedback of *dpp* and that some repression of *dpp* can occur in the absence of Ci binding, but it is still possible that Ci plays an indirect role on expression of our *dpp* reporters. As a final test for an indirect role for Ci in regulating *dpp*, we tested the ability of our mutated Ci binding sites reporters to respond negatively to BMP signaling in the absence of Ci. If Ci is playing an indirect role in negative feedback regulation of *dpp* transcription, then we would expect to see no BMP mediated repression of reporters with mutated Ci binding sites in the absence of Ci.

We examined expression of *dpp*<sup>APRD</sup>*lacZ*, *dpp*<sup>APRD-Ci5-M</sup>*lacZ*, and *dpp*<sup>APRD-Ci5</sup>*lacZ* in *ap>ci-RNAi* wing imaginal pouches without increased BMP signaling, expecting to see no change in expression of *dpp*<sup>APRD-Ci5-M</sup>*lacZ* and *dpp*<sup>APRD-Ci5</sup>*lacZ* in the absence of Ci. Surprisingly, expression of all three reporters, including those compromised in Ci binding, was greatly expanded without Ci (Fig 3.6A''-C''). *dpp*<sup>APRD-Ci5-M</sup>*lacZ* is uniformly expressed throughout the entire anterior dorsal compartment (Fig 3.6B''), while *dpp*<sup>APRD-Ci5</sup>*lacZ* shows a more uneven expression pattern in the anterior dorsal compartment and does not extend to the lateral regions of the wing pouch (Fig 3.6C''). These results were completely unexpected, as prior experiments have indicated that Ci<sup>A</sup> and Ci<sup>R</sup> regulate *dpp* by binding directly to the Ci consensus sequences that we had mutated (Hepker et al., 1999; Müller and Basler, 2000; Parker et al., 2011). This result suggests that Ci may also play an indirect role in regulating expression of all three reporter lines. Alternatively, Ci may still play a direct role through binding sequences at an unidentified and, most likely, degenerate site. Either one of these possibilities are exciting, as they represent novel mechanisms by which Ci could influence *dpp* expression.

We next tested the effect of increasing BMP signaling in the absence of Ci protein on expression of our reporter lines. In *ap>tkv<sup>QD</sup>*, *ci-RNAi* wing pouches, expression of *dpp*<sup>APRD</sup>*lacZ* and *dpp*<sup>APRD-Ci5-M</sup>*lacZ* was only slightly reduced along the anterior-posterior axis compared to *ap>ci-RNAi* pouches and we still observed expression throughout much of the anterior compartment (Fig 3.6A''',B'''). Expression of *dpp*<sup>APRD-Ci5</sup>*lacZ*, on the other hand, is almost completely lost in the dorsal compartment of *ap>tkv<sup>QD</sup>*, *ci-RNAi* wing pouches (Fig 3.6C'''). However, considering the low expression of *dpp*<sup>APRD-Ci5</sup>*lacZ* in *ap>ci-RNAi*



**Figure 3.6. Loss of Ci protein impacts *dpp* expression independently of Ci binding sites and does not impact BMP-mediated repression of *dpp*.** Expression of *dpp*<sup>APRD</sup>*lacZ* (A), *dpp*<sup>APRD-CIS-M</sup>*lacZ* (B), and *dpp*<sup>APRD-CIS</sup>*lacZ* (C) in *ap>GFP* (A-C), *ap>tkv*<sup>QD</sup> (A'-C'), *ap>ci-RNAi* (A''-C''), and *ap>GFP, tkv*<sup>QD</sup>, *ci-RNAi* (A'''-C''') wing pouches. A'-C'. As previously observed, expression of all reporters is reduced in response to increased BMP signaling. A''-C''. Expression of all reporters was reduced along the anterior-posterior boundary and expanded into the anterior compartment in the absence of Ci protein. In the dorsal anterior compartment of *ap>ci-RNAi* pouches, *dpp*<sup>APRD</sup>*lacZ* (A'') and *dpp*<sup>APRD-CIS-M</sup>*lacZ* (B'') are uniformly expressed while *dpp*<sup>APRD-CIS</sup>*lacZ* (C'') is unevenly expressed. A'''-C'''. In *ap>GFP, tkv*<sup>QD</sup>, *ci-RNAi* pouches with both increased BMP signaling and reduced Ci protein, expression of *dpp-lacZ* is further reduced from in *ap>ci-RNAi* discs, indicating that neither Ci binding or Ci protein is required for negative feedback regulation of *dpp*. This change in expression of *dpp*<sup>APRD</sup>*lacZ* (A''') and *dpp*<sup>APRD-CIS-M</sup>*lacZ* (B''') is subtle, but it is more obvious with *dpp*<sup>APRD-CIS</sup>*lacZ* (C''') expression.

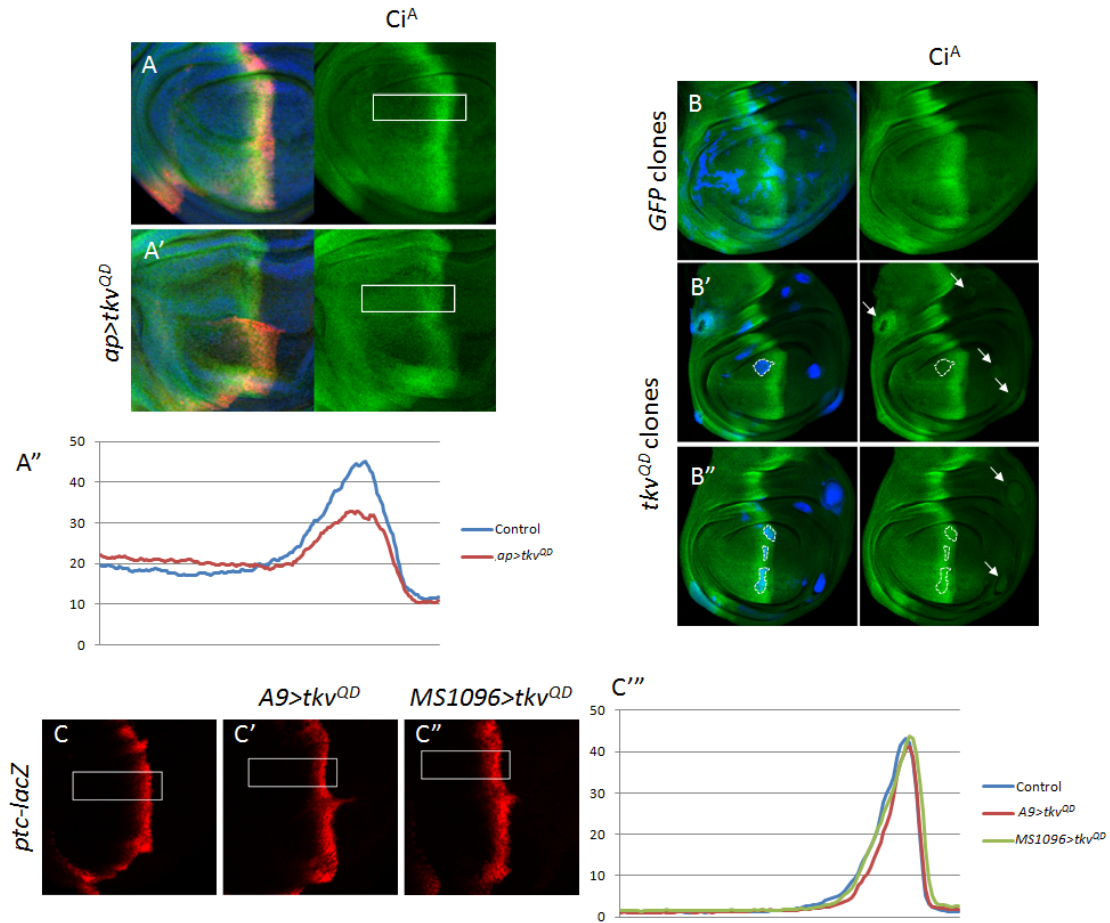
wing pouches (Fig 3.6C''), we do not believe that there is a stronger repression of *dpp*<sup>APRD-CIS</sup>*lacZ* expression compared to *dpp*<sup>APRD</sup>*lacZ* and *dpp*<sup>APRD-CIS-M</sup>*lacZ* in response to BMP signaling and without Ci protein. When we consider the strength of the response of any of these reporters to increased BMP signaling (Fig 3.6A'-C') as well as the expression of each in the absence of Ci (Fig 3.6A''-C''), we conclude that removing Ci causes no detectable changes in repression of each reporter in response to increased BMP signaling.

### BMP signaling causes a subtle change in the distribution of Ci<sup>A</sup> protein

Interestingly, we observed a slight but reproducible change in the distribution of Ci<sup>A</sup> in response to increased BMP signaling. Using an antibody that recognizes only the full-length and active form of Ci (Aza-Blanc et al., 1997; Motzny and Holmgren, 1995), we examined Ci<sup>A</sup> levels in cells with increased BMP signaling. BMP signaling was increased by driving dorsal compartment expression of *tkv<sup>QD</sup>* using the *ap-Gal4* driver or by inducing small *tkv<sup>QD</sup>* over-expression FLP-out clones. Reproducibly, increased BMP signaling levels in the dorsal compartment of the wing imaginal disc caused a slight reduction in the Ci<sup>A</sup> level along the anterior-posterior boundary without a change in Ci<sup>A</sup> levels in the lateral region of the disc (Fig 3.7A,A''). This is consistent with the reduction in *dpp-lacZ* expression seen in dorsal *ap>tkv<sup>QD</sup>* wing discs in that a slight reduction in Ci<sup>A</sup> levels could lead to a reduction in expression of *dpp* in these cells. However, a different response was observed on Ci<sup>A</sup> levels in clones of cells expressing *tkv<sup>QD</sup>* (Fig 3.7B), indicating that BMP signaling has a domain-specific effect on Ci. Clones that fall in both the anterior (arrows in Fig 3.7B') and posterior (arrows in Fig 3.7B',B'') lateral regions of the wing disc display a cell-autonomous increase in Ci<sup>A</sup> levels, consistent with the increase in lateral *dpp-lacZ* expression that we observed. Conversely, clones of cells that within the domains of high or moderate Ci activity displayed no changes in Ci<sup>A</sup> levels (outlined in Fig 3.7B',B'').

As the effects we observed on Ci<sup>A</sup> distribution were subtle, we wondered if this might affect expression of other Hh target genes or if it is a *dpp*-specific effect. To test whether BMP signaling can influence expression of other Hh targets, we examined expression of *ptc-lacZ*, a high-threshold target of Hh signaling, in wing pouches with endogenous and elevated BMP signaling levels. We used the *Gal4* drivers, *A9-Gal4* and *MS1096-Gal4*, to increase BMP signaling in the dorsal compartment or broadly across the wing pouch, respectively (for *Gal4* expression domains, see figures 6.1C, 2.1B). Under all signaling conditions, expression of *ptc-lacZ* was unchanged (Fig 3.7C), indicating that there is not a change in expression of all Hh signaling target genes with increased BMP signaling. Consistent with this, it has been reported that *ptc* may be less sensitive to changes in Ci level (Wu et al., 2011) or more sensitive to Ci<sup>R</sup> than Ci<sup>A</sup> (Parker et al., 2011), so a subtle change in Ci level or activity may not influence expression of *ptc-lacZ*.





**Figure 3.7. BMP signaling alters  $Ci^A$  protein levels but does not influence expression of the Hh target,  $ptc$ .** A-B. To test the effect of BMP signaling on  $Ci$  levels,  $Ci^A$  staining was observed in discs with endogenous and increased BMP signaling.  $Ci^A$  protein levels were visualized using  $Ci$ -2A1 antibody (provided by R. Holmgren), which recognizes the full length active form of  $Ci$  (shown in green). A.  $Ci^A$  in control (A, n=2) and  $ap>tkv^{QD}$  (A', n=4) wing pouches. Also shown is  $dpp^{p10638}lacZ$  (in red) and nuclei (in blue). A''. With increased BMP signaling, an overall reduction in  $Ci^A$  levels was observed along the anterior-posterior compartment boundary. B. FLP-out clones were generated (marked by GFP in blue) expressing GFP alone (B) or with  $tkv^{QD}$  (B', B''). GFP clones (B) display wildtype  $Ci^A$  distribution. Over-expression of  $tkv^{QD}$  has a variable effect on  $Ci^A$ , with clones in the lateral posterior (B'-B'') or anterior (B') compartments causing a slight increase in  $Ci^A$  levels (arrows) and clones in the center of the wing pouch (B'-B'') leaving  $Ci^A$  unaffected (outlined). C. BMP signaling does not reduce expression of  $ptc-lacZ$ , a reporter for expression of the high-threshold Hh target gene,  $ptc$ . A comparison of  $ptc-lacZ$  expression in wildtype (C, n=2),  $A9>tkv^{QD}$  (C', n=2), and  $MS1096>tkv^{QD}$  (C'', n=2) wing pouches reveals no reduction in expression of the reporter (C'').

While  $Ci$  is not required to mediate negative transcriptional regulation of  $dpp$ , these results suggest that it may still play a minor role in feedback regulation of  $dpp$ . BMP signaling may somehow influence the processing or activity of  $Ci$  and affect the ability of  $Ci$  to regulate  $dpp$ . Consistent with our previous domain-specific responses of BMP signaling on  $dpp$  expression, signaling also has a domain-specific effect on  $Ci$ . In the medial wing pouch, BMP decreases the level of  $Ci^A$ , possibly shifting the bias

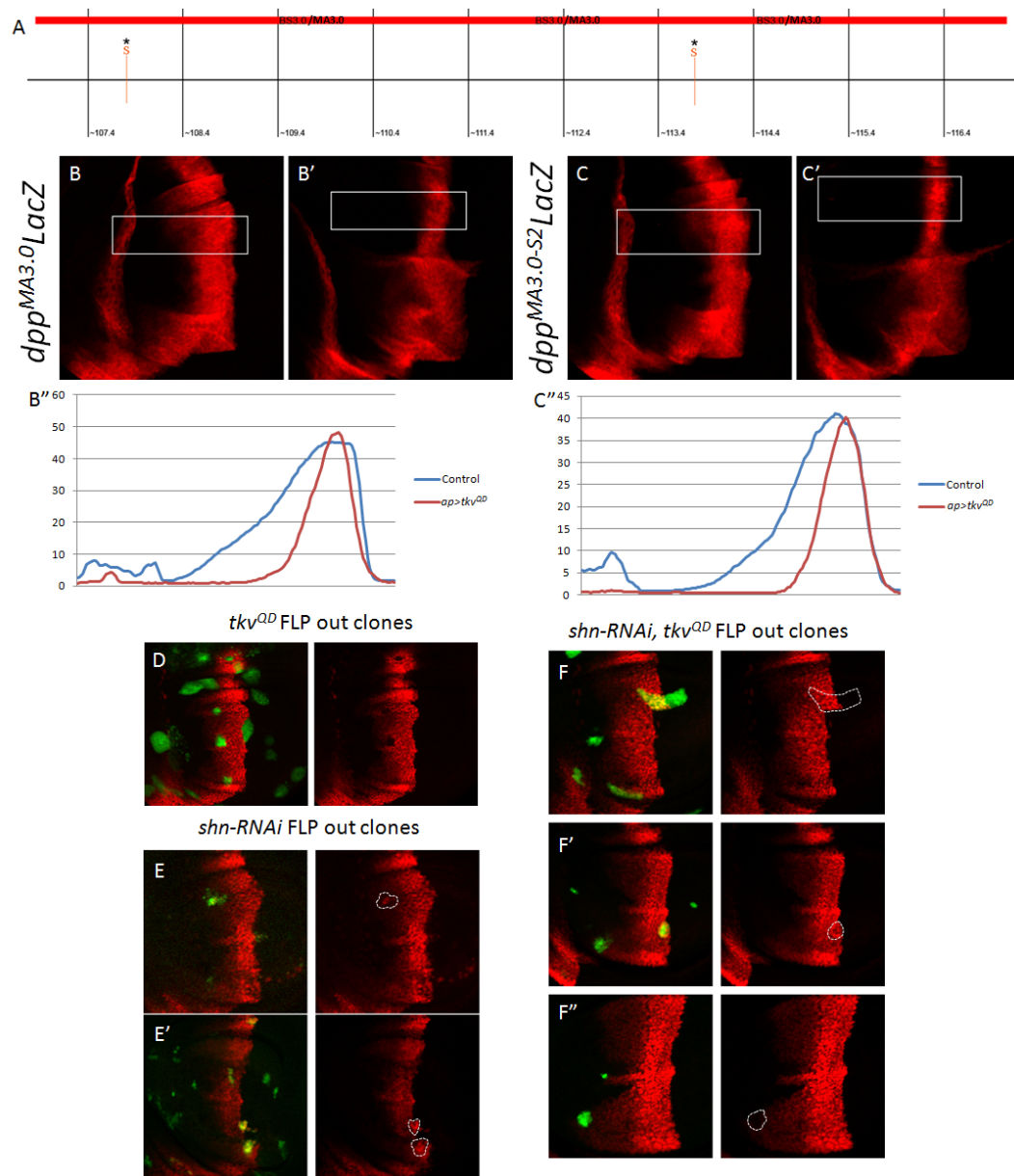
toward Ci<sup>R</sup> protein, whereas in the lateral wing disc, BMP signaling increases the level of Ci<sup>A</sup>, shifting the bias toward Ci<sup>A</sup> protein.

Taken together, our results indicate that the contribution of Ci to negative feedback regulation of *dpp* may be minor. Ci is important in generating the endogenous expression domain of *dpp* and, thus, remains an important regulator of *dpp*, but it may be dispensable in the negative feedback process of *dpp* regulation as in response to BMP signaling we observed repression of *dpp* in the absence of Ci protein and of *dpp-lacZ* lines that cannot bind Ci. However, it remains possible that Ci plays a minor role in this process considering the slight change in Ci<sup>A</sup> protein that we observed in response to increased BMP signaling and considering that in the absence of Ci protein we observe repression of the basal expression of *dpp* only. In addition, our experiments have revealed a potential indirect role for Ci in establishing the expression domain of *dpp*. This represents a novel function for Ci in regulating *dpp* and, potentially, an exciting addition to our understanding of *dpp* expression regulated by the Hh pathway.

#### **Shn is required for negative feedback regulation of *dpp* expression.**

Our experiments testing expression of *dpp-lacZ* reporters with a mutated Mad binding site suggest that Mad may play a direct role in regulating *dpp* expression, particularly in the lateral region of the wing imaginal pouch. However, these experiments also indicated that this site is not required for negative feedback regulation of *dpp* in the medial wing pouch. We considered other mechanisms through which Mad could have a direct repressive influence on *dpp* expression and looked to the co-repressor Shn. In addition to direct binding of Mad to DNA to activate transcription, Mad is well known to directly act as a repressor in a complex with the proteins Shn and Med (Dai et al., 2000; Gao et al., 2005; Pyrowolakis et al., 2004; Udagawa et al., 2000). In our sequence analysis of *dpp*<sup>BS3.0</sup>*lacZ* control sequences, we uncovered two highly conserved putative Shn/pMad/Med sites based on similarity to the consensus binding site for this complex, GNCGNCNNNNGTCT. These two sites are located approximately six kb from one another, with the 5'-most site found within the *dpp*<sup>Exel</sup>*lacZ* control sequences and the 3'-most site found within only the *dpp*<sup>BS3.0</sup>*lacZ* control sequences (Fig 3.8A). The presence of these sites suggests that Shn/pMad/Med could directly regulate *dpp* expression.





**Figure 3.8.** Shn is required for negative feedback regulation of *dpp*, but Shn/pMad/Med binding sites within the *dpp*<sup>BS3.0</sup>*lacZ* sequence do not mediate this response. **A.** Map depicting the *dpp* cis-regulatory region found within *dpp*<sup>BS3.0</sup>*lacZ* with Shn/pMad/Med consensus binding sites marked in orange. **B-C.** We observed expression of reporters with wildtype and mutant Shn/pMad/Med binding sites (in red) at endogenous and elevated levels of BMP signaling. **B.** Expression of *dpp*<sup>MA3.0</sup>*lacZ* (**B**), a re-creation of *dpp*<sup>BS3.0</sup>*lacZ*, in *ap>GFP* control (**B**, n=7) and *ap>GFP, tkv*<sup>QD</sup> (**B'**, n=4) wing pouches. **B''.** Expression of *dpp*<sup>MA3.0</sup>*lacZ* is reduced in a subset of its expression domain, giving a narrowed expression pattern. **C.** Expression of *dpp*<sup>MA3.0-S2</sup>*lacZ* (**C**), with mutant Shn/pMad/Med binding sites, in *ap>GFP* control (**C**, n=5) and *ap>GFP, tkv*<sup>QD</sup> (**C'**, n=2) wing pouches. **C''.** Expression of *dpp*<sup>MA3.0</sup>*lacZ* is expressed in a subset of its expression domain in a manner identical to *dpp*<sup>MA3.0</sup>*lacZ*. The Shn/pMad/Med binding sites are not necessary to regulate *dpp*. **D-F.** Clones of cells over-expressing either *tkv*<sup>QD</sup>, *shn-RNAi*, or both were generated and expression of *dpp*<sup>p10638</sup>*lacZ* (in red) was examined. Clones are marked by expression of GFP, shown in green. **D.** *dpp*<sup>p10638</sup>*lacZ* is almost completely reduced in *tkv*<sup>QD</sup>-expressing cells. **E.** Loss of *shn* has a modest effect on expression of *dpp*<sup>p10638</sup>*lacZ*, with 17% of clones recovered displaying a slight increase in expression of *dpp*<sup>p10638</sup>*lacZ* (outlined in **E**, **E'**) and all others displaying no change in *dpp*<sup>p10638</sup>*lacZ* expression. **F.** Clones of cells over-expressing both *tkv*<sup>QD</sup> and *shn-RNAi* also display only a modest change in *dpp*<sup>p10638</sup>*lacZ* expression, with 19% displaying a slight increase (**F'**), 15% displaying a slight decrease (**F''**), and 66% displaying no change (**F**) in expression. The persistence of *dpp*<sup>p10638</sup>*lacZ* expression in these clones indicates a requirement for Shn protein in mediating transcriptional regulation of *dpp*.

To test Shn for a role in regulating *dpp*, we asked whether negative feedback of *dpp* transcription could occur in the absence of Shn protein. We reduced Shn protein levels by creating clones of cells expressing *shn-RNAi* and compared the expression of *dpp<sup>p10638</sup>lacZ* in these clones to clones of cells expressing *tkv<sup>QD</sup>* alone or co-expressing *tkv<sup>QD</sup>* and *shn-RNAi*. As expected, expression of *tkv<sup>QD</sup>* causes a strong cell autonomous reduction in *dpp<sup>p10638</sup>lacZ* expression, regardless of clone location (Fig 3.8D). Clones of cells that express only *shn-RNAi* show either no change or a slight increase in the expression of *dpp<sup>p10638</sup>lacZ* (Fig 3.8E). Of the 36 clones that were recovered within the *dpp<sup>p10638</sup>lacZ* expression domain of the wing pouch, six (17%) displayed a slight increase in expression of *dpp<sup>p10638</sup>lacZ* (Fig 3.8E) while the remaining 30 (83%) left *dpp<sup>p10638</sup>lacZ* unchanged (data not shown). Interestingly, several clones recovered just posterior to the anterior-posterior stripe of *dpp<sup>p10638</sup>lacZ* expression, a region that does not express *dpp<sup>p10638</sup>lacZ* endogenously, displayed strong ectopic expression of *dpp<sup>p10638</sup>lacZ* (Fig 3.8E'). We recovered two clones within the peripodial expression domain of *dpp<sup>p10638</sup>lacZ* and both display a slight up-regulation of *dpp<sup>p10638</sup>lacZ* expression (not shown). As a reduction in Shn has only a subtle effect on expression of *dpp<sup>p10638</sup>lacZ*, these results suggest that Shn may play a minor role in determining the level and pattern of *dpp* expression in the wing pouch.

Clones of cells over-expressing both *shn-RNAi* and *tkv<sup>QD</sup>* displayed a variable but weak effect on expression of *dpp<sup>p10638</sup>lacZ*, thus behaving more like cells expressing *shn-RNAi* alone than like cells expressing *tkv<sup>QD</sup>*, which always display a strong repression of *dpp<sup>p10638</sup>lacZ* expression. Of the 26 clones recovered within the expression domain of *dpp<sup>p10638</sup>lacZ* in the wing pouch, five (19%) displayed a slight increase (Fig 3.8F'), four (15%) displayed a slight decrease (Fig 3.8F''), and 17 (65%) displayed no change in expression of *dpp<sup>p10638</sup>lacZ* (Fig 3.8F). In addition, five clones were recovered in the peripoidum, three of which (60%) display a reduction in the expression of *dpp<sup>p10638</sup>lacZ* and one of which (20%) displays an increase in expression of *dpp<sup>p10638</sup>lacZ* (not shown). We never observed the strong repression of *dpp<sup>p10638</sup>lacZ* seen in clones expressing *tkv<sup>QD</sup>* alone, indicating that Shn is required for strong repression of *dpp* transcription in response to increased BMP signaling levels.

**Binding of the Shn/pMad/Med complex to *dpp* cis-regulatory sequences is not required for negative feedback regulation of *dpp* transcription.**

Having identified a requirement for Shn in mediating feedback regulation of *dpp*, we next asked whether this effect was direct through the two Shn/pMad/Med binding sites that we had identified (Fig 3.8A). To test these sites for a function in mediating regulation of *dpp*, we created a *dpp-lacZ* reporter construct with both of the putative Shn/pMad/Med consensus binding sites disrupted within the context of the *dpp*<sup>BS3.0</sup>*lacZ* enhancer sequences, as this is the only reporter analyzed thus far that contains both sites. We mutated the last four base pairs of the consensus sequence to preclude binding of Med to the DNA, thereby disrupting the ability of a Shn/pMad/Med repressor complex to bind (Gao et al., 2005; Pyrowolakis et al., 2004). We created reporters with wildtype and mutated Shn/pMad/Med binding sites, denoted *dpp*<sup>MA3.0</sup>*lacZ* and *dpp*<sup>MA3.0-MS2</sup>*lacZ*, respectively.

Surprisingly, *dpp*<sup>MA3.0</sup>*lacZ* was expressed in a different pattern from the original *dpp*<sup>BS3.0</sup>*lacZ* line (Fig 3.8B, compare to Fig 2.2D,E). While *dpp*<sup>BS3.0</sup>*lacZ* is reproducibly expressed in only a fairly narrow stripe of anterior cells along the anterior-posterior boundary, *dpp*<sup>MA3.0</sup>*lacZ* is expressed in a broader expression domain that displays a graded distribution into the anterior compartment, reminiscent of the expression pattern of the enhancer trap *dpp*<sup>p10638</sup>*lacZ*. This suggests that the different expression patterns displayed by *dpp*<sup>BS3.0</sup>*lacZ* and *dpp*<sup>p10638</sup>*lacZ* may not be due to the specific enhancer sequences within *dpp*<sup>BS3.0</sup>*lacZ* but, rather, due to some aspect of transgene construction. The particular promoter or *lacZ* sequences used to generate *dpp*<sup>BS3.0</sup>*lacZ* and *dpp*<sup>p10638</sup>*lacZ* could account for this difference. Alternatively, it could be a position effect of transgene incorporation into the genome, as it is known that the insertion site can greatly influence expression levels (Spradling and Rubin, 1993).

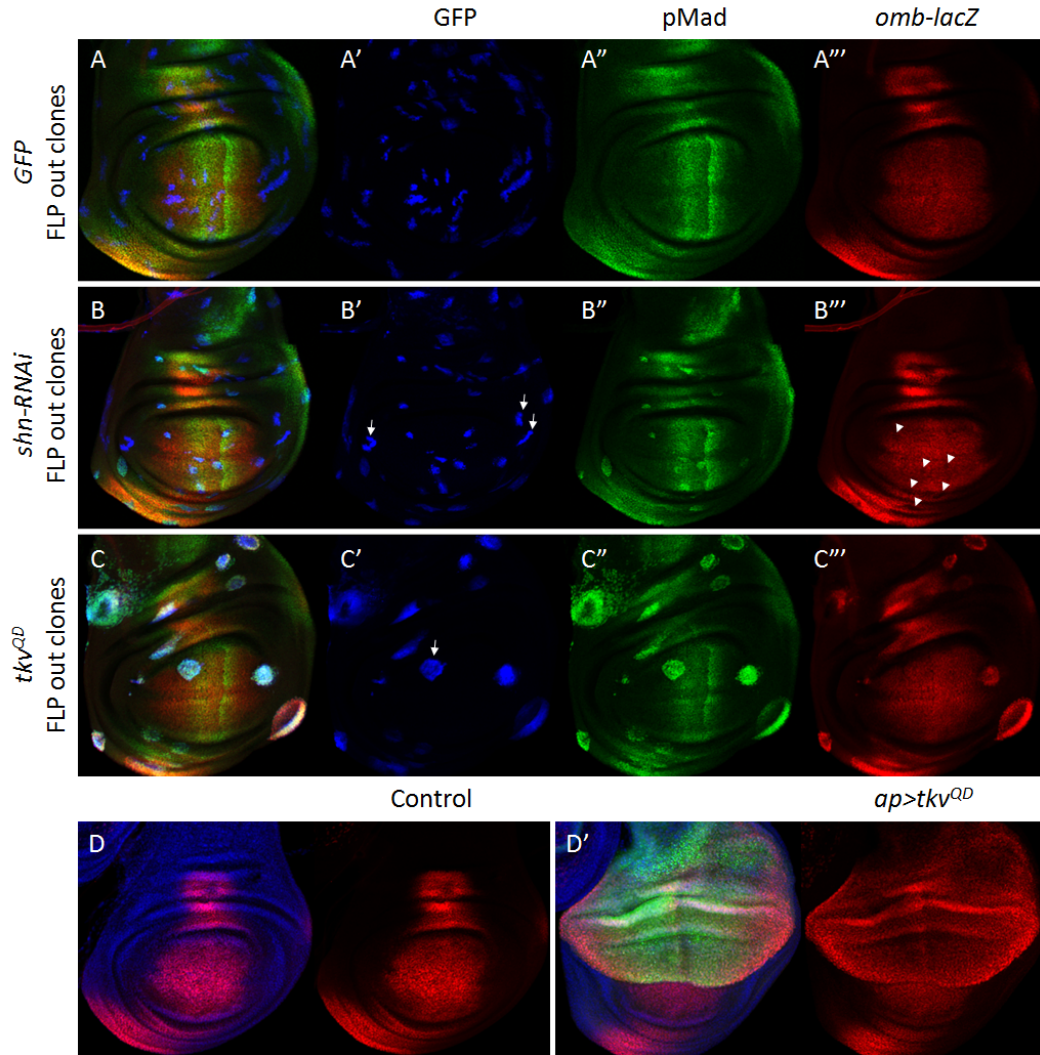
The expression of *dpp*<sup>MA3.0</sup>*lacZ* and *dpp*<sup>MA3.0-MS2</sup>*lacZ* in wildtype wing pouches is observed in seemingly identical expression patterns (Fig 3.8B,C), suggesting that these putative Shn/pMad/Med sites are not essential in determining the proper expression level or domain of *dpp* expression in the wing pouch under normal signaling conditions. Furthermore, increased BMP signaling reduces the expression of both reporters comparably, with each displaying repression in a subset of their expression domains (Fig 3.8B,C). These results suggest that binding of the Shn/pMad/Med complex to the *dpp* cis-regulatory

sequence is not required to mediate a repression of *dpp* in response to increased BMP signaling and that these sites may not be functional at all. Their mutation does not affect expression of the reporter line under wildtype or elevated BMP signaling conditions. In light of our observation that Shn protein is required for negative feedback of *dpp*, this result suggests that Shn either exerts its affect on *dpp* indirectly or that it binds elsewhere within the *dpp cis*-regulatory sequences.

### **A reduction in Shn increases pMad levels in the medial wing pouch**

We considered that possibility that Shn influences expression of *dpp* through indirect mechanisms and asked how loss of *shn* might influence cells. To characterize the effects of Shn removal, we examined pMad levels in clones of cells expressing *shn-RNAi*. We observed a cell autonomous increase in the amount of pMad within *shn-RNAi* clones located within the endogenous pMad gradient, including cells on the periphery of the gradient (Fig 3.9B"). Clones that fall outside of the pMad gradient do not lead to a change in pMad levels (Fig 3.9, outlined in B',B"), indicating that loss of *shn* is not sufficient to induce ectopic pMad. Importantly, *shn-RNAi* expressing clones display an increase in pMad without a concomitant decrease in *dpp*<sup>p10638</sup> *lacZ* expression, indicating that increased pMad alone is not sufficient to repress *dpp* expression.

An increase in pMad with reduced Shn was unexpected as, to the best of our knowledge, all current data indicate that Shn functions downstream of Mad phosphorylation in BMP signaling. It is possible that the increase in pMad is due to mis-regulation of a Shn/pMad/Med target. One possibility is the *Drosophila* i-Smad, *dad*, which is an indirect Shn/pMad/Med target and is directly regulated by both Brk and pMad (Weiss et al., 2009). Furthermore, *dad* expression has been reported to be lost in *shn* null clones (Torres-Vazquez et al., 2000). Dad negatively regulates BMP activity and is important in generating a robust BMP activity gradient (Ogiso et al., 2011) , so a reduction in *dad* may cause a cell-autonomous increase in BMP activity.



**Figure 3.9. The factor required for negative transcriptional regulation of *dpp* may be downstream of Shn, Omb and pMad.** There is a variable effect of BMP signaling on expression of *omb*, a gene product reported to affect negative transcriptional regulation of *dpp*. **A-C.** We examined levels of pMad (**A''-C''**, in green) and expression of *omb-lacZ* (**A'''-C'''**, in red) in clones of cells expressing *GFP* alone (**A**), *shn-RNAi* (**B**), or *tkv<sup>QD</sup>* (**C**). Clones were generated during second instar and examined during late third instar larvae. They are marked by *GFP* expression, displayed in blue. **A.** Control *GFP* clones displaying wildtype pMad (**A''**) and *omb-lacZ* expression (**A'''**). **B.** Clones of cells expressing *shn-RNAi* that fall within the pMad gradient lead to a cell-autonomous increase in pMad levels (**B''**) while those outside of the pMad gradient show no change in pMad levels (arrows in **B'**). Clones display a cell-autonomous decrease in *omb-lacZ* expression (**B'''**, arrowheads). **C.** Expression of *tkv<sup>QD</sup>* leads to an increase in pMad levels in all clones (**C''**), but only increases expression of *omb-lacZ* in clones at the periphery of the *omb-lacZ* expression domain (**C'''**). The clone marked by an arrow in **C'** does not display an increase in expression of *omb-lacZ*. **D.** We examined *omb-lacZ* expression (in red) in control (**D**) and *ap>GFP, tkv<sup>QD</sup>* (**D'**, *GFP* in green) wing discs. In response to increased BMP signaling, *omb-lacZ* expression is increased throughout the dorsal compartment, including the lateral wing disc and along the dorsal-ventral boundary. Nuclei are marked in blue.

### Omb is a potential mediator of *dpp* transcriptional regulation

Although we could not discern between a direct and indirect role for Shn, we considered possible candidates downstream of Shn that could be required for transcriptional regulation of *dpp*. We looked to

Omb, as a previously published report suggested a role for Omb in regulation of *dpp* (Umemori et al., 2007). It had been observed that *dpp*<sup>p10638</sup>*lacZ* displays an expanded expression domain in an *omb* mutant background and that *dpp* expression is reduced in *omb* over-expression clones. Importantly, over-expression of *UAS-tkv*<sup>QD</sup> in an *omb* mutant background did not alter expression of *dpp*, suggesting that Omb may be required for feedback regulation of *dpp*.

In examining our *shn-RNAi* clones, we noticed that clones that fell on the posterior side of anterior-posterior boundary were either ectopically expressing *dpp*<sup>p10638</sup>*lacZ* or were clones of anterior origin that migrated (Fig 3.9E'). The anterior and posterior regions of the wing disc are distinct developmental compartments that have distinct cell lineages and cell movements (Garcia-Bellido et al., 1973). Under endogenous developmental trajectories, all cells that reside in a particular compartment are derived from that compartment only. As *dpp*<sup>p10638</sup>*lacZ* should only express in anterior cells, expression of it in the posterior compartment suggested instability at the boundary. Omb has been found to play a role in maintaining the integrity of the anterior-posterior boundary (Shen and Dahmann, 2005; Shen et al., 2010; Shen et al., 2008), so the behavior of these clones led to us to ask if *omb* was altered in *shn-RNAi* clones. We examined expression of *omb-lacZ* in *shn-RNAi* clones and, indeed, there is a cell autonomous reduction of *omb-lacZ* expression with reduced Shn (Fig 3.9B''', arrowheads). The loss of *omb-lacZ* expression in *shn-RNAi* clones is consistent with a role for Omb in feedback regulation of *dpp*, as *dpp* transcription cannot be negatively regulated in these clones (Fig 3.8).

Conversely, if *omb* is the downstream target of Shn that is directly responsible for repression of *dpp*, then we might expect to see an increase in the expression of *omb-lacZ* in response to expression of *tkv*<sup>QD</sup>, particularly in the medial wing pouch overlapping the *dpp* expression domain. We asked if and where BMP signaling could alter expression of *omb* by examining expression of *omb-lacZ* in cells over-expressing *tkv*<sup>QD</sup> driven in the dorsal compartment by *ap-Gal4* and in FLP-out clones. Expression of *omb-lacZ* is up-regulated throughout the lateral and medial dorsal compartment of *ap>tkv*<sup>QD</sup> pouches, with this effect particularly evident in the lateral regions of the pouch (Fig 3.9D). However, the same was not seen in clones of cells over-expressing *tkv*<sup>QD</sup>, where only lateral clones and those on the periphery of the *omb*-

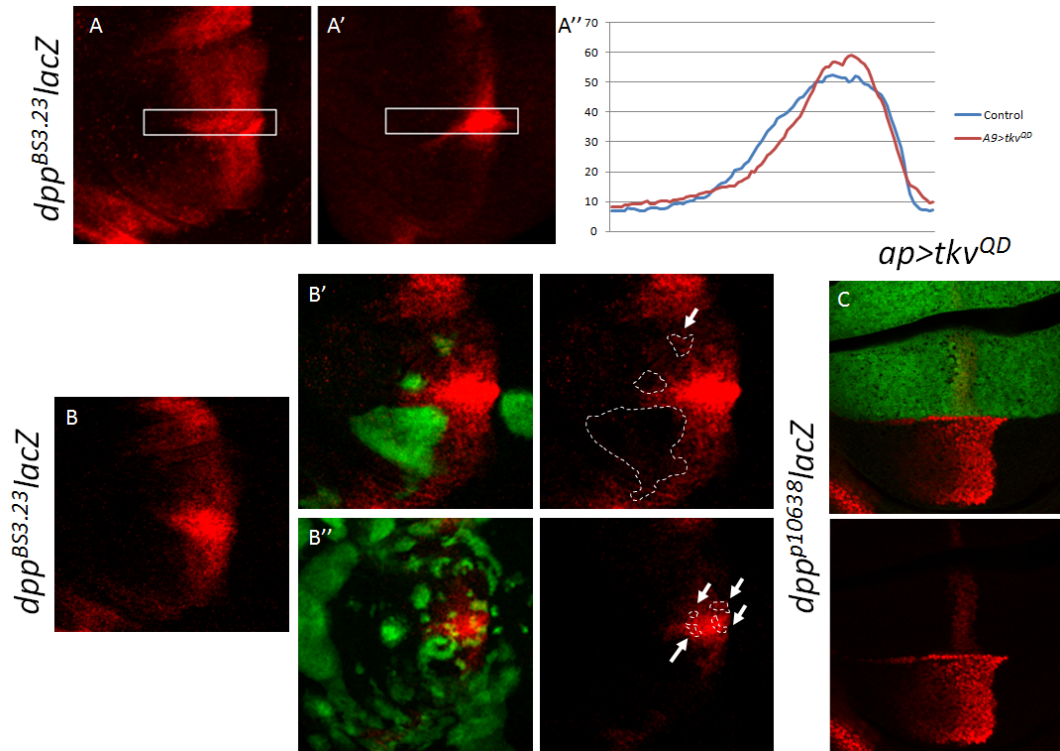
*lacZ* expression domain displayed an up-regulation of *omb-lacZ* expression. Clones that fell in the medial of the wing pouch did not show an increase in expression of *omb-lacZ* (Fig 3.9C''').

While it remains a possibility that BMP signaling could influence the activity of Omb post-transcriptionally, without an increase in *omb* expression in the medial wing pouch in response to increased BMP signaling, it seems unlikely that *omb* alone is sufficient to mediate negative feedback of *dpp*. Instead, Omb may be upstream of or work in cooperation with the regulator of *dpp* transcription. Omb has been reported to influence expression of several genes associated with BMP signaling (del Alamo Rodriguez et al., 2004). These include the BMP target genes *sal*, and *vg*, the BMP receptor, *tkv*, and the regulator of *tkv*, *master-of-thickveins* (*mtv*) (Funakoshi et al., 2001). Future experiments will test a requirement for these factors in regulating negative feedback of *dpp* transcription.

#### **Expression of *dpp* is differentially regulated at the dorsal-ventral compartment boundary**

In our examination of the *dpp* reporter *dpp*<sup>BS3.23</sup>*lacZ*, we noted that it responds differentially to BMP signaling at the dorsal-ventral compartment boundary compared to other regions of its expression domain. This reporter is unique in that under endogenous BMP signaling conditions it displays a very bright spot of expression at the intersection of the anterior-posterior and dorsal-ventral axes (Fig 3.10A,B), which is resistant to BMP signaling-induced repression (Fig 3.10A',B',B''). This is in contrast to the remainder of the *dpp*<sup>BS3.23</sup>*lacZ* expression domain, where a reduction in expression is observed in response to BMP signaling (Fig 3.1D). Similar results were observed in clones of cells expressing *tkv*<sup>QD</sup>. Clones that fall at or close to the dorsal-ventral boundary resist the negative feedback input and display expression of *dpp*<sup>BS3.23</sup>*lacZ* at levels equal to the surrounding wildtype tissue (Fig 3.10B',B'', arrows) while those that fall elsewhere display a cell-autonomous reduction in expression of the reporter (Fig 3.10B', outlined).

This behavior of *dpp*<sup>BS3.23</sup>*lacZ* at the dorsal-ventral compartment boundary is not our only observation of differential regulation of *dpp*. We noted several examples where *dpp* was positively regulated, rather than repressed, in the lateral regions of the wing pouch (see Fig 3.4), particularly along the dorsal-ventral boundary. Furthermore, expression of *dpp*<sup>p10638</sup>*lacZ* displays a curious non cell-



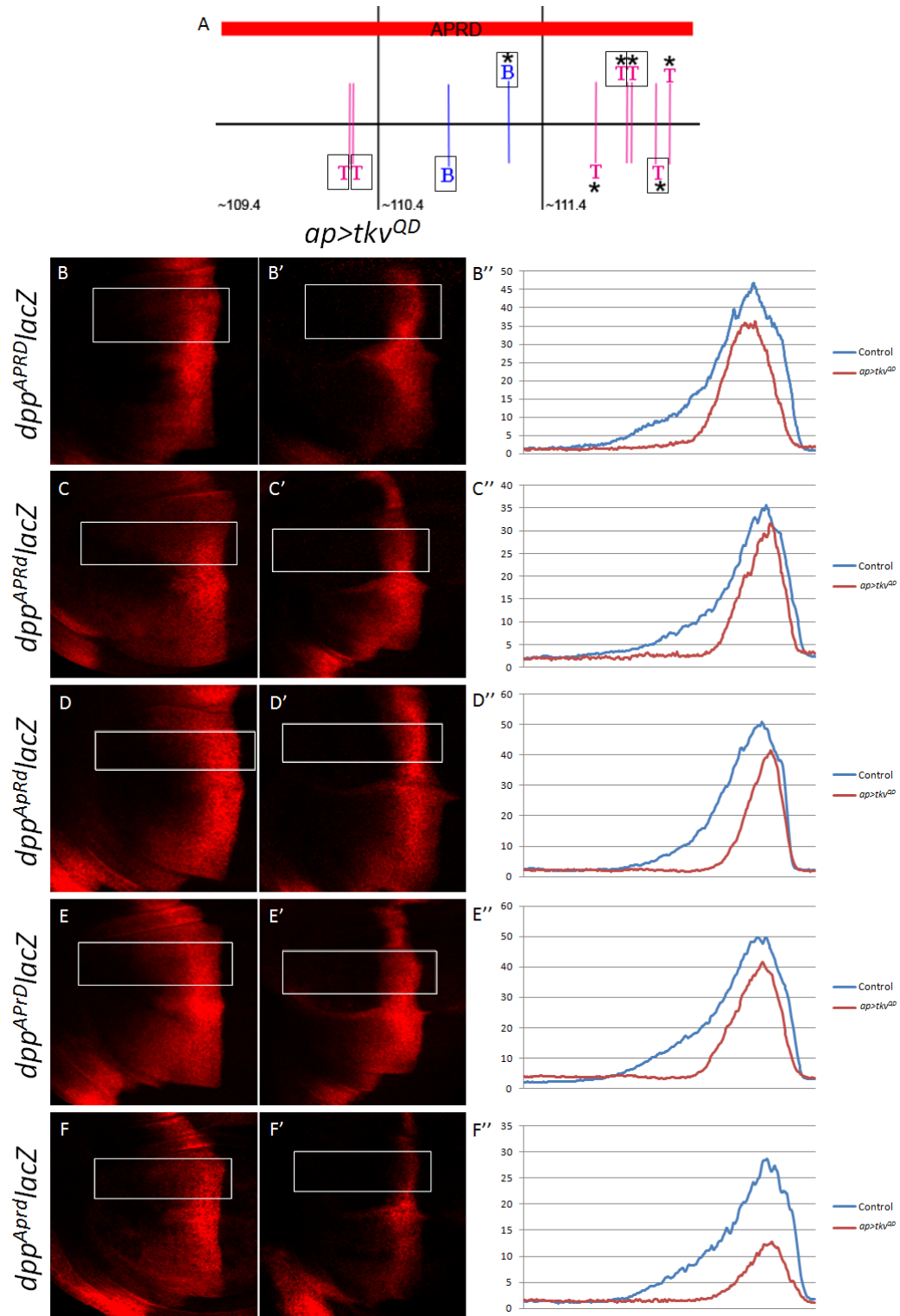
**Figure 3.10. Expression behavior of *dpp-lacZ* at the dorsal-ventral boundary is different than elsewhere in the wing disc.** Often, expression of *dpp-lacZ* reporters behave differently at the dorsal-ventral boundary. **A-B.** Expression of *dpp<sup>BS3.23</sup>lacZ* is less sensitive to BMP signaling at the dorsal-ventral boundary than elsewhere in its expression domain. The expression pattern of *dpp<sup>BS3.23</sup>lacZ* is unique in that it displays a very strong spot of expression at the dorsal-ventral boundary at the anterior-posterior boundary. We observed *dpp<sup>BS3.23</sup>lacZ* expression (in red) in wing pouches with increased BMP signaling, either in *ap>tkv<sup>QD</sup>* discs (**A**) or in *tkv<sup>QD</sup>* FLP-out clones (**B**). **A.** Expression of *dpp<sup>BS3.23</sup>lacZ* in wildtype (**A**, n=5) and *ap>tkv<sup>QD</sup>* (**A'**, n=5) wing pouches. **A''.** Expression is not reduced at the dorsal-ventral boundary with increased BMP signaling. **B.** *tkv<sup>QD</sup>* FLP-out clones display a cell-autonomous reduction in *dpp<sup>BS3.23</sup>lacZ* (**B'**, outline) unless they fall at or close to the dorsal-ventral boundary, where no reduction in expression was observed (arrows in **B'**, **B''**). **C.** We observed up-regulation of many *dpp* reporters at the dorsal-ventral boundary (also see figure 3.4). Expression of *dpp<sup>p10638</sup>lacZ* (in red) in an *ap>GFP*, *tkv<sup>QD</sup>* wing disc (GFP displayed in green) is up-regulated in the first one or two cells into the ventral compartment. The lack of co-localization between GFP and *dpp<sup>p10638</sup>lacZ* reveals this non cell-autonomous effect.

autonomous response to increased BMP signaling. In *ap>tkv<sup>QD</sup>* wing pouches, we observed increased *dpp<sup>p10638</sup>lacZ* expression in the first row of cells of the ventral compartment (Fig 3.10D), particularly in the medial region of the wing pouch. Together, these results suggest that expression of *dpp* at the dorsal-ventral boundary may be subject to additional regulatory cues.

#### Binding of dTCF or Brk is not required for feedback regulation of *dpp*

We considered possible factors that could influence expression of *dpp* at the dorsal-ventral boundary and decided to test the downstream effector of Wingless/Wnt signaling, dTCF, for several reasons. Extensive cross-talk has been reported between BMP and Wg signaling throughout development





**3.11. Mutation of dTCF or Brk binding sites within the *dpp* cis-regulatory region does not influence negative feedback regulation of *dpp*.** The expression patterns of reporter constructs with wildtype or mutant binding sites for dTCF and Brk, a generous gift from the Marsh lab, were examined with endogenous levels of BMP signaling. (Theisen et al., 2007) **A.** Map depicting the sequences present in the reporter constructs examined and the locations of tested binding sites for dTCF and Brk within this region (boxed). **B-F.** We examined the expression patterns of reporters in control (left panel) and *ap>tkv<sup>QD</sup>* (center panel) wing discs and examined  $\beta$ Gal levels (in

red). All reporters show an altered expression pattern with increased BMP signaling, indicating that these dTCF and Brk binding sites are not necessary to regulate *dpp*. **B.** Expression of *dpp<sup>APRD</sup>lacZ* in wildtype (**B**, n=1) and *ap>tkv<sup>QD</sup>* (**B'**, n=2) wing pouches. **B''.** Expression is reduced throughout its expression domain with increased BMP signaling. **C.** Expression of *dpp<sup>APRd</sup>lacZ*, with distal cluster of dTCF sites mutated, in wildtype (**C**, n=2) and *ap>tkv<sup>QD</sup>* (**C'**, n=1) wing pouches. **C''.** Expression of this reporter is reduced in a subset of its expression domain with increased BMP signaling. **D.** Expression of *dpp<sup>APRd</sup>lacZ*, with proximal and distal clusters of dTCF sites mutated, in wildtype (**D**, n=2) and *ap>tkv<sup>QD</sup>* (**D'**, n=1) wing pouches. **D''.** Expression of this reporter is reduced in a subset of its expression domain in response to increased BMP signaling. **E.** Expression of *dpp<sup>APRD</sup>lacZ*, with Brk sites mutated, in wildtype (**E**, n=2) and *ap>tkv<sup>QD</sup>* (**E'**, n=3) wing pouches. **E''.** With increased BMP signaling, expression of this reporter is reduced throughout its expression domain. **F.** Expression of *dpp<sup>APRd</sup>lacZ*, with Brk and dTCF sites mutated, in wildtype (**F**, n=2) and *ap>tkv<sup>QD</sup>* (**F'**, n=3) wing pouches. **F''.** Expression of this reporter is decreased throughout its expression domain with BMP signaling increased.

of many species (Eivers et al., 2009; Guo and Wang, 2009), so we had reason to suspect that the Wg pathway could interact with negative feedback regulation of *dpp*. In the wing disc, Wg is secreted from the cells along the dorsal-ventral compartment boundary and plays a role in patterning fates along the dorsal-ventral axis (Neumann and Cohen, 1997). In addition, Wg signaling directly regulates *dpp* expression in the leg imaginal disc where cooperation between dTCF, Armadillo, and Brk represses *dpp* (Theisen et al., 2007) and in the posterior spiracles where cooperation between dTCF, Mad, and Brk can both repress and activate *dpp* (Takaesu et al., 2008). Wg signaling also plays a role in regulating gene expression of BMP targets, such as *omb* (Grimm and Pflugfelder, 1996) and *vg* (Maves and Schubiger, 1998; Prasad et al., 2003) (Neumann and Cohen, 1997). Finally, binding of dTCF has been shown to be required for expression of *dpp<sup>BS3.23</sup>lacZ* in the wing disc (Hepker et al., 1999).

To test dTCF for a role in regulating *dpp*, we examined expression of *dpp-lacZ* reporters with mutated dTCF binding sites in control and *ap>tkv<sup>QD</sup>* wing pouches. We received several such reporter lines from the Marsh lab, who had had tested the role of both dTCF and Brk in regulating *dpp* expression in the leg disc (Theisen et al., 2007). While unlikely that Brk could be responsible for repression of *dpp* since its expression domain does not overlap with that of *dpp*, we considered the possibility that it may be expressed in the medial wing pouch and decided to test reporter lines with mutated Brk sites as well. We tested the following reporter lines: *dpp<sup>APRD</sup>lacZ*, which contains all wildtype binding sites; *dpp<sup>APRd</sup>lacZ*, which has mutations in three distal dTCF binding sites; *dpp<sup>APRd</sup>lacZ*, which has mutations in five dTCF binding sites; *dpp<sup>APRD</sup>lacZ*, which has mutations in two Brk binding sites; and *dpp<sup>APRd</sup>lacZ*, which has mutations in all dTCF and brk binding sites. The locations of all sites, as well as untested sites in this region that have been identified, is shown in figure 3.11A.

The expression pattern of all the reporters in wildtype wing pouches was observed in a similar wide gradient in the anterior compartment, suggesting that the dTCF and Brk sites do not play a role in generating the expression pattern of *dpp* in the wing pouch (Fig 3.11B-F). All reporters, regardless of the status of their dTCF or Brk sites were altered in response to increased signaling, indicating that binding of dTCF or Brk is not required for this process. *dpp*<sup>APRD</sup>*lacZ*, *dpp*<sup>APRd</sup>*lacZ*, *dpp*<sup>APRd</sup>*lacZ*, and *dpp*<sup>APrD</sup>*lacZ* exhibit reduced expression primarily in a subset of their expression domains (Fig 3.11B-E), while expression of *dpp*<sup>APrd</sup>*lacZ* is reduced throughout (Fig 3.11F). Furthermore, they all displayed a slight up-regulation in the lateral region and close to the dorsal-ventral boundary, suggesting that the mutated dTCF or Brk sites are not required for the observed dorsal-ventral boundary behavior. None of the sites tested are absolutely required to repress *dpp*. In future experiments, we would like to test for a requirement of the dTCF or Brk proteins themselves to more carefully analyze potential requirements for these proteins in particular regions of the wing pouch.

## Discussion

### Many regions of the *dpp* cis-regulatory sequence mediate negative feedback regulation of *dpp*

We employed two approaches to map the negative feedback behavior of *dpp* to particular regions of the *dpp* cis-control sequences: (1) analysis of conservation along 10kb of sequence known to mediate negative feedback of *dpp*, and (2) testing smaller reporter lines for their response to increased BMP signaling. We had initially hypothesized that these approaches would allow us to determine a minimal repression element, or CRM, specifically for negative feedback regulation of *dpp*. However, our analyses revealed that instead of distinct CRMs mediating feedback regulation of *dpp*, the *dpp* locus contains sequences distributed throughout the control regions that are competent to mediate negative feedback regulation of *dpp*.

The sequences contained within the *dpp*<sup>BS3.0</sup>*lacZ* reporter construct, totaling 10.2kb, display peaks of conservation along the entire length of the sequence. This suggests that there are many different regions of regulatory sequence with developmentally relevant functions. Since established CRMs critical for determining the expression domain of *dpp* in the wing disc display high conservation, it is possible that these regions of high conservation represent bona fide CRMs for expression of *dpp* at some point in development. When one considers the complexity of *dpp* expression as the animal develops, temporally and spatially, it is not surprising that so many CRMs may exist for *dpp*.

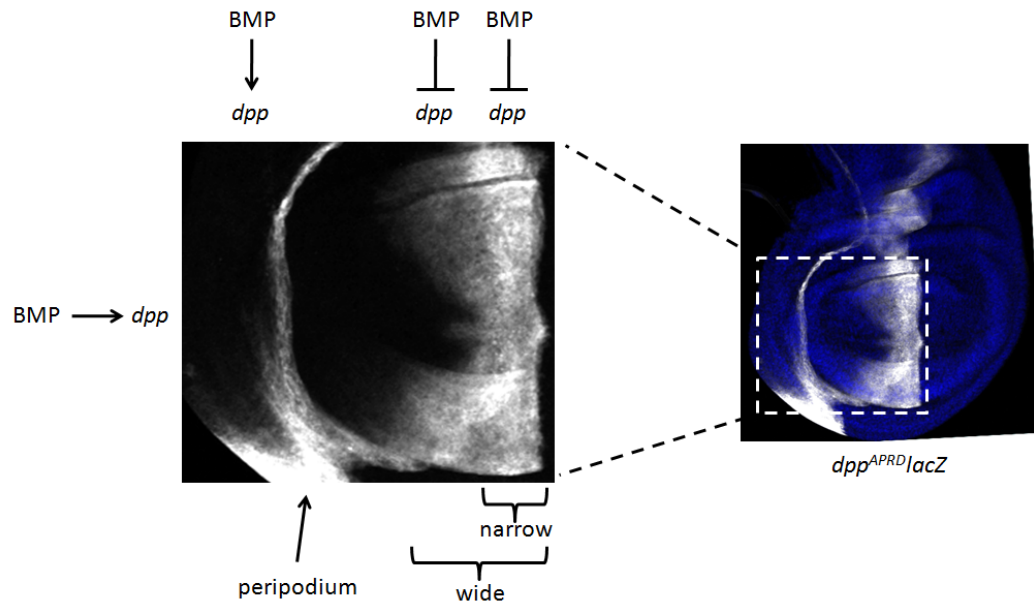
Analysis of different *dpp* reporter constructs consisting of either overlapping or non-overlapping regions of the cis-regulatory sequences revealed that several distinct regions of the control sequence can mediate negative feedback of *dpp*. Furthermore, it is likely that additional sequences outside of the *dpp*<sup>BS3.0</sup>*lacZ* control region mediate a transcriptional change in *dpp* in response to altered BMP signaling. It is of note that none of the reporter constructs that we examined exhibited as striking a repression in response to increased BMP signaling as *dpp*<sup>p10638</sup>*lacZ* did. This was not obvious until we generated *dpp*<sup>MA3.0</sup>*lacZ*, a re-creation of *dpp*<sup>BS3.0</sup>*lacZ*. This reporter displayed a wildtype expression pattern more similar to *dpp*<sup>p10638</sup>*lacZ* than *dpp*<sup>BS3.0</sup>*lacZ*, but was not repressed to the same extent in response to *tkv*<sup>QD</sup> expression as *dpp*<sup>p10638</sup>*lacZ* was (compare Fig 3.6B,B' to Fig 3.8B,B').

Together, these results indicate that regulatory sequences within the *dpp*<sup>disk</sup> region contain many functionally redundant and potentially repeated sequences to mediate the array of regulatory events that *dpp* is subject to. While this makes the study of *dpp* regulation complex, it also speaks to the tight regulation that *dpp* is under. With critical sequences located in several regions throughout the *dpp* locus, whether with unique functions for distinct developmental events or with redundant functions, this could contribute to robust expression of *dpp* by mediating a quick response that can successfully accommodate for signaling fluctuations. Furthermore, the repeated nature of these sequences may make the regulatory sequences of *dpp* less sensitive to genetic change.

### ***dpp* expression exhibits domain-specific responses to BMP signaling**

In our analysis of *dpp* reporter constructs we observed several different ways in which expression of the constructs were altered in response to increased BMP signaling levels. Within the endogenous expression domain of *dpp* along the anterior-posterior axis, reporter expression was reduced in response to increased BMP signaling. However, while some reporter constructs displayed a reduction in expression throughout their expression domain, others only displayed a reduction in expression in a subset of their expression domain. In the lateral region of the wing disc, particularly in the anterior compartment, BMP signaling led to an up-regulation in expression of *dpp* in cells that would normally not express it. Finally, we observed that many reporter constructs behaved differently at the dorsal-ventral boundary than elsewhere in the wing disc. These observations are summarized in figure 3.12. It is clear from our observations that one must pay careful attention to expression of *dpp* in several regions of the wing disc to completely understand its regulatory behavior. Furthermore, these results suggest that many independent mechanisms exist through which *dpp* is regulated. As with regulation of *dpp* in general, multiple functionally redundant control regions mediate all responses, as non-overlapping reporter lines display these particular behaviors.

We were surprised to observe the two different methods of repression along the anterior-posterior axis. Many reporters displayed what we consider to be a repression in a subset of its expression domain. Several pieces of data have indicated that expression of *dpp* in the anterior compartment may



**Figure 3.12. *dpp* displays domain-specific regulation in the wing pouch of the wing imaginal disc.** In the right panel, expression of *dpp<sup>APRD</sup>lacZ* is displayed in white in the context of the entire wing pouch, including the stripe of peripodial expression, with nuclei labeled (in blue). The left panel summarizes our observations of regulation with regards to the many spatial implications for regulation of *dpp*. Along the anterior-posterior boundary expression of *dpp-lacZ* is observed in a narrow domain of high expression and a wider domain of lower expression. The wide expression domain narrows at the dorsal-ventral compartment boundary. In response to increased BMP signaling, we observed repression of *dpp-lacZ* either throughout its expression domain or specifically in the wide domain. Along the dorsal-ventral axis, expression of many reporter constructs were either insensitive to changes in BMP signaling or displayed an up-regulation in these cells. Finally, in the lateral regions of the wing disc, increased BMP signaling increases expression of *dpp-lacZ*.

occur in two overlapping domains along the length of the anterior-posterior axis. First, expression of several reporters display a step-like distribution into the anterior compartment, with a narrow domain of high expression close to the anterior-posterior boundary and a wide domain of weaker expression that extends farther into the anterior compartment (see figure 3.1F). Expression in the wide domain is not always uniform along the dorsal-ventral axis and is narrow close to the dorsal-ventral boundary and widens as it gets further from it (see figure 3.1E). Expression within the wide domain may be especially sensitive to changes in BMP signaling levels, as this domain of *dpp* expression is almost always decreased in response to BMP signaling. However, some reporters, such as *dpp<sup>D</sup>GFP*, are expressed narrowly along the anterior-posterior boundary, suggesting that they could lack regulatory sequences to direct expression in the wide domain of *dpp* (see figure 3.3A).

Regulation of *dpp* in the lateral region of the wing disc is quite different from in the medial wing disc. Previously in the lab, through experiments using the *hh<sup>MRT</sup>* allele of *hh*, which causes ectopic

expression of *dpp* in the anterior compartment, we determined that *dpp* is not negatively regulated in the lateral region of the wing disc. Our more recent data has indicated that not only are lateral cells less sensitive to negative regulation than medial cells, but that expression of *dpp* reporters may actually be up-regulated in response to BMP signaling in this region.

We also reproducibly observed behaviors near the dorsal-ventral boundary that differed from elsewhere within a reporter's expression domain. Lateral cell-autonomous up-regulation was often strongest in the cells closest to the dorsal-ventral boundary. Non cell-autonomous up-regulation at the dorsal-ventral boundary, particularly in the medial region of the pouch, was also observed in several reporter constructs, most noticeably *dpp<sup>p10638</sup>lacZ*. Finally, expression of at least one reporter construct, *dpp<sup>BS3.23</sup>lacZ*, was insensitive to BMP signaling in cells located at the intersection of the dorsal-ventral and anterior-posterior axes. While the specific mechanisms by which *dpp* is differentially regulated at the dorsal-ventral axis are unclear, we are particularly interested in the possibility that this represents an influence by the Wg signaling pathway. In other tissues, the downstream Wg effector proteins, dTCF and arm, have been found to cooperate with Brk and Mad to regulate *dpp*, both positively and negatively (Takaesu et al., 2008; Theisen et al., 2007). While our preliminary analysis on a role for dTCF in this process have been inconclusive, we would like to explore this possibility further by testing the effect of reducing dTCF or arm levels. We are particularly interested in addressing the contributions of these proteins to *dpp* regulation in different regions of the wing disc, as they could interact with the BMP pathway in a different manner in the lateral and medial regions of the disc.

In other developmental contexts, such as in the developing embryo and in the eye imaginal disc, positive regulation of *dpp*, rather than negative regulation, is the norm (Biehs et al., 1996; Chanut and Heberlein, 1997; Hursh et al., 1993; Lee et al., 2007; Pignoni and Zipursky, 1997; Wiersdorff et al., 1996; Yu et al., 1996a; Yu et al., 1996b). At this time, it is unclear what advantage that positive regulation in the lateral wing disc or at the dorsal-ventral boundary imparts on the animal, but it is possible that positive regulation is actually the "default" response of *dpp* to BMP signaling in many cellular contexts. It is important to keep in mind that the *dpp<sup>disk</sup>* regulatory region controls expression of *dpp* at many points in development in response to several different inputs, the regulatory sequences for which are often

redundant or overlapping. Each particular tissue-specific response that we observe may reflect a combination of effects from sequences that actively impart a particular expression behavior as well as from sequences that act to counter-act or compensate for sequences that mediate expression in other contexts. In the lateral wing disc, without factors present to repress *dpp*, it is possible that the up-regulation we observe in the lateral wing disc is an artifact of the propensity of BMP signaling to activate *dpp* expression in other developmental contexts. At the dorsal-ventral boundary, it is possible that the effects we observe are due, in part, to sequences that mediate a BMP/Wg interaction in other contexts. In the case of *dpp*<sup>BS3.23</sup>*lacZ*, it may express so strongly at the dorsal-ventral boundary due to the absence of compensatory regulatory sequences.

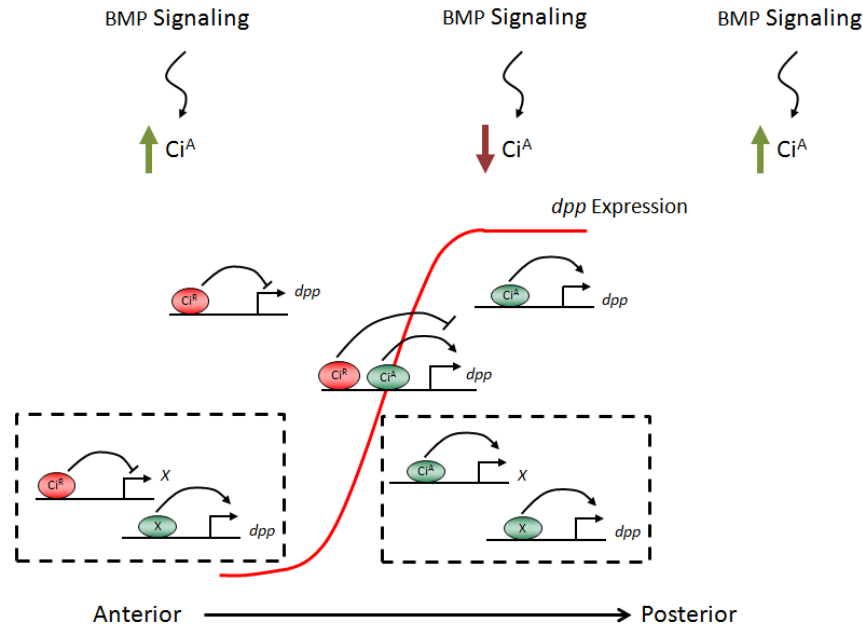
#### **Ci is not required for negative feedback regulation of *dpp***

Ci is the best-understood regulator of *dpp* expression in the wing pouch where it plays a dual role in *dpp* regulation. Ci<sup>A</sup> activates expression of *dpp* along the anterior-posterior boundary and Ci<sup>R</sup> represses expression of *dpp* throughout the remainder of the anterior compartment. As it can both activate and repress *dpp*, Ci was an attractive candidate to be the factor responsible for negative feedback regulation of *dpp*. However, by removing both binding of Ci to the *dpp* control sequence and by removing the Ci protein itself, we have determined that it is not absolutely required for negative regulation of *dpp*.

These experiments do not, though, indicate that Ci plays no role in feedback regulation of *dpp*; It could still play a minor and non-essential role in this process. Our experiments examining expression of *dpp*<sup>p10638</sup>*lacZ* without Ci have demonstrated that negative regulation of *dpp* can occur independent of Ci, but these results do not speak to the efficiency of *dpp* repression in the absence of Ci. In discs without Ci, *dpp* levels are already reduced in comparison to wildtype. In response to increased BMP signaling, the reduction in reporter expression that we observe in the absence of Ci may be less than what we observe in its presence. Distribution and levels of Ci<sup>A</sup> were also found to be slightly altered in response to BMP signaling (Fig 3.13), a response that could influence expression of *dpp*.

In the course of these analyses we made several interesting observations that impact our current understanding of how Ci regulates *dpp*. First, we noted that expression of *dpp* reporters that cannot be





**Figure 3.13. Ci both directly and indirectly regulates expression of *dpp*.** Schematic diagram of *dpp* expression in the wing pouch, primarily in the anterior compartment. Based on our previous knowledge of regulation of *dpp* by Ci, the expression of *dpp* along the anterior-posterior boundary is the result of a balance of activating influence by *Ci<sup>A</sup>* (in green) and repressing influence by *Ci<sup>R</sup>* (in red). Our results suggest an additional method of regulation by Ci, displayed in the dashed boxes, by which Ci can indirectly regulate *dpp*. This model explains our observation that *dpp-lacZ* reporters that cannot bind Ci display a uniform expression pattern in the absence of Ci protein. In addition, we have demonstrated that BMP signaling causes a slight change in the levels of *Ci<sup>A</sup>*. In the lateral regions of the wing pouch, in both the anterior and posterior compartments, *Ci<sup>A</sup>* levels are slightly increased by BMP signaling. In the medial region of the wing pouch, *Ci<sup>A</sup>* levels are slightly decreased by BMP signaling.

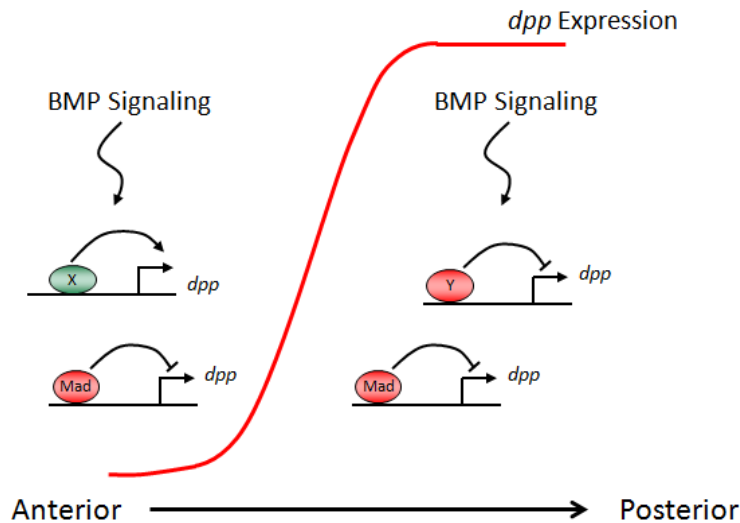
regulated by Ci display drastically different expression patterns depending on the *cis*-regulatory context of the mutations. In comparison to a wildtype reporter construct, expression of *dpp<sup>D-Ci3</sup>GFP* is reduced along the anterior-posterior axis but also increased throughout the remainder of the anterior compartment (see figure 3.3B). This has explained through the loss of both activation and repression by Ci, leaving only basal activation of *dpp* (Müller and Basler, 2000). Expression of our *dpp<sup>APRD-Ci5</sup>lacZ* and *dpp<sup>APR-Ci3</sup>lacZ* reporters, however, is actually narrowed and increased compared to wildtype (see figure 3.3F,I). In the context of larger regions of *dpp cis*-regulatory sequence, the unknown basal activator of *dpp* is either less efficient in activating *dpp* throughout the anterior compartment or the additional sequences present in these larger reporter constructs bind factors that generate a different expression domain of *dpp*. It would be of interest in future studies to determine the identity of the basal activator and determine the sequences that result in such an altered expression domain of *dpp* in the absence of Ci.

Finally, we made the intriguing observation that expression of *dpp* reporters with mutant Ci binding sites, including *dpp*<sup>APRD-Ci5-M</sup>*lacZ*, *dpp*<sup>APRD-Ci5</sup>*lacZ* (see figure 3.7), and *dpp*<sup>APR-Ci3</sup>*lacZ* (see figure 7.32) were sensitive to the loss of Ci protein. Each of these constructs displayed an expansion of their expression domain in the absence of Ci. This was entirely unexpected, as these reporters should not bind Ci and all current data indicates that Ci directly regulates *dpp*. Previous work has examined expression of *dpp* in the absence of Ci protein or expression of *dpp* reporters in the absence of Ci binding, but no one has combined these methods before. Our observation could be explained by one of two scenarios: (1) Ci binds and regulates *dpp* at another location within the control sequences, perhaps at an unappreciated or degenerate binding site, or (2) Ci has an indirect role in regulating *dpp*. The second possibility, displayed in figure 3.13, is particularly interesting as it represents a novel mechanism by which Ci may regulate *dpp*.

#### **Mad may both directly and indirectly regulate *dpp***

Our experiments have revealed a direct role for Mad in repressing *dpp*. The control sequences of *dpp*<sup>BS3.0</sup>*lacZ* that we examined contain five consensus binding sites for Mad, three of which are highly conserved. Mutation of one of these sites in the context of mutant Ci binding sites leads to a greatly expanded expression domain of *dpp-lacZ* into the anterior compartment. Furthermore, this reporter is expressed at slightly higher levels than its wildtype counterpart. When we examined expression of the *dpp*<sup>APRD-Ci5-M</sup>*lacZ* reporter in cells with increased BMP signaling, we observed an even further increase in expression in the anterior compartment. Together, these results suggest that Mad may play a role in keeping *dpp* repressed throughout the anterior compartment.

Our results suggest that BMP signaling plays a dual role in regulating expression of *dpp* in the wing imaginal pouch (displayed in figure 3.14). In the medial region of the wing pouch BMP signaling leads to a repression of *dpp* expression. In the lateral region, BMP signaling leads to an up-regulation of *dpp* that is enhanced by a reduction in the ability of Mad to bind *dpp* control sequences. As of now, it is unclear how binding of Mad can counteract BMP induced up-regulation of *dpp* in the lateral wing pouch, but the answer may lay in the action of particular cofactors or the function of different Mad binding sites



**Figure 3.14. BMP signaling has a dual role in regulation of *dpp*.** Schematic diagram of *dpp* expression in the anterior compartment of the wing disc. BMP signaling can both activate and repress *dpp* in the wing imaginal disc. In the lateral region of the anterior compartment, increased BMP signaling leads to an up-regulation of *dpp* expression. Along the anterior-posterior compartment boundary, increased BMP signaling represses expression of *dpp*. Direct binding of Mad has a repressive effect on *dpp* expression throughout the anterior compartment, with loss of Mad binding leading to a further enhancement of the lateral up-regulation.

within the *dpp* control sequences. It is possible that these individual sites perform different regulatory functions.

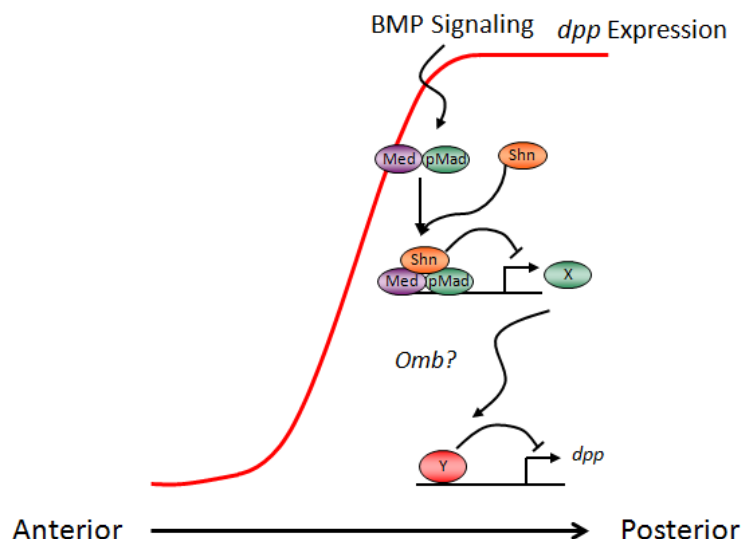
Future experiments will further address the role of Mad in regulating *dpp* in the wing pouch. As we have only tested one Mad site thus far, we would like to investigate the function of the other Mad binding sites present in the *dpp* control sequences by mutating them as well. These sites will be tested in the context of wildtype Ci binding sites, as our current studies only examined one Mad binding site mutation in reporters that also had mutant Ci binding sites. Finally, it will be important to address the requirement for Mad in this process by examining the expression of *dpp* in the absence of Mad protein.

To the best of our knowledge, Mad requires the activity of repressive co-factor to directly behave as a transcriptional repressor, one of which is Shn. In the wing disc, the action of a Shn/pMad/Med complex plays an important role in determining the expression domains of several BMP target genes (Campbell and Tomlinson, 1999; Moser and Campbell, 2005; Müller et al., 2003; Vuilleumier et al., 2010). Two consensus binding sites for Shn/pMad/Med (Gao et al., 2005; Pyrowolakis et al., 2004) were identified within the *dpp*<sup>B53.0</sup>*lacZ* control region. However, when we mutated these sites in a manner that should disrupt binding of the repressive complex, we did not observe any effect on expression of the

reporter under endogenous or elevated BMP signaling levels. We concluded that direct binding of the Shn/pMad/Med complex to consensus sequences within *dpp*<sup>BS3.0</sup>*lacZ* is not required to regulate expression of *dpp*, but will still consider the possibility that Shn/pMad/Med may bind elsewhere in the *dpp* locus.

Removal of Shn protein, however, did influence regulation of *dpp*. In clones of cells without Shn protein, increased BMP signaling does not lead to a significant reduction in expression of *dpp*<sup>p10638</sup>*lacZ*. This result suggests that the Shn/pMad/Med complex is required for repression of *dpp*, but it may play an indirect role in mediating this response. In future experiments, we will test several factors downstream targets of Shn/pMad/Med for a role in regulating *dpp*.

There has been one published report suggesting that Omb may play a role in regulating *dpp* (Umemori et al., 2007). As *omb* is downstream of Shn (Sivasankaran et al., 2000), we performed a preliminary experiment testing the transcriptional response of *omb-lacZ* in clones of cells without *shn* or with increased BMP signaling. If Omb regulates *dpp*, then we expect its activity or levels to change in both cases. While loss of Shn does reduce *omb-lacZ* expression, increased BMP signaling does not increase expression of *omb-lacZ* in the medial wing disc. Based on this observation, it seems unlikely that Omb



**Figure 3.15. Shn is indirectly required for negative feedback regulation of *dpp* in the medial wing disc.**

Schematic diagram of *dpp* expression in the anterior compartment of the wing disc. Shn is required for negative feedback regulation of *dpp* to occur but it does not directly regulate *dpp*. In response to increased BMP signaling, a complex of Shn, pMad, and Med influences the expression of a factor that influences *dpp*, either directly or indirectly. A potential indirect regulator of this process is Omb.

alone is responsible for repression of *dpp* unless BMP signaling can somehow influence activity of Omb protein. Instead, we expect that a factor downstream of Omb or that works in cooperation with Omb is responsible (fig 3.15). There are several downstream factors of Omb that we are interested in examining including the BMP targets *sal* and *vg* and the regulator of *tkv*, known as *mtv* (del Alamo Rodriguez et al., 2004). In our future experiments, we will ask whether *dpp* can be negatively regulated in the absence of either *sal*, *vg*, *mtv*, or *omb*.

### **BMP signaling is a robust system**

Taken together, the results presented in this study demonstrate complex mechanisms through which *dpp* is regulated in the wing imaginal disc. Proper expression and regulation of *dpp* represents the combined activity of many factors acting across a large region of *cis* control sequence. These factors not only determine where *dpp* will be expressed, but also how much *dpp* will be expressed. Our experiments have revealed multiple spatial considerations for *dpp* regulation in the wing disc as well as potential roles for both Mad and Shn/pMad/Med in regulating *dpp*. In addition, we have uncovered a potentially novel indirect role for Ci in regulating *dpp*. We believe that the output of all of these regulatory components is the carefully regulated expression of a BMP ligand that contributes to a robust signaling pathway.

Robustness refers to the ability of a signaling pathway to compensate for fluctuations in signaling output and keep activity levels at a particular levels or within an effective range of levels (Barkai and Leibler, 1997; Barkai and Shilo, 2009; Eldar et al., 2004; Freeman, 2000; Kerszberg, 2004; Kerszberg and Wolpert, 2007). BMP signaling is known to attain robustness through the regulatory actions of BMP targets such as *dad* and *pent* (Ben-Zvi et al., 2011; Hamaratoglu et al., 2011; Ogiso et al., 2011; Vuilleumier et al., 2010). Negative regulation of *dpp* may represent an additional mechanism through which BMP signaling can maintain robust signaling in the face of environmental, developmental, or genetic perturbations. We propose that regulation of *dpp* transcription plays an important role in maintaining the pMad activity gradient during development and are interested in determining the contribution of negative regulation to this process. We expect that in the absence of *dpp* feedback regulation, BMP signaling could become unstable and pMad levels could be altered inappropriately.

To understand the implications for negative feedback of *dpp* in maintaining the pMad activity gradient, it will be important for us to determine the temporal aspects of the mechanism. To be most effective, we expect this system to work quickly to restore signaling to endogenous levels. In addition, we would expect it to be active throughout development of the wing disc, particularly during and before the stages when cells are most sensitive to pMad levels. We are also interested in testing *gbb* for a similar mechanism of negative feedback regulation. If, indeed, this mechanism is important for maintaining the pMad gradient then we might expect some regulation of *gbb* expression as well. Preliminary data in the lab in an S2 cell system has suggested that *gbb* is repressed by BMP signaling as well (Takuya Akiyama, personal communication). Together, these results will help determine the contribution that negative feedback of BMP ligands plays in maintaining the pMad gradient during the development of the wing imaginal disc.

## Chapter 4:

### Discussion and Future Directions

Morphogen activity gradients generate transcriptional responses and influence cell fate specification in a concentration-dependent manner. The pathways that generate the morphogen activity must have a level of built-in robustness to ensure that particular thresholds of morphogen activity are encountered by cells at the appropriate time in development. Fluctuations in activity may occur in response to inherent developmental processes, such as tissue growth or proliferation, or they may result from environmental or genetic influences. Regardless of the source of variability in activity, an effective morphogen system must be able to sense these disturbances and accommodate by adjusting pathway output; they must, in essence, be highly robust systems.

In development of the *Drosophila* wing imaginal disc, the BMP signal transduction pathway plays a critical role in patterning along the anterior-posterior axis of the developing wing pouch through activity of its downstream transducer, pMad (Khalsa et al., 1998; Lecuit et al., 1996; Nellen et al., 1996; Singer et al., 1997). In this pathway, mechanisms exist that ensure robust signaling through transcriptional regulation of intracellular and extracellular negative regulators (Ogiso et al., 2011; Szuperák et al., 2011; Vuilleumier et al., 2010). In our work, we have identified and characterized an additional method of BMP regulation through transcriptional regulation of the ligand *dpp*. Within the endogenous *dpp* expression domain along the anterior-posterior boundary of the wing pouch, a negative feedback loop represses *dpp* in response to increased BMP signaling and up-regulates *dpp* in response to decreased BMP signaling. We believe that this form of feedback regulation may play an important role in “fine tuning” the output of BMP signaling throughout development.

#### **Negative feedback regulation of *dpp* is BMP-specific and correlates with pMad levels**

By experimentally manipulating the expression level or activity of pathway components, we have revealed that expression of *dpp* is sensitive to changes in activity or level of several BMP pathway components, including ligand, type I receptor, and transducer. Over-expression of *Mad* results in a cell-autonomous decrease in *dpp* expression, indicating that this effect is in response to Mad-mediated canonical BMP signaling. Furthermore, we have identified a correlation between increased levels of pMad



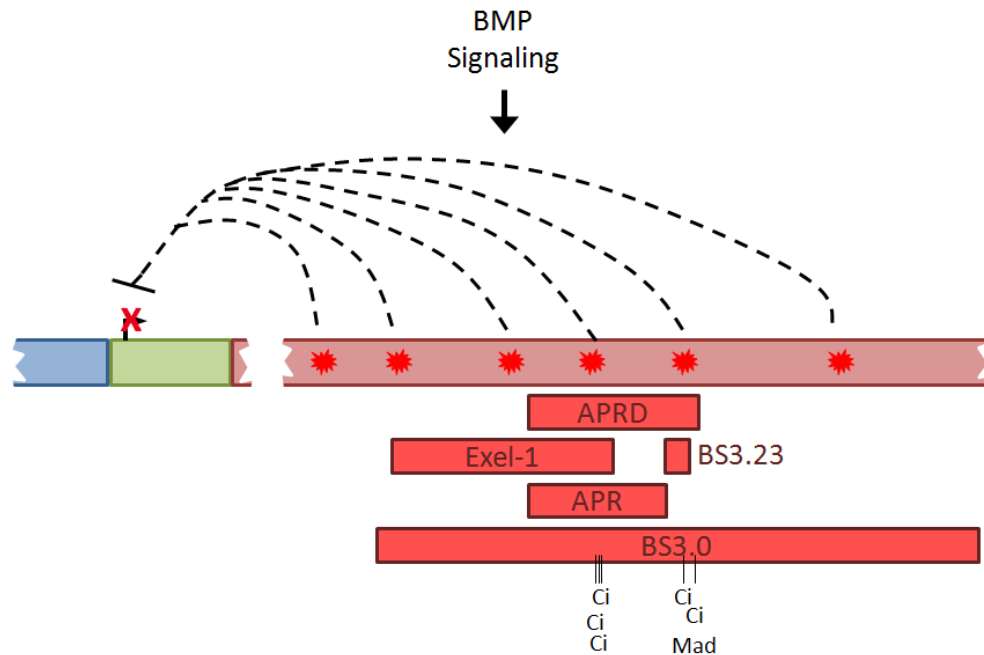
and decreased transcription of *dpp*. The response in expression of *dpp*, thus, appears to be pMad-dose dependent.

In future experiments, we would like to further explore this observed dose-dependency and better characterize this response. In particular, we will test whether there is a dose-dependent response to decreases in activity or level of *Mad* as well. By experimentally decreasing several components of the pathway, such as *gbb*, *tkv*, *sax*, or *Mad*, and observing the resulting pMad level and expression of *dpp<sup>p10638</sup>lacZ*, we can test for a correlation between decreased pMad and increased *dpp* expression. These components can be decreased by creating loss-of-function clones for each factor, as previously done, or by expressing *RNAi* lines directed against the factor.

All experiments thus far that revealed a dose-dependency of pMad on *dpp* expression were based on correlations; pMad was increased in response to over-expression of *gbb*, *dpp*, or *tkv<sup>QD</sup>* and we observed a correlation between increased pMad and decreases *dpp-lacZ* expression. As a second, more direct, method to test the dose-dependent response of *dpp* expression to *Mad* manipulation, we can specifically control the amount of *Mad* expressed and examine the resulting *dpp-lacZ* expression and pMad. This will confirm that the responses we see are specifically due to the *Mad* level and not a specific response to altered receptor or ligand level. To control levels of *Mad*, we will generate *Mad* and *Mad-RNAi* FLP-out clones and raise larvae over a range of temperatures after clone induction. The Gal4 protein used in these experiments is more active at higher temperatures and less active at lower temperatures; by raising the larvae at multiple temperatures we can generate a range of expression strengths. We expect that higher levels of *Mad* expression will lead to stronger repression of *dpp<sup>p10638</sup>lacZ* expression and lower levels of *Mad* expression will lead to stronger up-regulation of *dpp<sup>p10638</sup>lacZ* expression.

#### **There is redundancy within the *dpp cis*-regulatory sequences in mediating feedback regulation of *dpp***

The *dpp* locus contains over 50kb of *cis*-regulatory sequence, specific regions of which direct expression of *dpp* in a temporal- and tissue-specific manner throughout *Drosophila* embryonic and larval development (Blackman et al., 1991; Jackson and Hoffman, 1994; Kopp et al., 1999; Masucci et al., 1990; St Johnston et al., 1990). We observed that a *dpp-lacZ* reporter line, *dpp<sup>B53.0</sup>lacZ*, containing 10kb of



**Figure 4.1. Negative feedback regulation of *dpp* is mediated by many *cis*-regulatory sequences.** Our experiments have revealed a functional redundancy within *dpp* enhancer regions in mediating negative feedback of *dpp* expression. Several reporter constructs containing sequences from within the *dpp*<sup>disk</sup> region were tested and found to display altered expression patterns in response to increased BMP signaling, indicating that multiple sequences direct negative regulation of *dpp*. In response to increased BMP signaling, binding of repressors to these sites (demonstrated in red) interact with the transcriptional machinery and repress expression of *dpp*. These sites may represent multiple different factors mediating regulation of *dpp*, one factor binding at many sites, or a combination of both.

control sequence displayed a transcriptional response to increased BMP signaling, indicating that the factors sufficient to mediate repression must bind within this region. To map the sequences that mediate this response, we examined the expression patterns of reporter lines containing smaller subsets of the 10kb region in response to increased BMP signaling. These experiments demonstrated that multiple reporters containing non-overlapping *cis*-regulatory regions are sufficient to mediate repression in response to increased BMP signaling, indicating a functional redundancy within these sequences in mediating negative feedback regulation. Therefore, the factor or factors that regulate *dpp* in response to BMP signaling must be able to bind the *cis*-regulatory sequences at multiple locations (Fig 4.1).

Due to the functional redundancy in mediating negative feedback, it is likely that transcription factors responsible for negative feedback bind the *dpp* enhancer regions at multiple, distinct locations. If this is the case, we expect that the sequences that the factor binds will be repeated throughout the length of the regulatory sequence. To help us determine candidate factors, we will identify these repeated

sequences by creating a dot plot for the 10kb *dpp*<sup>BS3.0</sup>*lacZ* enhancer sequence. To do this, we will use the Purstell matrix analysis function in MacVector to compare this 10kb region to itself. The matrix generated from this analysis will allow us to identify repeated sequences. Once identified, we will examine these repeated regions to identify repeated consensus binding sites and use bioinformatics tools, such as Target Explorer and RedFly (Gallo et al., 2011; Sosinsky, 2003), to identify potential transcription factor binding sites and candidate factors that bind these regions. It is likely that several factors contribute directly to feedback regulation of *dpp*, in which case we may identify several distinct repeated modules, all of which can be analyzed by similar methods.

### **Multiple factors influence expression of *dpp***

The functional redundancy that we observed in the *cis*-regulatory sequences of *dpp* is underscored by our observations that multiple factors influence expression of *dpp*. We tested several candidate factors that we suspected may play a role in regulation of *dpp* based on their known roles in BMP signaling. Given the well-established role that Ci plays in both activating and repressing *dpp*, we suspected that it would be required to regulate *dpp*. However, by both removing Ci protein and precluding binding of Ci to *dpp* reporter constructs, we determined that Ci is not required for negative feedback regulation. This does not, however, necessarily mean that Ci is not involved in this process. We observed a slight change in Ci<sup>A</sup> protein with increased BMP signaling, so Ci could still play a minor role in this process. In addition, we made the surprising discovery that *dpp* reporter constructs with non-functional Ci binding sites are still sensitive to the presence of Ci, suggesting that Ci can either indirectly regulate *dpp* or that Ci regulates *dpp* at degenerate or non-consensus binding sites. Future experiments will explore these possibilities.

Shn, a Mad cofactor, was also tested as a potential negative regulator of *dpp* transcription. In the medial region of the wing disc where *dpp* is expressed, a complex of Shn, pMad, and Med represses expression of the BMP target genes *brk* and *pent* (Marty et al., 2000; Vuilleumier et al., 2010). Given its function as a repressor and its activity in the medial wing disc, as well as the presence of Shn/pMad/Med binding sites in the *dpp* enhancer sequence, we hypothesized that Shn mediates negative regulation of

*dpp*. Indeed, this protein is required for *dpp* to be repressed in response to increased BMP signaling, but the requirement may be indirect as mutating the Shn/pMad/Med binding sites does not influence negative regulation. While it remains possible that the Shn/pMad/Med complex could act at an alternate site, we conclude that a factor downstream of Shn likely regulates *dpp*. Several factors are known to be directly or indirectly regulated by Shn. We are particularly interested in a role for Omb, as this factor has also been implicated in regulation of *dpp* (Umemori et al., 2007). Omb itself has been reported to influence the expression of *sal*, *vg*, and *mtv* (del Alamo Rodriguez et al., 2004). Future experiments will test these factors, as well as *omb*, for a role in regulating *dpp* by examining the expression of *dpp* in response to increased BMP signaling in the absence of each factor.

#### **BMP signaling plays a dual role in regulating *dpp* in the lateral wing disc**

In addition to negative regulation of *dpp* within its endogenous expression domain, we observed positive feedback in the lateral regions of the wing disc and along the dorsal-ventral boundary. At this point, it is unclear what role this positive feedback could be playing, especially since *dpp* is not expressed in these cells under endogenous signaling conditions. Interestingly, *dpp* is known to be positively regulated by BMP signaling in several other tissues, including the embryonic midgut, the eye imaginal disc, and the pupal veins. It is possible that positive feedback regulation of *dpp* is the “default” regulation that occurs in response to BMP signaling and that *dpp* is negatively regulated in the medial wing pouch due to the combination of particular factors that are present or active in this region. In these cells, repressive co-factors allow BMP signaling to repress, rather than activate, expression of *dpp*.

Unexpectedly, we uncovered a potential direct role for Mad binding in regulating *dpp*, particularly in the lateral region of the wing pouch. Mutation of a previously untested consensus Mad binding site (Hepker et al., 1999) resulted in increased expression of *dpp-lacZ* throughout the anterior compartment, particularly in response to increased BMP signaling. Mutation of this site did not, however, impact negative regulation of *dpp* within its endogenous expression domain. Our results indicate that in the lateral wing disc, BMP signaling plays a dual role in regulating *dpp*. BMP signaling increases expression of *dpp* in these cells, possibly by indirect mechanisms, but direct binding of Mad at that site may repress

*dpp*. We propose that, at this site, direct binding of Mad to *dpp* control regions counteracts the BMP mediated up-regulation of *dpp* that was observed.

In our future endeavors, we will further explore the role of Mad binding in both negatively and positively regulating *dpp*. The mutation created in the Mad binding site was introduced into a *dpp-lacZ* reporter that also had several Ci binding sites mutated. It will be important to address the function of this site in the context of an otherwise wildtype reporter. This is of particular importance considering that this Mad site overlaps with an existing Ci site, raising the possibility that Ci and Mad could interact in this location. As evidence from the mammalian system has suggested that these two proteins may interact (Liu et al., 1998), future experiments will address potential interactions between Mad and Ci in regulating *dpp*.

In addition, we have identified and will test several other Mad consensus sites that could be influencing expression of *dpp*. As mutation of the single Mad binding site leads to an altered expression pattern of *dpp-lacZ*, we believe this site to be active in regulating *dpp*. However, to more directly test binding of Mad to these sequences, we will perform gel shift assays between Mad protein and *dpp* enhancer sequences with wildtype and mutated Mad binding sites.

As a final test for the role of Mad in regulating *dpp*, we would like to determine the transcriptional response of *dpp* to loss of Mad protein. As described above, we can remove Mad by creating *Mad* loss-of-function or *Mad-RNAi* clones. Combining Mad binding site mutant reporters with a loss of *Mad* expression will help us determine the direct and indirect roles that Mad plays in regulation of *dpp* in the lateral wing disc. We expect, given the different responses of *dpp* expression to BMP signaling in different regions of the wing disc, that the specific role of Mad will display domain-specific behavior.

### **Negative feedback regulation of *dpp* may contribute to robustness of BMP signaling**

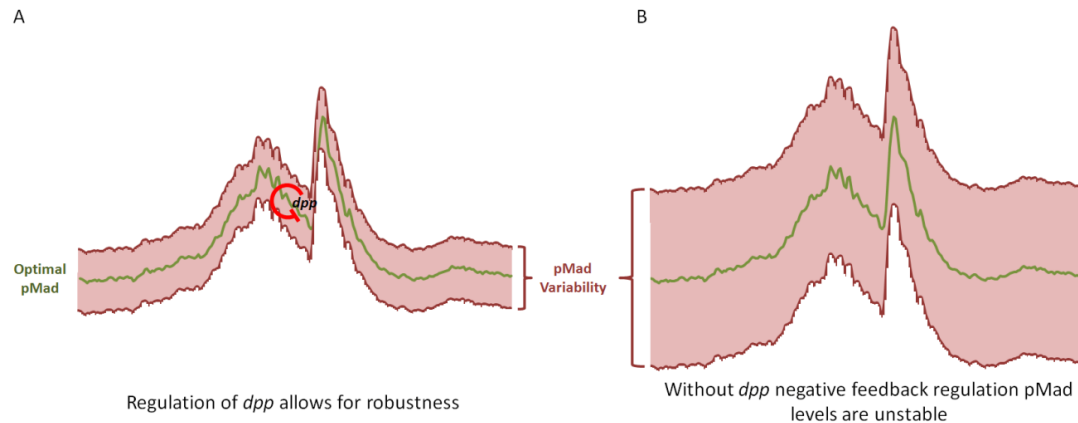
Our observation that repression of *dpp-lacZ* is pMad dose-dependent suggests that this system can sense different levels of BMP signaling activity and respond accordingly. What is not clear, at present, is the specific role that negative feedback regulation of *dpp* plays in BMP activity robustness. We believe that this mechanism “fine tunes” expression of *dpp* in response to fluctuations in pMad level and that it

plays an important role in ensuring that correct levels of pMad are met and maintained throughout development.

We propose a model in which negative feedback regulation of *dpp* serves to maintain pMad levels within a normal distribution or range that allows for proper development (Fig 4.2). At any point during wing imaginal disc development, pMad activity may fall within a certain range of variability, centering on an optimal or mean pMad level (Ogiso et al., 2011). The specifics of this gradient may change over time, both in the distribution of the gradient as well as in the amplitude of the gradient, but at any point an optimal level exists. Feedback regulation of *dpp* transcription keeps the pMad activity within the acceptable and narrow range; if pMad falls below the optimal level transcription of *dpp* will increase and if pMad raises above the optimal level transcription of *dpp* will decrease (Fig 4.2A). If, for some reason, *dpp* could not be negatively regulated, the BMP signaling pathway would not be able to efficiently accommodate for deviations from optimal levels of pMad signaling and pMad activity would become unstable. As a result, a greater variability in pMad activity would be observed (Fig 4.2B).

Indeed, we have made one observation that suggests a possible role for negative feedback of *dpp* in maintaining robust pMad levels. Our observations of increased pMad in *shn-RNAi* clones may represent a potential instability in pMad levels in the absence of *dpp* repression. We observed that clones of cells expressing *shn-RNAi* were unable to reduce expression of *dpp*<sup>p10638</sup>*lacZ* while also displaying an increase in pMad levels (Fig 3.8F, 3.9B"). While we suspect that misregulation of *dad* may be partially responsible for this observation, the increase in pMad that we observed is stronger than expected from loss of *dad* alone (Ogiso et al., 2011). Instead, we think our observation is due to a combination of *dad* misregulation and the absence of *dpp* negative feedback. This result points to an important role of *dpp* negative feedback in maintaining pMad levels during development of the wing imaginal disc.

To directly test the role for *dpp* feedback regulation in maintaining pMad robustness, we will make use of a sensitized background in which *dpp* cannot be properly regulated, the *dpp*<sup>d</sup> alleles (Spencer et al., 1982; St Johnston et al., 1990). These alleles are chromosomal inversions, translocations, or deletions that remove regions of the *dpp*<sup>disk</sup> enhancer sequences directing expression of *dpp* in the wing imaginal discs and, as we have found, allowing for negative feedback of *dpp* expression. We have



**Figure 4.2. Model of the contribution of *dpp* negative feedback regulation to robustness of BMP signaling. A.**

Expression of *dpp* can be negatively regulated by BMP signaling. As a result of this process, pMad levels are close to the optimal level (in green) and remain within a functional range (in red). To accommodate for variability in pMad, as pMad levels increase *dpp* is repressed and as pMad levels decrease *dpp* is up-regulated. **B.** When *dpp* cannot be negatively regulated by BMP signaling, the pathway loses its ability to accommodate for fluctuations in pMad activity. The observed pMad variability is now much higher, bringing levels far higher and lower than the optimal level and outside of a functional range.

available to us several such alleles with breakpoints at various locations within the control region, effectively serving as a series of truncations in the enhancer region. Given the functional redundancy of regions that mediate negative feedback of *dpp*, each allele should vary in its ability to mediate repression of *dpp* in the wing disc. Our viability experiments described in chapter two (Tables 2.1, 2.2) suggest to us that this is, indeed, occurring. We observed that reducing *gbb* levels in a *dpp<sup>d5</sup>/dpp<sup>hr</sup>* background led to a rescue in viability, while reducing *gbb* levels in a *dpp<sup>d6</sup>/dpp<sup>hr</sup>* background exacerbated lethality, suggesting that *dpp<sup>d5</sup>* animals could negatively regulate *dpp* while *dpp<sup>d6</sup>* animals could not.

We will analyze the pMad gradient in wing pouches in control and in various *dpp<sup>d</sup>* backgrounds to determine the pMad variability in each genotype. Based on our hypothesis, we should observe a pMad distribution with greater variability in *dpp<sup>d</sup>* animals that cannot mediate feedback regulation of *dpp* than in wildtype animals. Furthermore, we expect those *dpp<sup>d</sup>* alleles with larger enhancer regions removed to display higher pMad variability than those with smaller enhancer regions removed. As *dpp<sup>d</sup>* animals already have reduced *dpp* expression in the wing pouch, differences in basal pMad activity for each genotype may complicate our interpretations. If so, we can, instead, test the response of each genotype to increased expression of BMP ligand. In our previous studies we observed that, in a wildtype background, over-expression of *gbb* has little effect on pMad while over-expression of *dpp* has a

moderate effect on pMad. We expect that in a *dpp<sup>d</sup>* background over-expression of *gbb* and *dpp* will now lead to a much greater increase in pMad levels.

Also key to understanding the role of *dpp* negative feedback regulation in BMP robustness is an appreciation of the temporal aspects of this process. The pMad gradient scales throughout development, suggesting the action of regulatory mechanisms that influence pMad as the tissue size increases (Ben-Zvi et al., 2011; Ben-zvi et al., 2008; Hamaratoglu et al., 2011; Wartlick et al., 2011). As influencing transcription of *dpp* could affect pMad throughout the tissue, negative feedback regulation of *dpp* could act as a mechanism to allow proper scaling of the pMad gradient as development proceeds. For all of our experiments thus far, we observe pMad and *dpp* expression in late third instar wing imaginal discs. In future experiments, we would like to explore the contribution of *dpp* negative feedback regulation to robustness of pMad not just in this late stage, but throughout development. To test this, we can observe the effect of increasing BMP ligand at specified time points in both wildtype and *dpp<sup>d</sup>* backgrounds. The ability of the animal to accommodate for increased ligand and the resulting pMad gradient will help us to understand when negative feedback regulation of *dpp* can occur and what the contribution of this process is in ensuring robust BMP signaling in the wing imaginal disc.



Chapter 5:  
Introduction to border cell migration

### **Cell migration in development**

Cell migration is a complex and highly regulated process essential for formation of many different cell types and structures during the development of multicellular organisms (Aman and Piotrowski, 2010). In several organ systems, proper organogenesis relies on the migration of cells from a distant location to the organ in question, where they will differentiate into one or several of the cell types that contribute to the final structure. This migration process can involve individual cells that migrate independently from one another or groups of cells that move together as one structure. In either case, this process involves three distinct steps: (1) detachment from the initial cellular environment, (2) migration along a particular path within the embryo, and (3) arrival and incorporation at the target site. Each step of this process must be highly regulated to ensure that cells migrate at the appropriate time, along an appropriate path, and to the appropriate location. If this process is not performed correctly during development, entire cell types or structures may not form properly. It is also important that this process be restricted to particular times during the life of the animal, as inappropriate activation of embryonic migration mechanisms in an adult animal can have disastrous results, such as is the case with metastatic cancers.

Perhaps one of the best studied examples of directed cell migration is that of vertebrate neural crest migration (Kulesa and Gammill, 2010). The neural crest is a population of dorsal ectodermal cells originating from the boundary between cells of the neural tube and the overlying ectoderm. As the neural tube closes, a population of these cells undergoes an epithelial-to-mesenchymal transition (EMT) and delaminates from the surrounding ectodermal tissue. These cells will then migrate as mesenchymal cells in distinct paths to various regions of the developing embryo. Once these cells have reached their destination, they will take on one of several different cell fates including, but not limited to, cartilage, components of the peripheral nervous system, and melanocytes. The processes underlying the initial specification and migration steps of the neural crest have been intensely studied. Coordinated action of many different components ensures that these cells reach their proper destination.

Other examples of directed cell migration involve, not clusters of mesenchymal cells, but cohesive groups of cells. In recent years, a new model system has emerged to study collective cell

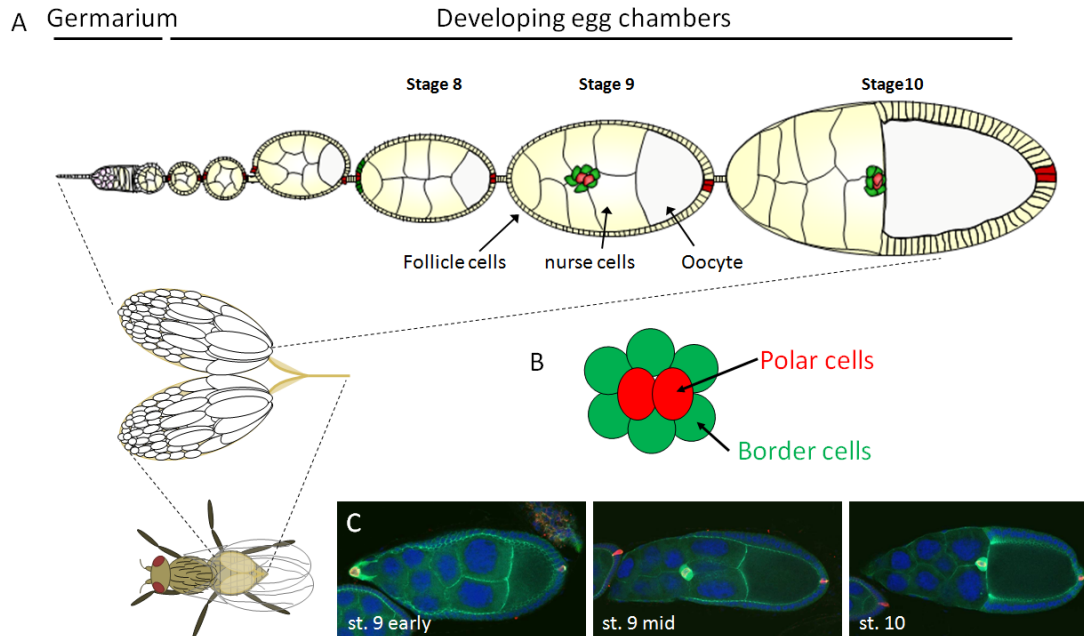
migration of a group of cells known as the border cells during *Drosophila* oogenesis. In this system, a collection of cells migrates as a group within an egg chamber of the *Drosophila* ovary.

### ***Drosophila* border cell migration**

The *Drosophila* ovary is comprised many individual ovarioles, within which oogenesis takes place (Fig 5.1A) (Bastock and St Johnston, 2008; Wu et al., 2008). At the anterior-most end of the ovariole is the germarium, containing the germline stem cells as well as somatic stem cells, and at the posterior-most end is the mature oocyte, ready for fertilization. Within one ovariole are several egg chambers at various stages in oogenesis, connected to one another by stalk cells. As egg chambers travel through the ovariole towards the posterior end, they progress through oogenesis.

Each egg chamber begins as 16-cell germ cell cyst surrounded by a single layer of somatic follicle cells. Through a series of incomplete cleavages, the germ cells remain connected to one another through structures known as ring canals, allowing the transfer of cytoplasm between them. Of these cells, one will go through meiosis to become the oocyte and the others will become the polyploid nurse cells that provide nourishment to the developing oocyte. As oogenesis proceeds, the oocyte will be pushed to the posterior end of the egg chamber, accumulate yolk, and grow at the expense of the nurse cells until they have been completely displaced. In addition, the surrounding follicle cells, which are initially cuboidal, will take on a columnar structure over the oocyte at the posterior end and flatten out to cover the nurse cells at the anterior end.

The process of border cell migration is initiated by the specification of the pole cells (Jang et al., 2007; Montell, 2003). Early in development, two cells at each end of the egg chamber are specified as pole cells. At stage eight of oogenesis, the anterior-most pole cells will direct the surrounding follicle cells to take on the border cell fate. During stage nine, the border and polar cells will delaminate from the follicle cell epithelium and begin to migrate toward the oocyte. Moving as an intact cluster of four to eight border cells and two polar cells (Fig 5.1B), the border cell cluster will travel between the nurse cells towards the posterior pole of the egg chamber (Fig 5.1C). By stage ten, these cells would have reached the oocyte and flattened against it. This stage is characterized by a sharp and straight boundary between



**Figure 5.1. *Drosophila* border cell migration.** **A.** *Drosophila* oogenesis proceeds within the ovarioles that make up the ovary. Within each ovariole is a posterior germarium, containing the germline stem cells and somatic stem cells, and several egg chambers at various stages of oogenesis. Two polar cells (in red) are specified early in oogenesis at each pole of the egg chamber. At stage eight, border cells are specified in the region surrounding the anterior polar cells. During stage nine, the border cells and polar cells detach from the surrounding follicle cell epithelium and migrate between the nurse cells until they reach the oocyte at stage ten. (Ovariole image courtesy of T. Akiyama). **B.** The border cell cluster consists of two polar cells and between four and eight border cells. **C.** Confocal images of migrating border cells at early stage nine, before the cluster has delaminated (left panel); in mid stage nine, as they migrate between the nurse cells (center panel); and at stage ten when the cluster has reached the oocyte (right panel). Nuclei are labeled in blue, border cells are labeled in green, and polar cells are marked in red. (Images courtesy of T. Akiyama).

the anterior half of the egg chamber, containing nurse cells covered by flattened follicle cells, and the posterior half of the egg chamber, containing the large oocyte surrounded by columnar follicle cells (Fig 5.1C). Once the border cell cluster has reached the oocyte, it will travel dorsally until it comes to rest above the oocyte nucleus. Here, they will contribute to formation of the micropyle, the entry point for sperm and a critical structure for fertilization. In addition, they will contribute to the initiation of Torso Receptor tyrosine kinase signaling in the oocyte through expression of *torso-like*.

### Molecular mechanisms of border cell migration

The output of this process, proper border cell migration, is the result of the coordinated action of many cues. The initial signal to specify border cell fate is JAK/STAT signaling, initiated by the *Drosophila* Jak/Stat ligand, Unpaired (Upd) (Silver and Montell, 2001). *Upd* is specifically expressed by polar cells early

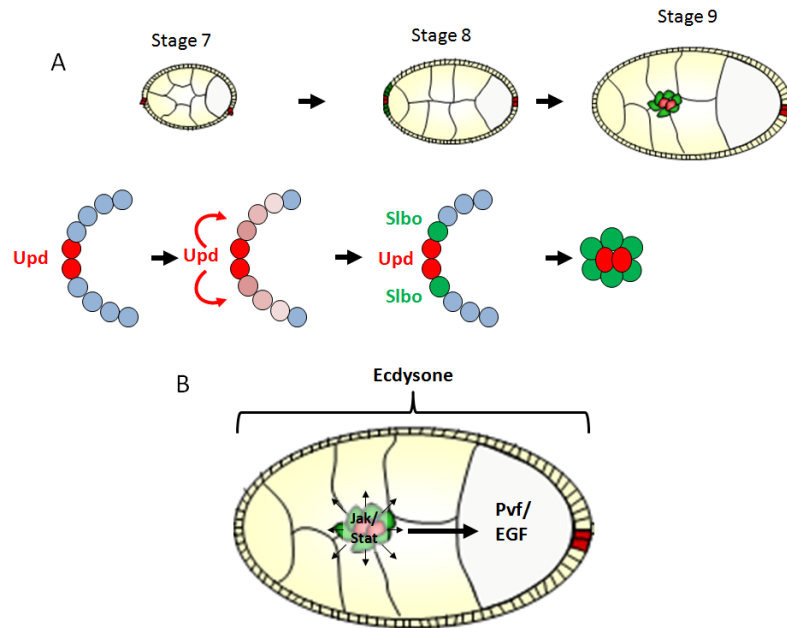
in oogenesis (McGregor et al., 2002) where it is received by the surrounding follicle cells (Fig 5.2A).

Through a system of feedback inhibition and refinement, only the cells closest to the polar cells will be specified as border cells (Fig 5.2A) (Starz-Gaiano, 2008).

In addition to its role in specification of border cell fate, Jak/Stat signaling is also required throughout border cell migration (Beccari et al., 2002; Silver et al., 2005). A critical target of Jak/Stat signaling for border cell migration is the transcription factor, *slbo* (Montell et al., 1992). *Slbo* is a C/EBP homolog and regulates expression of several genes critical for proper migration (Liu and Montell, 2001). One target in particular, DE-cad, is absolutely essential for border cell migration (Pacquelet and Rorth, 2005). DE-cad is a homophilic cell-cell adhesion molecule that is required in both the border cells and the nurse cells for proper cell migration (Geisbrecht and Montell, 2002). Loss of DE-cad in either population causes migration defects, indicating that contact between the border cells and the nurse cells is a highly regulated and important aspect of proper border cell migration (Niewiadowska, 1999).

Several other pathways and factors play a role in allowing for proper migration as the cluster travels through the nurse cells. The ETS transcription factor, Yan, regulated by Jak/Stat, RTK, and Notch signaling, is involved in turnover of DE-cad (Schober et al., 2005). The planar cell polarity pathway is important for maintaining actin-rich cellular projections that allow efficient migration through the nurse cells (Bastock and Strutt, 2007). Signaling through Rac GTPases allows for proper migration (Geisbrecht and Montell, 2004; Wang et al., 2010). In addition, the JNK pathway plays a critical role in maintaining polarity and association of the migrating border cell cluster (Llense and Martín-Blanco, 2008; Melani et al., 2008). While migrating, these cells retain distinct apical-basal polarity and require proper localization of several polarity components, such as Par6 and bazooka (Abdelilah-Seyfried, 2003; McDonald et al., 2008; Pinheiro and Montell, 2004). If polarity of these cells is disrupted, migration delay and dissociation of the cell cluster can result.

Also tightly regulated is the timing of border cell migration, a process that has been found to be largely in response to signaling by the steroid hormone Ecdysone (Fig 5.2B)(Bai et al., 2000; Jang et al., 2009). This pathway plays an important role in the global regulation of border cell migration as well as in



**Figure 5.2. Molecular mechanisms of border cell migration.** **A.** Jak/Stat signaling plays an important role in specifying border cell fate. Early in oogenesis, polar cells secrete the ligand for Jak/Stat signaling, Unpaired (Upd). Upd affects the surrounding follicle cells in a concentration dependent manner. By stage eight, Jak/Stat activity has been refined and the closest cells to the polar cells that have encountered the highest levels of Jak/Stat activity will express *slbo* and take on border cell fate. The cluster of border cells and polar cells can now delaminate and travel through the egg chamber. **B.** In addition to its role in specification of border cells, Jak/Stat is also required throughout border cell migration. Global regulation of border cell migration, in particular its timing, is achieved through the steroid hormone Ecdysone. Migration cues provided by Pvf and EGF direct the border cell cluster to the oocyte at the posterior end of the egg chamber. Image modified from (Montell, 2003)

other processes of oogenesis. It is important for coordinating the movement of the border cells within the context of the other events that take place during oogenesis.

One other consideration in border cell migration is the particular migratory path that these cells take (Fig 5.2B). The border cells travel between the nurse cells straight toward the oocyte and rarely deviate from this path. Several factors have been identified to play a role in chemoattraction of the border cell cluster, including PVR and EGFR (McDonald, 2003) (Poukkula et al., 2011) (Assaker et al., 2010; Bianco et al., 2007; Duchek, 2001; Janssens et al., 2010; Prasad and Montell, 2007). *Pvf*, expressed by the oocyte and related to the chemokines *Vegf* and *Pdgf* (McDonald, 2003), is important in guiding the border cell cluster to the oocyte. Signaling through the receptor tyrosine kinase EGFR and its ligand, Gurken, plays a role in guidance to the oocyte as well as in dorsal guidance once the border cell cluster has reached the oocyte (Bianco et al., 2007) (Fig 5.2B).

### **BMP signaling in border cell migration**

For border cell migration to occur properly, the coordinated action of many pathways is required. Preliminary work in our lab has recently identified a role for the BMP signaling pathway in regulation of border cell migration (Takuya Akiyama, personal communication). The BMP signal transduction pathway is a member of the TGF- $\beta$  super-family of signal transduction molecules which also includes the activin/TGF- $\beta$  signaling pathway in *Drosophila*. BMP signaling is important in a variety of cellular and developmental processes, including pattern formation, growth, and apoptosis (Kitisin et al., 2007; Wu and Hill, 2009). In the border cell cluster, the BMP ligand *gbb*, but not the ligand *dpp* (Twombly et al., 1996), is specifically expressed in the border cells, suggesting a role for BMP signaling in these cells. Indeed, both pMad, the activated form of the BMP transducer, and *dad*, a target of BMP signaling, are seen within the border cell cluster in early stage nine. pMad levels are high in the polar cells prior to detachment of the border cell cluster and are seen in both polar and border cells right after delamination. However, by mid-stage nine, pMad is completely absent from both the border cells and the polar cells. Despite the loss of pMad as the cells migrate, *dad-lacZ* expression can be observed throughout migration. Furthermore, reducing BMP signaling in either polar cells or border cells leads to migration defects of the border cell cluster. These defects include delayed border cell migration and abnormal localization of DE-cadherin. Reduction of BMP signaling specifically in the polar cells causes a reduction in Stat levels in the surrounding border cells prior to delamination, suggesting that BMP signaling within the polar cells may regulate Jak/Stat signaling early in the process of border cell specification.

### ***Drosophila* activin/TGF- $\beta$ signaling**

Given the role that BMP signaling plays in border cell migration, we asked if the closely related signaling pathway, activin, might also play a role. The BMP and activin pathways are both members of the TGF- $\beta$  superfamily of signaling molecules that signal through similar mechanisms, often sharing the same pathway components (Fig 1.3) (Moustakas and Heldin, 2009; Wharton and Derynck, 2009; Whitman and Raftery, 2005). In *Drosophila*, TGF- $\beta$  signaling falls into two pathways: the BMP pathway and the

activin/TGF- $\beta$ /nodal pathway (Rafferty and Sutherland, 1999). In activin signaling, the signal is initiated by one of four ligands, Daw, Act- $\beta$ , Myo, or Mav, which signal through a heterotetrameric receptor complex consisting of two type I and two type II serine/threonine kinase receptors (Arora et al., 1994; Gelbart, 1989; Lo and Frasch, 1999; Nguyen et al., 2000; Parker et al., 2006; Wharton et al., 1991). In *Drosophila*, there is one type I receptor unique to the activin pathway, Baboon (Babo), and two type II receptors which are shared between the activin and BMP pathways, Punt and Wit (Aberle et al., 2002; Childs, 1993; Wrana et al., 1994). Upon ligand binding, the constitutively active type II receptor phosphorylates Baboon which, in turn, phosphorylates the intracellular transducer dSmad2 (also known as Smox) (Brummel et al., 1999; Henderson and Andrew, 1998). phospho-dSmad2 forms a complex with the co-Smad, Medea (Med), accumulates in the nucleus, and affects target gene expression.

Despite their similar mechanisms of transduction, the activin and BMP signaling pathways are often responsible for different, even antagonistic, functions. Depending on the cellular context, these pathways have been found to either cooperate with or antagonize one another. This antagonism is particularly evident in their roles regulating cell migration. For example, in the process of epithelial-to-mesenchymal transition (EMT) that occurs during renal development, these pathways have opposite functions: TGF- $\beta$  promotes EMT while BMP7 can reverse TGF- $\beta$ -induced EMT (Zeisberg et al., 2003). Furthermore, TGF- $\beta$  signaling through ALK5, the homolog of *Drosophila baboon*, inhibits migration and proliferation of endothelial cells while BMP signaling through Alk1, the homolog of *Drosophila sax*, facilitates these processes (Goumans et al., 2003; Goumans et al., 2002).

In addition to many important functions in development, TGF- $\beta$  signaling is known to be associated with improper cell migration and plays a critical role in the development of many metastatic cancers (Massague, 2008). Interestingly, under normal conditions TGF- $\beta$  actually acts as a tumor suppressor, maintaining tissue homeostasis by regulating processes such as proliferation and apoptosis. However, TGF- $\beta$  signaling is capable of eliciting a vast array of cellular responses depending on the particular cellular contexts. Cancers that can interfere with the tumor suppressive function of TGF- $\beta$  also,



often, exploit TGF- $\beta$  signaling to use one of its other functions to its advantage. As a result, TGF- $\beta$  signaling is often associated with cancer development and with the transition to a metastatic cancer.

Given the established role for TGF- $\beta$  signaling in influencing cell migration and on our observations of BMP signaling in the border cell cluster, we asked if *Drosophila* activin signaling might also influence the migration of the border cell cluster. In the experiments presented in chapter six, we test our hypothesis that activin signaling plays a role in regulating border cell migration. We took a reverse genetics approach to test our hypothesis and have revealed a requirement for activin signaling in achieving proper border cell migration. Our experiments seek to identify a mechanism through which this may be achieved, in particular through interaction with other pathways known to regulate migration, including Jak/Stat, JNK, and BMP signaling.

## Chapter 6:

Investigating the role of activin signaling in border cell migration

I performed all experiments presented here, with the exception of the following data: Figure 6.3A (Takuya Akiyama).

### Abstract

The migration of the border cell cluster in the developing *Drosophila* ovary is a highly regulated example of collective cell migration. In this process, a group of cells consisting of two polar cells surrounded by four to eight border cells delaminates from the anterior follicle cell layer of the egg chamber and migrates posteriorly to the developing oocyte. Many signaling pathways have been found to play a role in regulating border cell migration, but no role for TGF- $\beta$  signaling has been determined. The TGF- $\beta$  super-family of signaling molecules is well-known to be important in other systems of directed cell migration. Furthermore, it is often associated with improper cell migration, such as the case with metastatic cancers. In the experiments presented here, we have addressed a role for activin/TGF- $\beta$  signaling in regulation of *Drosophila* border cell migration. By taking a reverse genetics approach and specifically reducing levels of activin pathway components within the border cell cluster, we have revealed a function for activin signaling in migration of the border cells. A reduction of activin receptors or transducers in either polar cells or border cells results in delayed border cell migration, suggesting that activin signaling is functional in both cell types. We have also determined that reduced activin signaling influences the distribution of a critical factor in migration, DE-cadherin, in the border cell cluster. Preliminary results reveal that reduced activin signaling does not influence the activity of JAK/STAT, JNK, or BMP signal transduction pathways. Future experiments will address the mechanism by which activin affects border cell migration by testing other candidate factors that activin signaling may interact with to influence border cell migration.

## Introduction

Regulated cell migration plays an essential role in the formation and growth of many structures throughout development. During the process of *Drosophila* oogenesis, a group of cells known as the border cell cluster follows a tightly regulated migratory path from the anterior end of the egg chamber to the posterior oocyte (Montell, 2003). This cluster consists of two central cells, called polar cells, surrounded by between four and eight cells known as border cells. Migrating as a group, these cells must detach from the follicular epithelium and then migrate posteriorly and dorsally before coming to rest above the nucleus of the oocyte. Migration of the border cell cluster in the developing *Drosophila* ovary is a highly regulated model system in which to study collective cell migration.

Early in development of the *Drosophila* egg chamber, a pair of polar cells is specified at each anterior and posterior poles of the egg chamber. The anterior polar cells secrete Upd, the ligand for Jak/Stat signaling, which is required for specification of border cells in the surrounding follicular epithelium (Silver and Montell, 2001; Starz-Gaiano, 2008). During stage 9 of oogenesis, the border cells and polar cells delaminate from the follicular cell epithelium and migrate as a cluster between the nurse cells. By stage 10, the border cell cluster has reached the anterior end of the oocyte, at which point it travels dorsally and comes to rest above the oocyte nuclei. Here, they will contribute to formation of the micropyle, a structure required for sperm entry.

The polar cells themselves are non-migratory and rely on the surrounding migratory border cells to pull them along, a feat that is largely accomplished by persistent Jak/Stat signaling during migration (Beccari et al., 2002; Silver et al., 2005). Jak/Stat signaling in the border cells leads to expression of *slbo*, a factor that regulates the expression of several genes required for proper border cell migration, one of which is DE-cad (Montell et al., 1992; Pacquelet and Rorth, 2005). DE-Cad is a homophilic cell-cell adhesion molecule that, through interactions with nurse cell DE-cad, allows the border cell cluster to migrate between the nurse cells (Niewiadomska, 1999). Also important during migration is JNK signaling (Llense and Martín-Blanco, 2008), which is important in maintaining cell polarity and cluster integrity as the cells migrate.

Preliminary work in our lab has recently identified a role for the BMP signaling pathway in regulation of border cell migration (data not shown, Takuya Akiyama, personal communication). Reducing BMP signaling in either polar cells or border cells leads to migration defects of the border cell cluster. These defects include delayed border cell migration and abnormal localization of DE-cadherin. Reduction of BMP signaling specifically in the polar cells causes a reduction in Stat levels in the surrounding border cells prior to delamination, suggesting that BMP signaling emanating from the polar cells may regulate Jak/Stat signaling. In addition, we have observed pMad, an indicator of active BMP signaling, in polar cells and border cells early in border cell specification and migration.

Given the role that BMP signaling plays in border cell migration, we asked if the closely related signaling pathway, activin/TGF- $\beta$ , might also play a role. The BMP and activin pathways are both members of the TGF- $\beta$  superfamily of signaling molecules. These pathways signal through similar mechanisms, often sharing the same pathway components (Whitman and Raftery, 2005). *Drosophila* activin signaling is initiated by binding of activin ligand to a heterotetrameric receptor complex consisting of two type I and two type II serine/threonine kinase receptors. In *Drosophila*, there is one type I receptor unique to the activin pathway, Baboon (Babo), and two type II receptors which are shared between the activin and BMP pathways, Punt and Wit. Upon ligand binding, Baboon is phosphorylated which, in turn, phosphorylates the intracellular transducer dSmad2, allowing it to affect target gene expression.

Activin and BMP signaling pathways are known to both cooperate and antagonize one another in different cellular contexts, particularly in the process of cell migration. For example, antagonistic roles for these pathways have been identified in epithelial-to-mesenchymal transition (EMT) during renal development (Zeisberg et al., 2003). TGF- $\beta$  signaling itself has also often been found to be associated with improper cell migration, such as the case with metastatic cancers (Massagué, 2008; Wharton and Derynck, 2009). Based on this and on our observations of BMP signaling in the border cell cluster, we asked if activin signaling might also influence the migration of the border cell cluster.

We took a reverse genetics approach to test the activin pathway for a role in regulating the migration of the border cell cluster. Activity of the pathway was reduced specifically in the polar cells or

the border cells through use of *UAS-RNAi* constructs directed against components of the pathway or dominant-negative forms of the type I receptor, Babo. We then assayed stage ten egg chambers for migration defects. These experiments revealed that a reduction in activin signaling in either border or polar cells leads to delayed migration of the border cell clusters as well as mis-localization of DE-cadherin, indicating that activin signaling is required for proper border cell migration.

To identify the mechanism through which activin may influence migration of the border cell cluster, we took a biased approach and tested several candidate signaling pathways for possible interactions with activin signaling, including the Jak/Stat, BMP, and JNK signaling pathways. Thus far, activity of none of the tested factors have been found to be influenced by activin signaling, suggesting that activin signaling impacts border cell migration through alternate mechanisms. Future experiments will seek to identify the mechanism through which activin signaling can influence migration of the border cell cluster.

## Materials and Methods

### *Drosophila melanogaster* strains, crosses, and husbandry

Flies were raised on standard cornmeal/yeast medium at 25°C in plastic bottles or glass vials. Flies used for virgin collection were raised at 18°C or 22°C in plastic bottles. Crosses were performed using virgin females. All crosses were performed in vials. Unless otherwise noted, crosses involving *slbo-Gal4* were raised at 29°C until eclosion. To avoid complications from *upd-gal4* expressed in other tissues and times in development, crosses involving *upd-Gal4* were raised at 18°C until eclosion. After eclosion, all flies were cleared from vials daily and moved to 29°C to age for two days before dissection. At 29°C, the Gal4 protein is highly active, ensuring the strongest possible expression of our *UAS* constructs and the strongest possible knock-down through *RNAi*.

### Reducing activin function in the border cell cluster

We used the following *RNAi* constructs:

|                            |                                                                                        |
|----------------------------|----------------------------------------------------------------------------------------|
| <i>dSmad2-RNAi-TRiP:</i>   | <i>y<sup>1</sup>v<sup>1</sup></i> ; ; <i>p{TRiP.JF02320} AttP2/TM3, Sb<sup>1</sup></i> |
| <i>babo-RNAi-V1:</i>       | <i>w</i> ; ; <i>UAS-babo-RNAi</i> (VDRC line # 853)                                    |
| <i>babo-RNAi-M1:</i>       | <i>UAS-babo RNAi 10E2,6E2/CyO Act GFP</i> (a gift from Dr Mike O'Connor)               |
| <i>babo-RNAi-M2:</i>       | <i>UAS-babo RNAi 1A3,58B3/TM6c - Sb, Tb</i> (a gift from Dr Mike O'Connor)             |
| <i>babo-DN</i> constructs: |                                                                                        |
| <i>baboA-DN:</i>           | <i>w; UAS-baboA-ΔI 5A2,8A2 ; UAS-baboA-ΔI 16A3,20A3</i>                                |
| <i>baboB-DN:</i>           | <i>w; UAS-baboB-ΔI 7A2,6A2; UAS-baboB-ΔI 11E3,11H3</i>                                 |
| <i>Flag-dSmad2:</i>        | <i>Gla/CyO ; UAS-Flag-dSmad2</i> (a generous gift from Dr. Chi-Hon Lee)                |
| <i>Gal4</i> lines:         | <i>w ; slbo-Gal4 UAS-GFP/CyO</i>                                                       |
|                            | <i>w Upd-Gal4</i>                                                                      |

The following crosses were performed:

*RNAi* or *babo-DN* males were crossed to *w ; slbo-Gal4 UAS-GFP/CyO* females to reduce activin signaling in the border cells

*yw* males were crossed to *w ; slbo-Gal4 UAS-GFP/CyO* females as controls

*RNAi* or *babo-DN* males were crossed to *w Upd-Gal4* females to reduce activin signaling in the polar cells

*yw* males were crossed to *w Upd-Gal4* females as controls

*Gla/CyO ; UAS-Flag-dSmad2* males were crossed to *w ; slbo-Gal4 UAS-GFP/CyO* females to observe dSmad2 in border cells

*Gla/CyO ; UAS-Flag-dSmad2* males were crossed to *w Upd-Gal4* females to observe dSmad2 in border cells

### **Dissections and immunostaining**

Adult females were anesthetized with triethylamine and dissected in PBS for no longer than 30 minutes to remove ovaries. Once isolated, ovaries were stored on ice in 1.5mL tubes in PBS until fixation. All steps were performed in 1.5mL tubes. Tissue was fixed for 30 minutes in 4% paraformaldehyde in PBS at room temperature (RT) on a rotator. Fix was removed and ovaries were washed three times with PBT (PBS with 0.3% Triton X-100 and 0.02% sodium azide) for 20min at RT on a rotator. Tissue was incubated in block (PBS, 0.3% Triton X-100, 0.02% sodium azide, 10% normal goat serum) for 60 min at RT on a shaker.

Ovaries can be stored, and often were, in block at 4°C until ready for staining. Tissue was incubated in primary antibody at appropriate concentration in block for 2 days at 4°C. After incubation, samples were washed three times with PBT for 20min at RT on a rotator and then incubated in secondary antibody at appropriate concentration in block for 2 days at 4°C. Next, tissue was washed three times with PBT for 20min at RT on a rotator and stored in block at 4°C until mounting. Ovaries could be stored for several weeks to a few months. Tissue was mounted just before imaging, as the GFP signal quickly fades once mounted. To mount, egg chambers were separated from ovarioles using forceps and a needle and transferred to a glass slide. For analysis of migrating defects, stage 10 egg chambers were removed. For Stat, pMad, and pJun staining, stage 10 and earlier were removed. Once transferred to a clean slide, block was quickly removed with a pipette and covered with 18μL mounting solution (80% glycerol, 0.1% N-propyl-gallate in water) and a #1.5 cover slip.

Antibodies and concentrations used were:



anti-DE-cadherin (rat DE-cad DCAD2, 1:25, Developmental Studies Hybridoma Bank), anti- fasciclin III (mouse FasIII 7G10, 1:200, Developmental Studies Hybridoma Bank), anti-Flag (mouse monoclonal Flag, 1:100, Sigma F3165), anti-pMad (rabbit monoclonal Smad3 phospho (pS423/425), 1:1000, Epitomics), anti-pJun (mouse monoclonal p-c-Jun (KM-1) sc-822, 1:200, Santa Cruz) and anti-STAT (rabbit stat, 1:1000, a generous gift from Dr Denise Montell).

Secondary antibodies used were Alexa Fluor 633 goat anti-rat IgG, Alexa Fluor 633 goat anti-mouse IgG, Alexa Fluor 555 donkey anti-mouse IgG, Alexa Fluor 488 goat anti-mouse IgG, Molecular probes. All were used at 1:500 concentrations. Nuclei were labeled with Hoechst-33432 at 1:1000. GFP was visualized by direct immunofluorescence.

### **Confocal microscopy and image analysis**

Images used to analyze migration defects were taken with a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope (Figs 6.1A, 6.3AB) at 20X. Images used to analyze STAT, pMad, Flag, and pJun staining were taken with a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope (Figs. 6.4-6.7) at 20X or 63X.

Confocal images were taken as z-stacks with one 1 $\mu$ M (Leica) or optimal (Zeiss, usually .95 $\mu$ M) slice sizes at either 512X512 or 1024X1024 pixels. All images were taken sequentially. Scan speed of 6 was used (Zeiss). All images are shown as single sections.

### **Data analysis**

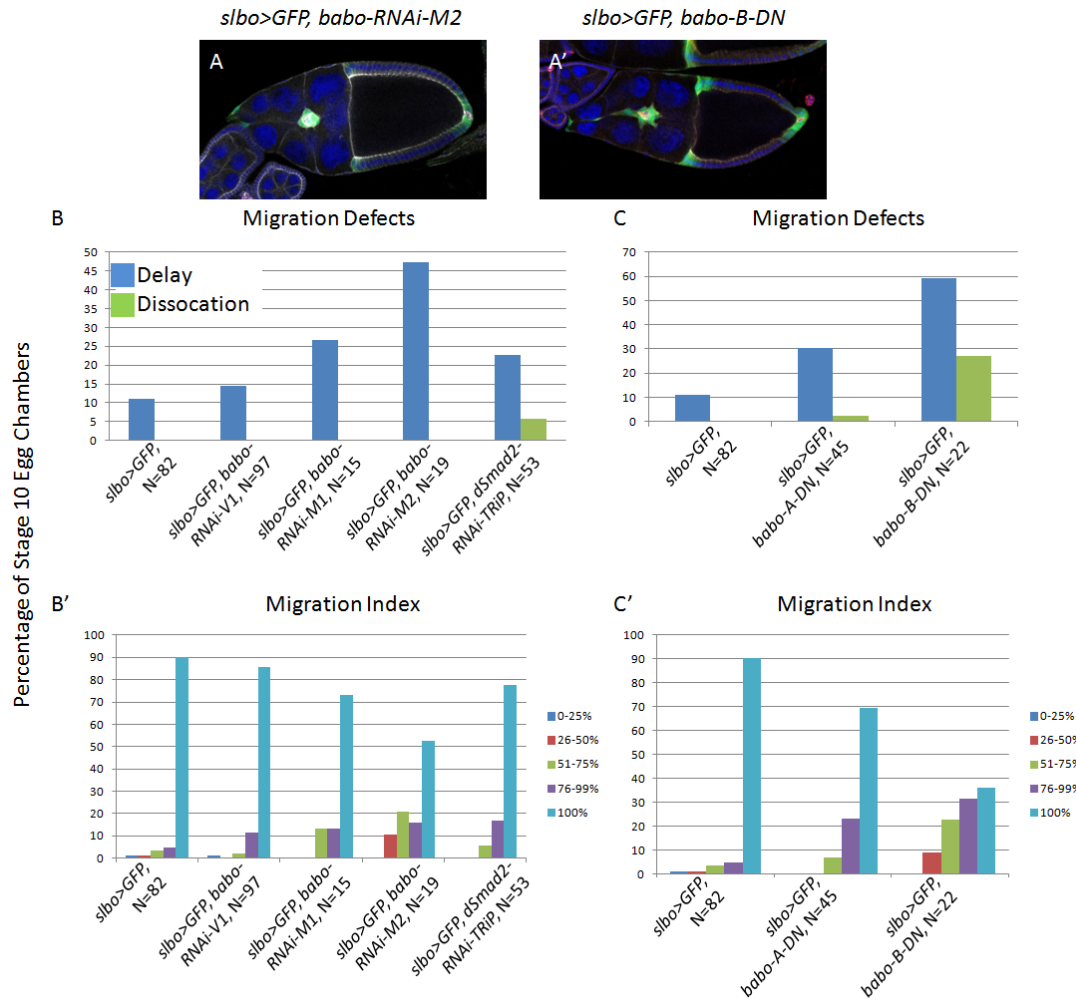
All stage ten egg chambers observed were scored as “no defect,” “migration delay,” or “dissociation.” The percentage of each phenotype observed was determined out of the total number of stage ten egg chambers observed. To determine the migration index, we calculated the distance travelled for each border cell cluster by stage ten as a percentage of the length of the egg chamber. The distance travelled was determined by measuring the distance from the anterior tip of the egg chamber to the posterior end of the border cell cluster. The distance of the egg chamber was determined by measuring the distance from the anterior tip of the egg chamber to the anterior end of the oocyte. Measurements were done using Fiji and ImageJ software. Values were rounded to the closest whole number and classified into one of five classes: 0-25%, 26-50%, 51-75%, 76-99%, and 100%.

## Results

### Reduced activin signaling in the border cells or the polar cells leads to migration defects

To test activin for a role in regulating border cell migration, we took a reverse genetics approach. We compromised activin signaling in border cells or polar cells and assayed for migration defects in stage ten egg chambers. To achieve this, we drove expression of *UAS-RNAi* constructs directed against an activin pathway component, either *babo* or *dSmad2*, specifically in the polar cells by the *upd-Gal4* driver or in the border cells by the *slbo-Gal4* driver. As a second method for reducing activin function, we over-expressed a dominant-negative form of Babo and monitored phenotypes (Ng, 2008). We tested dominant-negative versions of two Babo isoforms: Babo-A, which signals in response to an unknown ligand, and Babo-B, which signals in response to Myo and Act- $\beta$  (Michael O'Connor, personal communication, M O'Connor). As a caveat to this second approach, over-expression of receptor could have unintended consequences on other signaling pathways, such as the BMP pathway, by causing unwanted receptor interactions which we will bear in mind when interpreting our results.

We examined stage ten egg chambers and assayed for delayed migration, observed as a stage ten egg chamber whose border cell cluster has not yet reached the oocyte (Fig 6.1A), and for cluster dissociation, observed as clusters that have lost tight association between cells and appear elongated or that have two or more cells left behind between the nurse cells (Fig 6.1A'). At this stage, a normal border cell cluster should have reached the oocyte intact and flattened against it. For each genotype tested, we determined the penetrance of each phenotype by calculating the percentage of observed stage ten egg chambers displaying a particular migration defect. To characterize the degree of migration delay observed, we calculated the migration index, a measure of how far into the egg chamber that a border cell cluster has travelled by stage ten. All genotypes were compared to *Upd-Gal4/+* and *Slbo-Gal4 UAS-GFP/+* egg chambers as controls. Several *RNAi* lines were tested to determine those which displayed the strongest phenotypes (Fig 7.41-7.42) but, we chose to focus on three *babo* lines; *babo-RNAi-V1*, *babo-RNAi-M1*, and *babo-RNAi-M2*, all of which are isoform non-specific; and one *dSmad2* line; *dSmad2-RNAi-TRiP*.



**Figure 6.1. Reduced activin signaling in the border cells leads to migration defects.** Activin signaling was reduced specifically in the border cells through use of the *slbo-Gal4* driver and stage ten egg chambers were examined. We observed two phenotypes, (A) delayed migration of the border cell cluster, and (A') dissociation of the border cell cluster. In A, nuclei are shown in blue, DE-cad is shown in white, GFP is shown in green, and FasIII is shown in red to label polar cells. We calculated the percentage of stage ten egg chambers that display each phenotype out of the total number of stage ten egg chambers examined (B-C). For those with delayed migration, we determined the migration index distribution for each genotype (B'-C'), representing the percentage of the egg chamber that the border cell cluster has travelled by stage ten. Values were classified into five categories based on the percentage of the egg chamber travelled. B. RNAi constructs directed against *babo* or *dSmad2* were expressed in the border cells, giving an increase in delayed migration and dissociation compared to the *slbo>GFP* control. B'. There is variability in the distance that border cell clusters travel with reduced activin signaling, but most make it at least 51% of the way through the egg chamber. C. Activin signaling was reduced in the border cells by expression of two dominant-negative isoforms of Baboon: *baboA-DN* and *baboB-DN*. Expression of either leads to delayed migration and dissociation. C'. Migration indexes reveal that most border cell clusters are able to travel at least half-way through the egg chamber.

Loss of activin signaling specifically in border cells, through expression of *RNAi* or *babo-DN* by *slbo-Gal4*, causes delayed border cell migration (Fig 6.1B,C, in blue). Specifically, in our *RNAi* over-expression studies, the percentage of egg chambers with delayed migration varied: 14% of *slbo>babo-*

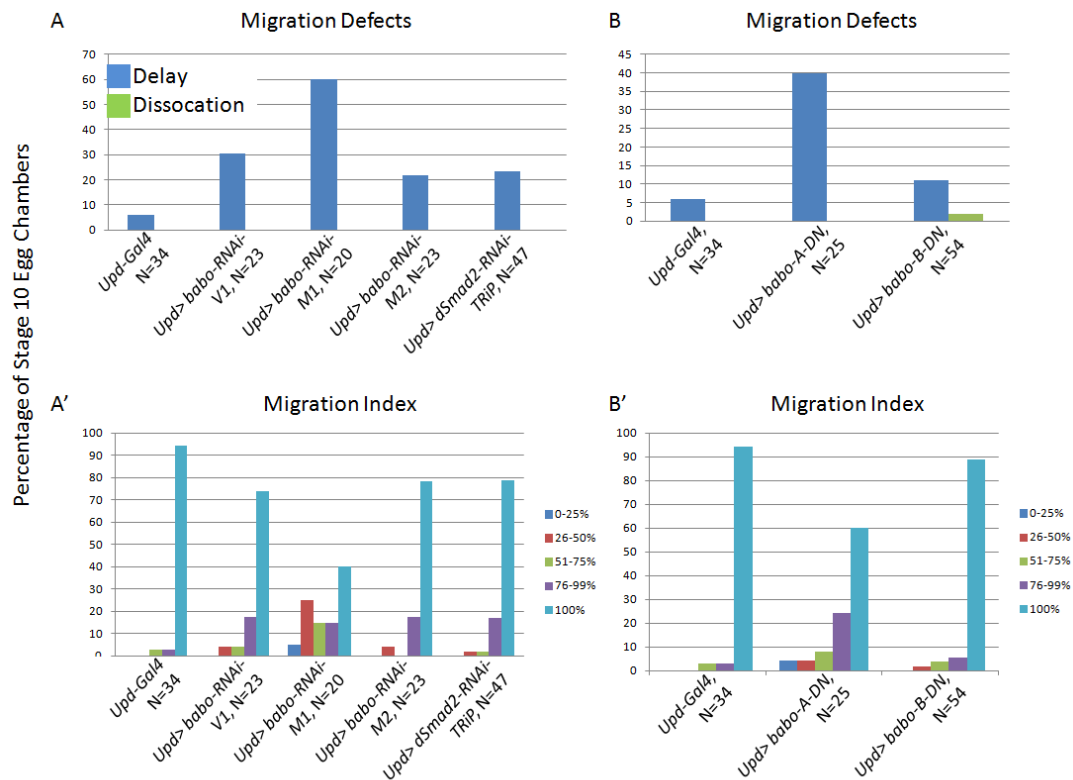
*RNAi-V1*, 27% of *slbo>babo- RNAi-M1*, 47% of *Slbo>babo- RNAi-M2*, and 23% of *slbo>dSmad2- RNAi-TRiP* stage ten egg chambers (Fig 6.1B). These all represent an increased from *slbo>GFP* control animals, in which 11% of the egg chambers displayed a delay in migration. Observation of egg chambers in which dominant-negative *babo* was expressed in border cells revealed migration delays as well, with 30% of *slbo>babo-A-DN* egg chambers and 59% of *slbo>babo-B-DN* egg chambers displaying delayed migration (Fig 6.1C). Migration indexes reveal variability in the degree of the delay (Fig 6.1B',C'). In addition, we observed a defect in maintaining border cell cluster association during migration in these genotypes, with 6% of *slbo>dSmad2- RNAi-TRiP*, 2% of *slbo>babo-A-DN*, and 27% of *slbo>babo-B-DN* stage 10 egg chambers displaying this phenotype (Fig 6.1B,C, in green).

A reduction of activin signaling in the polar cells also led to migration defects (Fig 6.2). When we expressed our *RNAi* constructs by *upd-Gal4*, we observed migration delays but not dissociation defects. 30% of *upd>babo-RNAi-V1*, 60% of *upd>babo-RNAi-M1*, 22% of *upd>babo-RNAi-M2*, and 33% of *upd>dSmad2-RNAi-TRiP* egg chambers displayed delayed migration, an increase from the 6% of *upd-Gal4/+* egg chambers that display delayed migration (Fig 6.2A). Over-expression of dominant negative receptor also led to a delay in migration, with 40% of *upd>babo-A-DN* and 11% of *upd>babo-B-DN* egg chambers displaying this phenotypes (Fig 6.2B). As with our *slbo* experiments, we observed a variability in migration indexes (Fig 6.2A',B').

Together, these results indicate that activin signaling must play a role in regulating migration of the border cell cluster, as reducing signaling in either the border cells or the polar cells leads to a delay in migration. Furthermore, the increase in cluster dissociation that we observed from reducing activin signaling specifically in the border cells suggests that signaling within these cells may play a role in maintaining epithelial integrity, cellular polarity, or cell-cell contacts.

Interestingly, we observed variability in the prevalence of each phenotype between different *RNAi* constructs. We interpreted these different values as differences in the strength of knock-down generated from each RNA line. However, our results for any one particular *RNAi* line were not always consistent between border cells and polar cells. For example, more *slbo>babo-RNAi-M2* egg chambers have delayed border cell clusters than *slbo>babo-RNAi-M1* egg chambers, but the reverse is true when

the same *RNAi* lines are driven by *upd-Gal4* in the polar cells. This is curious, especially considering that these two constructs target the same sequence that is common to all isoforms of Babo (Michael O'Connor, personal communication). The reason for this difference is unclear at the moment. We also observed different responses to over-expression of the two dominant-negative isoforms in polar and border cells. Higher incidences of delay were observed in border cells expressing *babo-B-DN*, whereas more chambers displayed delay with polar cells expressing *babo-A-DN*. These results may suggest different roles for the different Baboon isoforms in border cells and polar cells, perhaps as a result of the particular ligands present in this system.



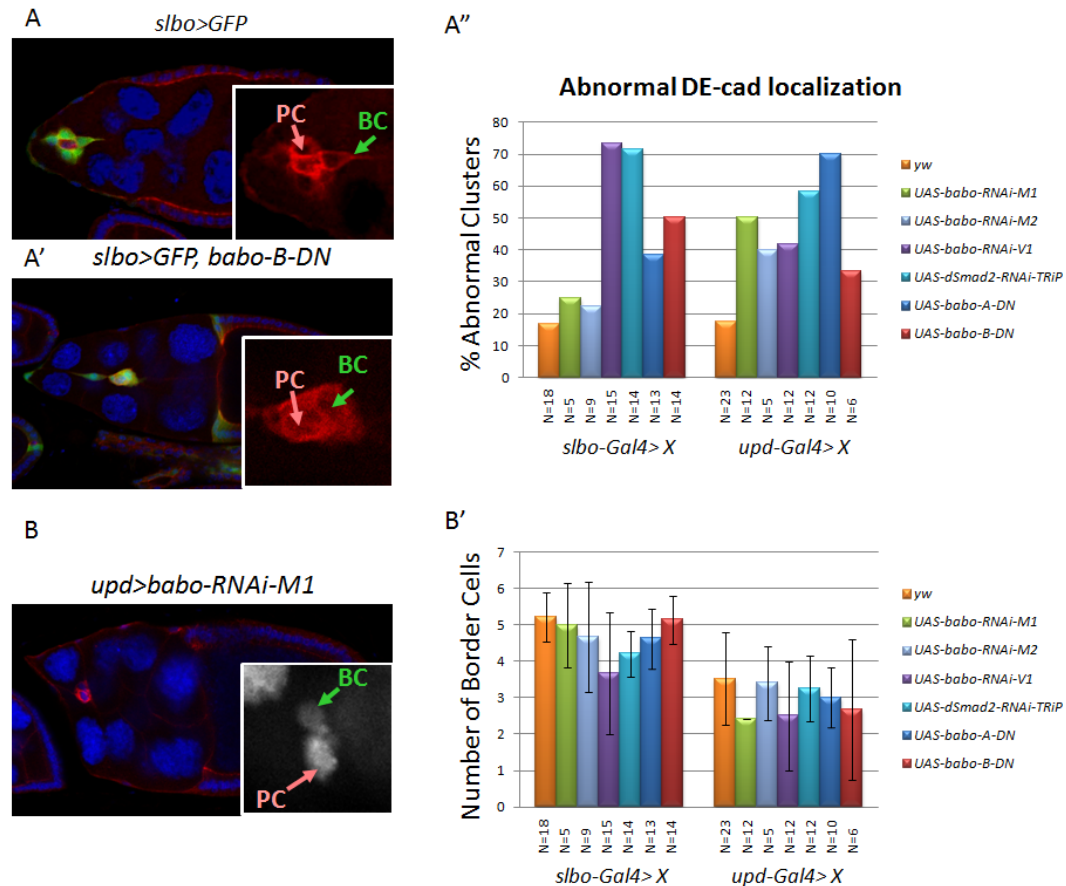
**Figure 6.2. Reduced activin signaling in the polar cells leads to migration defects.** Activin signaling was reduced specifically in the polar cells through use of the *upd-Gal4* driver and stage ten egg chambers were examined. **A.** *RNAi* constructs directed against *babo* or *dSmad2* were expressed in the polar cells, leading to an increased prevalence of delayed migration but not dissociation in comparison to the *upd-Gal4* control. **A'.** There is variability in the distance that border cell clusters travel with reduced activin signaling, with most observed in the first half of the egg chamber. **B.** Activin signaling was reduced in the polar cells by expression of two dominant-negative isoforms of Baboon: *baboA-DN* and *baboB-DN*. Expression of either leads to delayed migration. **B'.** Migration indexes reveal variability in the distances that border cell clusters can travel, with most reaching at least half-way through the egg chamber.

### Activin signaling influences DE-cadherin localization but not border cell specification.

As a second test of border cell cluster integrity, we observed the distribution of *Drosophila* E-cadherin (DE-cad) in border cell clusters from each of our genotypes. In a wildtype border cell cluster, DE-cad is observed in a rosette structure, with high levels surrounding the polar cells and between the border cells (Fig 6.3A). Proper localization of DE-cad is essential for migration as it plays an important role in forming cell-cell contacts with the surrounding nurse cells, allowing the border cell clusters to travel between these cells (Niewiadomska, 1999). DE-cad was observed in delayed stage ten egg chambers with reduced activin signaling and mid-stage nine egg chambers for controls. In all of the genotypes tested, some degree of abnormal DE-cad localization was observed, recognized as a loss of localization between the border cells or polar cells or as higher levels surrounding the border cell cluster (Fig 6.3A'). The prevalence of this effect varied, from 22% of *slbo>babo-RNAi-M2* to 73% of *slbo>babo-RNAi-V1* egg chambers and 33% *upd>babo-B-DN* to 70% of *upd>babo-A-DN* egg chambers, but all displayed an increase from control, indicating that in both the border cells and the polar cells activin signaling plays a role in generating proper expression or distribution of DE-cad.

One known regulator of DE-cad expression and localization is the Jak/Stat pathway, which regulates *DE-cad* expression through action of a downstream target, *slbo* (Niewiadomska, 1999). In addition to its role in regulating DE-cad, Jak/Stat is also responsible for border cell specification (Starz-Gaiano, 2008). We considered the possibility that activin signaling could influence DE-cad by interacting with the Jak/Stat signaling pathway and, if so, it may also influence border cell specification. To test this, we counted border cells in delayed stage ten egg chambers with reduced activin signaling and in mid-stage nine egg chambers from *upd-Gal4/+* and *slbo>GFP/+* control animals (Fig 6.3B'). A wildtype border cell cluster is expected to contain anywhere from four to eight border cells. *Slbo>GFP/+* animals fall within this range, with the average border cell number observed to be approximately five. *Upd-Gal4/+* animals, on the other hand, displayed a reduction in border cell number from expected, with an average of only three border cells (see Fig 6.3B for an example). This effect may be due to reduced *upd* activity, as this *Gal4* line was constructed through direct *p[GawB]* insertion into the *upd* locus and may represent a weak hypomorphic allele of *upd* (Tsai and Sun, 2004). We determined the border cell counts in egg chambers

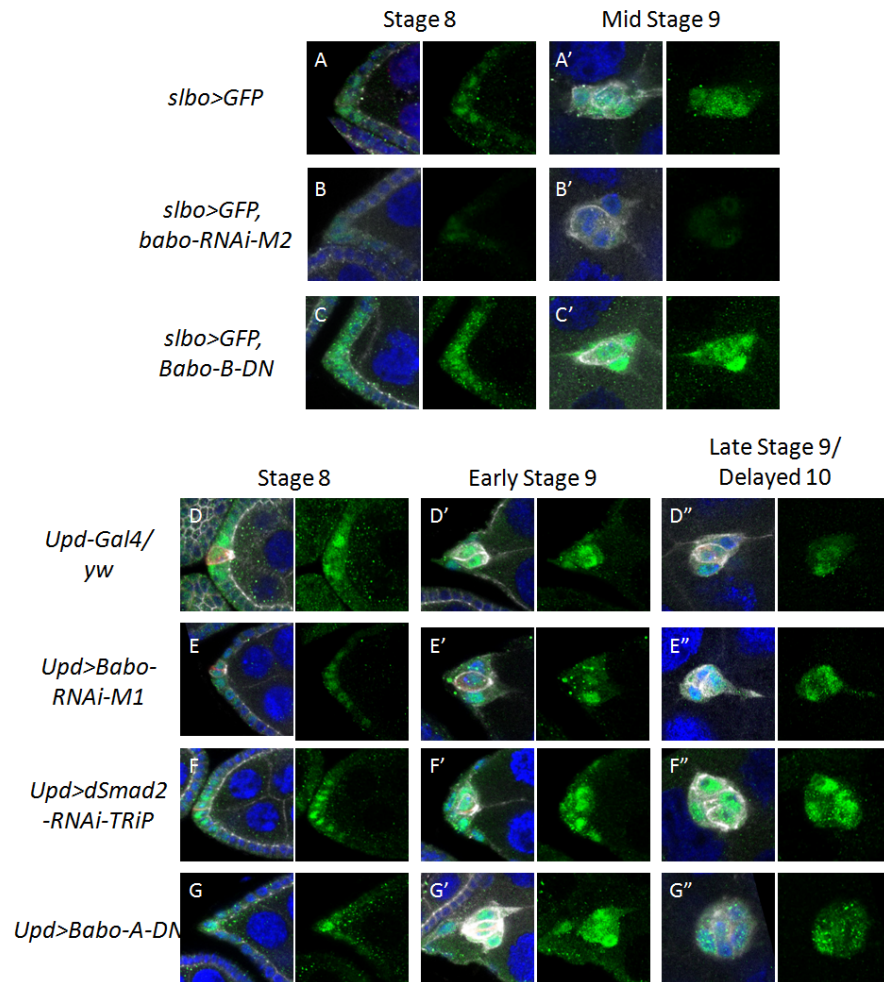
with reduced activin signaling and, in all cases tested, did not observe a change in border cell number in comparison to the appropriate control. Based on these results, it is unlikely that activin signaling influences the Jak/Stat pathway at or before border cell specification. Alternatively, our *Gal4* drivers may not turn on early enough to observe such a change. *Upd-Gal4* is expressed quite early, but *slbo>GFP* is not expressed until after the border cells have been specified.



**Figure 6.3. Reduced activin signaling leads to defects in DE-cadherin localization but not in border cell specification.** **A.** Reduced activin signaling in either the border or polar cells affects localization of DE-cad. **A.** Wildtype DE-cad distribution surrounding the polar cells and the border cells (image courtesy of T. Akiyama). **A'.** Abnormal DE-cad distribution with higher levels surrounding the border cells or uniform distribution around all cells. DE-cad is shown in red, nuclei are shown in blue, and GFP is shown in green. **A''.** Percentage of stage nine egg chambers (for control genotypes) or delayed stage ten egg chambers (with disrupted activin signaling) with abnormal DE-cad distribution. Reducing activin signaling in border cells or polar cells influences DE-cad localization. **B.** Egg chamber with reduced border cell number. DE-cad is shown in red, and nuclei are shown in blue (overlay) or white (inset). The expected number is between four and eight border cells. **B'.** Border cells in stage nine egg chambers (for control genotypes) and in delayed stage ten egg chambers (with disrupted activin signaling) were counted and scored. Overall, no reduction in border cell number was observed in border cell clusters in which activin signaling was reduced in border cells (left). The *Upd-Gal4* line has approximately three border cells, but no further reduction is observed with disrupted activin signaling (right).

### Reduced activin signaling in the border or polar cells does not influence Stat activity

Despite our observation that reducing activin signaling in the border cell cluster does not influence border cell specification, it is still possible that activin could be influencing the activity of Jak/Stat. Continuous Jak/Stat signaling is required throughout migration to regulate expression of *slbo* and maintain proper expression of *DE-cad* (Silver et al., 2005). Given that we observed migration delays and altered DE-cad distribution but not a change in border cell number, we asked whether activin may



**Figure 6.4. Reducing activin signaling in the border cells or the polar cells does not affect Jak/Stat signaling.** Activin signaling was reduced in border cells (A-C) or polar cells (D-G) and Stat protein was observed (in green). DE-cad is shown in white (A-G), nuclei in blue (A-G), and FasIII in red to mark polar cells (D-G). Nuclear Stat indicates active signaling. **A-C.** Reducing activin signaling in border cells does not influence Stat protein distribution. **A.** Control animals displaying nuclear staining in stage eight and mid stage nine egg chambers. **B-C.** When activin signaling is reduced in border cells in *slbo>babo-RNAi-M2* (B) or *slbo>babo-B-DN* (C) animals, Stat is still nuclear localized. Lower levels in B may be due to poor staining (also visible in DE-cad). **D-G.** Reducing activin signaling in polar cells does not influence Stat protein distribution. **D.** *upd-Gal4* control animals display Stat protein within the nucleus of cells in early and migrating clusters. **E-G.** Reduction in activin activity in polar cells in *upd>babo-RNAi-M1* (E), *upd>dSmad2-RNAi-TRiP* (F), and *upd>babo-A-DN* (G) egg chambers does not influence Stat distribution.



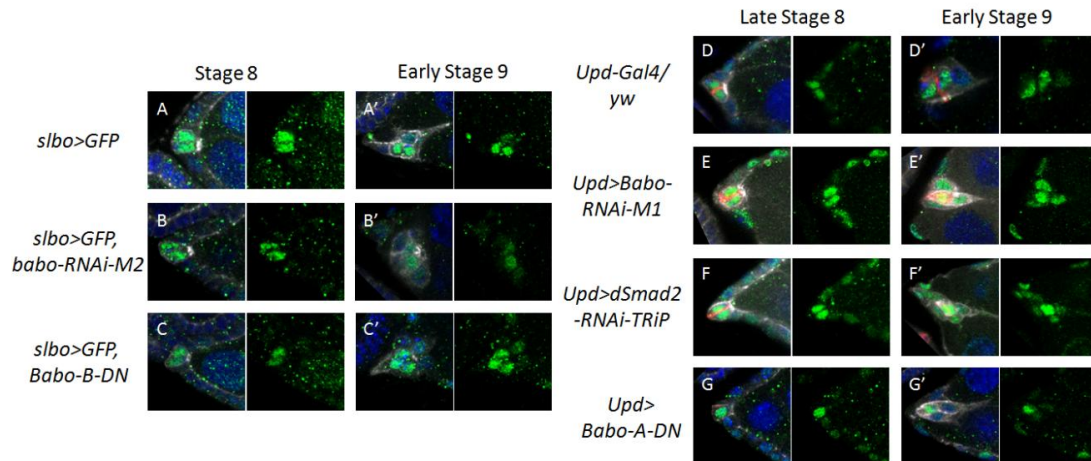
interact with Jak/Stat signaling during migration. To test this, we observed levels of the Jak/Stat transducer, STAT, at various times during oogenesis in border cell clusters with activin signaling reduced.

The Stat antibody that we used recognizes both unphosphorylated and phosphorylated protein, so nuclear localization indicates Jak/Stat pathway activity (Chen et al., 2002; Starz-Gaiano, 2008). We reduced activin signaling in the border cells and the polar cells and examined Stat protein in stage eight and nine egg chambers. For egg chambers with reduced activin signaling in the polar cells, we also examined Stat in delayed stage ten egg chambers. For each experiment, images were taken at the same settings as control images to allow for comparison of Stat levels between genotypes. Control Stat protein is seen within the nuclei at all stages, indicating active signaling (Fig 6.4A,D). In all genotypes tested with reduced activin signaling, Stat protein is still present at comparable levels to control and is still nuclear localized. These data suggest that activin does not interact with the Jak/Stat pathway at the level of regulating Stat protein levels or activity. The activin signaling pathway must be influencing border cell migration by a different mechanism.

#### **Reduced activin signaling in the border or polar cells does not influence BMP activity**

We next considered other ways in which activin signaling could influence border cell migration. Work in our lab (Takuya Akiyama, personal communication) has revealed a role for BMP signaling in regulation of border cell migration. Reduced BMP signaling in either border or polar cells leads to delayed migration. Given this data, and given the known history of activin/BMP interaction, we asked whether activin signaling in border cells can influence BMP signaling in these cells by observing pMad protein in the border cell cluster.

In control tissue, pMad levels throughout border cell migration are dynamic. It is at high levels in the polar cells before delamination (Fig 6.5A,D), at high levels in polar cell nuclei and low levels in border cell nuclei right after the cluster leaves the epithelium (Fig 6.5A',D'), and completely absent by mid stage nine (data not shown). When we reduced activin signaling in both border cells and polar cells, we did not observe a change in the pMad distribution (Fig 6.5B-C,E-G). pMad was seen in the same distribution and at the same levels as in control tissue, indicating that reduced activin signaling does not influence pMad. It

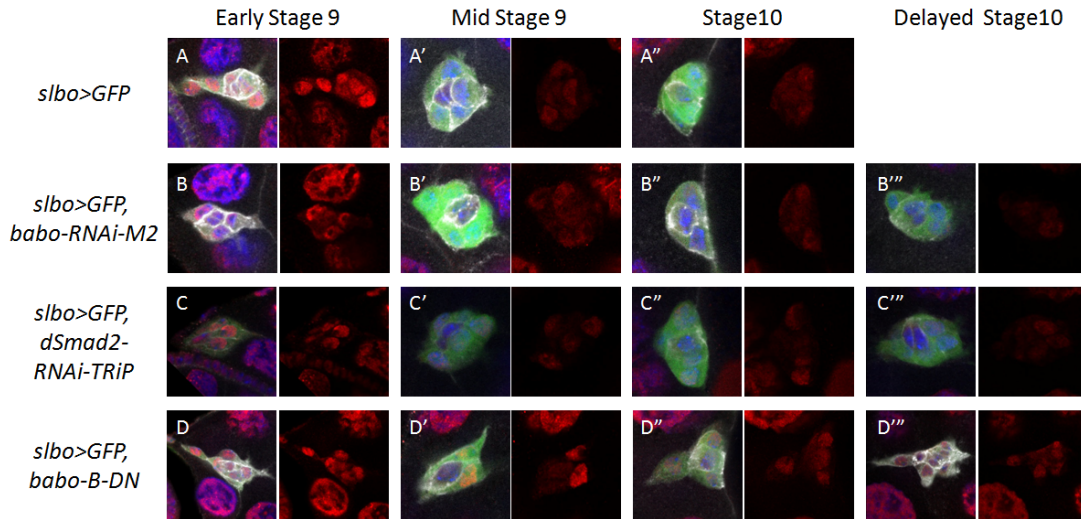


**Figure 6.5. Reducing activin signaling in the border cells or the polar cells does not affect pMad levels.** To test for an interaction with BMP signaling, activin signaling was reduced in border cells (**A-C**) or polar cells (**D-G**) and pMad protein was observed (in green) in stage eight and nine egg chambers. DE-cad is shown in white (**A-G**), nuclei in blue (**A-G**), and FasIII in red to mark polar cells (**D-G**). **A-C.** Reducing activin signaling in border cells does not influence pMad protein. **A.** Control animals displaying high nuclear pMad in polar cells in stage eight and nine and low nuclear pMad in border cells in stage nine. **B-C.** Reduced border cell activin signaling in *slbo>babo-RNAi-M2* (**B**) or *slbo>babo-B-DN* (**C**) egg chambers does not influence pMad. **D-G.** Reducing activin signaling in polar cells does not influence pMad protein distribution. **D.** *upd>Gal4* control animals display similar pMad distributions as *slbo* controls. **E-G.** Reduction of activin activity in polar cells in *upd>babo-RNAi-M1* (**E**), *upd>dSmad2-RNAi-TRiP* (**F**), and *upd>babo-A-DN* (**G**) egg chambers does not influence pMad distribution.

is still possible that the BMP and activin pathways may interact in regulating border cell migration, but with BMP signaling upstream of activin signaling.

#### Reduced activin signaling in the border cells does not influence JNK activity

We observed that reducing activin signaling in the border cells leads to an increase in dissociation of the border cell cluster during migration. JNK Signaling has been shown to play a role in maintaining cohesion of the cluster during migration, with altered levels leading to dissociation of the cluster (Llense and Martín-Blanco, 2008). We hypothesized that reduced activin signaling leads to dissociation of the cell cluster through an interaction with the JNK pathway. To test this, we stained egg chambers with wildtype and reduced activin signaling in the border cells for pJun, an antibody that recognizes the phosphorylated and active form of the JNK transducer, Jun. In control tissue, pJun is nuclear localized in the border cell cluster throughout its migration, as well as in the nurse cell nuclei (Fig 3.6A). We observed similar patterns when activin signaling was reduced in the border cells, indicating the reduced activin signaling does not influence activity of the JNK pathway (Fig 3.6B-D).



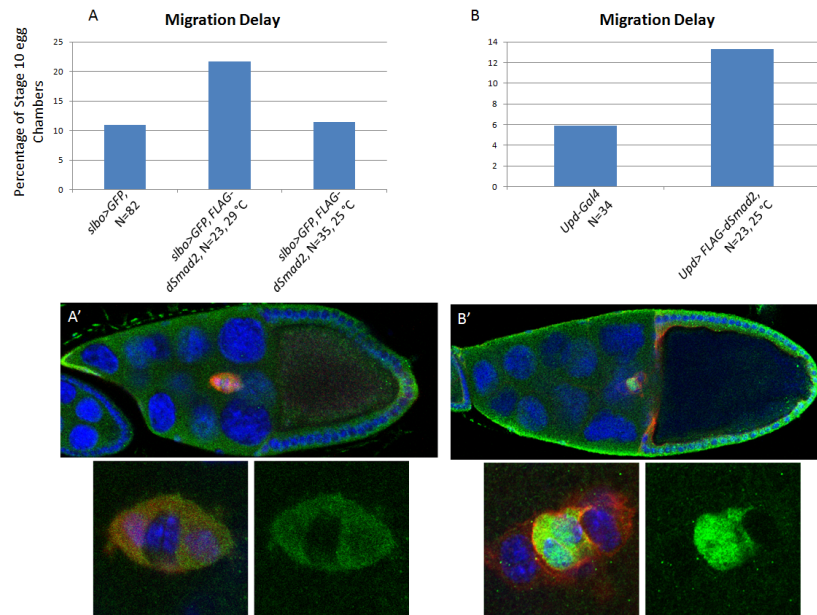
**Figure 6.6. Reducing activin signaling in the border cells does not affect JNK signaling.** pJun levels were observed in *slbo>GFP* (A) control egg chambers and *slbo>babo-RNAi-M2* (B), *slbo>dSmad2-RNAi-TRiP* (C), and *slbo>babo-B-DN* (D) egg chambers with reduced activin function in border cells. pJun is shown in red, DE-cad in white, GFP in green, and nuclei in blue. **A.** In control border cells, pJun is present within the nuclei of the border cells and the polar cells, as well as within the nurse cell nuclei. **B-D.** Reduced activin signaling in border cells does not affect pJun levels. All genotypes examined displayed comparable expression levels and patterns to wildtype.

#### Tagged dSmad2 is not nuclear localized in border or polar cells

It is important to note that all of our observations thus far are based on phenotypes observed with knock-down of activin components or with over-expression of a dominant-negative form of the receptor. We have not been able to directly observe activin pathway activity in these cells and, to the best of our knowledge, a reliable and effective antibody for immunohistochemistry of dSmad2 or p-dSmad2 does not exist. Direct observation of activin activity would be exceedingly helpful to conclusively say where and when the activin pathway is active in border cell migration.

In an attempt to observe activin activity, we obtained a *UAS-Flag-dSmad2* construct that should, in theory, allow us to observe activin activity as nuclear-localized *Flag-dSmad2* (Ting et al., 2007). To test this, we expressed *UAS-Flag-dSmad2* under the control of *slbo-Gal4* and *upd-Gal4* and assayed for migration delays and Flag distribution. As this experiment results in over-expression of *dSmad2*, we were concerned that the increase in levels of dSmad2 could result in nuclear localization of dSmad2 and migration defects. Indeed, expression of this construct led to migration delay in 22% of *slbo>Flag-dSmad2* stage ten egg chambers observed, suggesting that over-expression of *dSmad2* alone can influence

migration, possibly by increasing activin signaling (Fig 6.7A). To reduce the level of over-expression, we raised flies at 25 °C instead of 29 °C, a temperature where the *Gal4* should be less active. While this did result in fewer migration delays for *slbo>Flag-dSmad2* (11%) egg chambers, *upd>Flag-dSmad2* animals displayed significant delay (13%) compared to *upd-Gal4/+* controls (6%) (Fig 3.7A,B). When we observed Flag protein in *slbo>Flag-dSmad2* and *upd>Flag-dSmad2* egg chambers that had been raised at 25 °C, we observed Flag staining in both the cytoplasm and the nucleus (Fig 3.7A',B'). Flag is neither excluded from the nucleus nor concentrated in it. Based on these results, we cannot draw any conclusions about the signaling capacity of dSmad2 in border cells or polar cells.

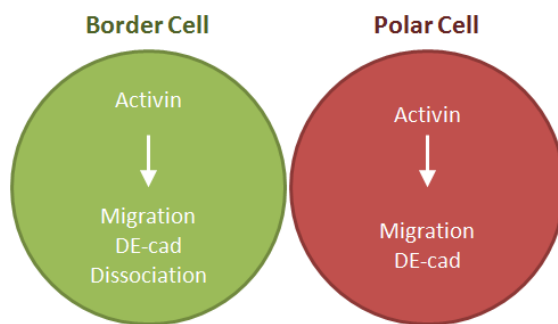


**Figure 6.7. Flag-dSmad2 does not localize to the nucleus in border cells or polar cells.** To test for activity of dSmad2 protein in the border cells and polar cells, we expressed *UAS-Flag-dSmad2* specifically in the border cells by *slbo-Gal4* (A) or specifically in the polar cells by *upd-Gal4* (B) and examined the resulting migratory behavior of the border cell clusters and the pattern of Flag protein. If active, we expect to see nuclear localization. **A.** Expression of *Flag-dSmad2* specifically in the border cells led to high levels of delayed migration using our standard method of raising animals at 29°C. Instead, animals were raised at 25°C to decrease the amount of *UAS-Flag-dSmad2* over-expression. **A'.** Flag protein is observed specifically in the border cells and absent from the central polar cells, but no specific cytoplasmic or nuclear localization was observed. Flag is displayed in green, nuclei in blue, and GFP in red. **B.** In *upd>Flag-dSmad2* animals raised at 25°C, delayed migration is observed. **B'.** Flag is specifically observed in the polar cells, but is localized to neither the cytoplasm nor the nucleus. Flag is shown in green, DE-cad in red, and nuclei in blue.

## Discussion and Future Directions

### Activin signaling influences border cell migration

Our experiments have revealed a functional role for activin signaling in regulating migration of the border cell cluster during *Drosophila* oogenesis. By observing the migration behavior of border cell clusters with compromised activin signaling in either the border cells or the polar cells, we have revealed that proper activin signaling is required to generate wildtype migration behavior. Reduced activin signaling in either cell type leads to a higher incidence of delayed migration as well as a change in the



**Figure 6.8. Activin signaling influences migration of the border cell cluster.** Our results have revealed that reduced activin signaling in either the border cells (in green) or the polar cells (in red) leads to defects in border cell migration. These observations indicate that activin signaling must play a role in the process of border cell migration. When activin signaling is compromised in the border cells, we observed delayed migration, abnormal DE-cad localization, and dissociation of the border cell cluster. Reduced activin signaling specifically in the polar cells results in delayed migration and abnormal localization of DE-cad.

distribution of DE-cad in the border cell clusters (Fig 6.8). Furthermore, as reduced activin signaling in the border cells lead to a higher incidence of border cell cluster dissociation, activin signaling may play a unique role in these cells by maintaining polarity and association of the cells within the cluster. Our future experiments will identify the specific role that activin plays in border cell migration and the mechanisms through which it accomplishes regulation of border cell migration.

### Activin signaling does not influence several of the known regulators of border cell migration

To identify the mechanisms of activin signaling in border cell migration, we took a biased approach and tested for interactions between activin signaling and several other pathways known to be involved in regulation of border cell migration. In particular, we were interested in an interaction with the Jak/Stat pathway, as this pathway is important early in both border cell specification and later maintain migration through the nurse cells (Silver et al., 2005; Silver and Montell, 2001; Starz-Gaiano, 2008). We analyzed Stat protein levels in border cells and polar cells with reduced activin signaling and, in both

cases, did not observe any changes in Stat activity. It is unlikely that these two pathways interact, but it is still possible that they interact at a step other than at Stat levels. For example, Stat and dSmad2 could directly interact to regulate target gene expression. Future experiments will test for a genetic interaction between these two pathways.

We next considered a possible interaction between activin and BMP signaling in regulating migration of the border cell cluster. Work in our lab has identified a role for BMP signaling in proper cell migration, with reduced BMP signaling leading to an increase migration delay. We tested whether activin is upstream of BMP signaling by observing pMad levels in cells with reduced activin activity. However, we did not observe a change in pMad when activin signaling was compromised in either the border cells or the polar cells, suggesting that activin does not influence BMP activity in this context.

Importantly, our data suggest that activin and BMP signaling perform similar but not identical functions in border cell migration. Unlike other systems where these pathways have opposite functions, it appears that both activin and BMP signaling are important in facilitating border cell migration. However, it appears that they do so through different mechanisms. While a reduction in polar cell BMP signaling shows a clear reduction in Stat levels, the same is not true for activin signaling. Moreover, pMad staining is not altered with reduced activin signaling, indicating that the activin pathway is not upstream of BMP signaling in regulation of border cell migration. It appears, instead that the BMP and activin signaling either act in parallel to regulate migration of the border cell cluster or that BMP signaling acts up-stream of activin signaling. Future experiments will test for a genetic interaction between the pathways by examining migration behavior when both pathways are compromised.

Finally, our observation that reducing activin signaling in border cells leads to increased cluster dissociation led us to question whether activity of the JNK pathway was being interrupted. This pathway is required for maintenance of polarity within the cells of the cluster and for maintaining cohesion of the cluster as it migrates (Llense and Martín-Blanco, 2008). We examined pJun levels in border cells and polar cells with reduced activin signaling but did not observe any changes compared to control border cell clusters. This result suggests that activin signaling may influence cluster cohesion by an alternate method.

In our future experiments, we will continue to test candidate factors through which activin signaling may influence border cell migration. Several other pathways play a role in migration of these cells, and we will look to what is known regarding this process to determine possible mediators. Some pathways and processes that we will consider exploring include the ecdysone pathway (Bai et al., 2000; Jang et al., 2009), the planar cell polarity pathway (Bastock and Strutt, 2007), the Rac GTPase (Wang et al., 2010), and the ETS transcription factor Yan (Schober et al., 2005), all of which are required for correct migration of the border cell cluster.

#### **Activity of activin signaling in the border cell cluster**

In future experiments, it will be important to determine when and where activin signaling is active during the migration process. Our experiments thus far indicate to us that removing activin has an effect, but does not tell where and when the pathway is acting. Without a reagent to detect dSmad2 activity, we may primarily have to infer function from mutant phenotypes and expression patterns.

As a first step, it will be important to determine the expression patterns of several components of the activin signaling pathway within the egg chamber. This can be performed through a variety of methods, including antibody staining for various components, observation of *UAS-GFP* expression driven by *Gal4* drivers under the control of regulatory sequences for the various components, or in situ hybridization. Determining the expression pattern of *babo* will tell us specifically which cells the activin pathway signals through. In addition, we are particularly interested in determining which activin ligands are active in this process and which cells express ligand, be it border cells, polar cells, nurse cells, or follicle cells. Based on our observations, activin signal transduction must occur within the border cell cluster as removal of receptor or transducer in these cells affects migration, but we would like to address potential interactions with the surrounding germline and somatic tissue.

#### **Border cell migration as a model system for collective cell migration**

While many model systems are used to study cell migration, most focus on migration of individual mesenchymal cells and the process of epithelial-to-mesenchymal transition (EMT). Border cell

migration, however, is the best-understood model for collective cell migration, in which a cohesive group of cells migrates as a group. Given its well established role in cancer and metastasis, a function for activin/TGF- $\beta$  signaling in this process is a particularly exciting addition to our body of knowledge regarding border cell migration (Massagué, 2008). In addition, a role for activin signaling in border cell migration presents itself as a model system in which to further our understanding of how TGF- $\beta$  may contribute to migratory behavior of cells, specifically in the process of collective cell migration (Jang et al., 2007). The experiments presented here and our future work will contribute to both the fields of border cell migration and to the field of cancer biology as a whole.



## Chapter 7: Appendices

I performed all experiments presented here, with the exception of the following data:  
Figure 7.3C-E (Juliane Kellner), figure 7.10 (Lorena Soares), and Table 7.1 (Erdem Bangi)

## Preface

This chapter includes summaries of several experiments that do not address the central questions in chapters 2, 3, or 6 but serve, rather, as supplementary material.

Experiments are listed with the rationale for each experiment, the approach to each experiment, and the conclusions that could be drawn.

## Appendix 7.1 – Increased BMP signaling in the wing imaginal disc leads to a pMad dose-dependent reduction in *dpp* expression

### Rationale:

Our results presented in chapter two indicate that increased BMP signaling can repress expression of the *dpp-lacZ* reporter lines *dpp<sup>p10638</sup>lacZ* and *dpp<sup>BS3.0</sup>lacZ* in a pMad dose-dependent manner. In those experiments, we used three *Gal4* drivers to over-express components of the pathway: *ap-Gal4*, *MS1096-Gal4*, and *omb-Gal4*. In these experiments we test several other *Gal4* drivers that express in the wing disc to get a more complete picture of the effect of increased BMP signaling in particular cells of the wing pouch.

### Approach:

The *Gal4* drivers *dpp<sup>blk</sup>-Gal4*, *ptc-Gal4*, and *A9-Gal4* were used to over-express *UAS-gbb*, *UAS-dpp*, and *UAS-tkv<sup>QD</sup>* and expression patterns of *dpp<sup>p10638</sup>lacZ* and *dpp<sup>BS3.0</sup>lacZ* and levels of pMad protein were observed. GFP expression in different *Gal4* domains with *dpp<sup>p10638</sup>lacZ* expression and pMad staining in these backgrounds was examined by performing the following crosses:

*UAS-GFP ; dpp<sup>blk</sup>Gal4/T(2;3) SM5-TM6b* males to *yw ; dpp<sup>p10638</sup>lacZ/SM6a* virgin females to generate *UAS-GFP/ dpp<sup>p10638</sup>lacZ ; dpp<sup>blk</sup>-Gal4/+* larvae

*ptc-Gal4 UAS-GFP ; +/T(2;3) SM5-TM6b* males to *yw ; dpp<sup>p10638</sup>lacZ/SM6a* virgin females to generate *ptc-GAL4 UAS-GFP/ dpp<sup>p10638</sup>lacZ* larvae

*A9-Gal4/Y ; UAS-GFP/+* males to *yw ; dpp<sup>p10638</sup>lacZ/SM6a* virgin females to generate *A9-GAL4/+ ; UAS-GFP/ dpp<sup>p10638</sup>lacZ* larvae

Expression patterns of *dpp<sup>p10638</sup>lacZ* or pMad staining with over-expression of *UAS-tkv<sup>QD</sup>* were examined by performing the following crosses:

*UAS-GFP ; dpp<sup>blk</sup>Gal4/T(2;3) SM5-TM6b* males to *w ; dpp<sup>p10638</sup>lacZ ; UAS-tkv<sup>QD</sup>/T(2;3) SM5-TM6b* virgin females to generate *UAS-GFP/ dpp<sup>p10638</sup>lacZ ; dpp<sup>blk</sup>-Gal4/ UAS-tkv<sup>QD</sup>* larvae

*ptc-Gal4 UAS-GFP ; +/T(2;3) SM5-TM6b* males to *w ; dpp<sup>p10638</sup>lacZ ; UAS-tkv<sup>QD</sup>/T(2;3) SM5-TM6b* virgin females to generate *ptc-GAL4 UAS-GFP/ dpp<sup>p10638</sup>lacZ ; UAS-tkv<sup>QD</sup>/+* larvae, which did not survive to the third instar larval stage

*A9-Gal4/Y ; UAS-GFP/+* males to *w ; dpp<sup>p10638</sup>lacZ ; UAS-tnv<sup>QD</sup> /T(2;3) SM5-TM6b* virgin females to generate *A9-GAL4/+ ; UAS-GFP/ dpp<sup>p10638</sup>lacZ ; UAS-tnv<sup>QD</sup> /+* larvae

Expression patterns of *dpp<sup>BS3.0</sup>lacZ* with over-expression of *UAS-tnv<sup>QD</sup>* were examined by performing the following crosses:

*A9-Gal4/Y ; dpp<sup>BS3.0</sup>lacZ/+* males to *UAS-tnv<sup>QD</sup>* females to generate *A9-Gal4/+ ; dpp<sup>BS3.0</sup>lacZ/UAS-tnv<sup>QD</sup>* larvae

Expression patterns of *dpp<sup>p10638</sup>lacZ* or pMad staining with over-expression of *UAS-gbb<sup>9.7</sup>* were examined by performing the following crosses:

*UAS-GFP ; dpp<sup>blk</sup>Gal4/T(2;3) SM5-TM6b* males to *yw UAS-gbb<sup>9.7</sup> ; dpp<sup>p10638</sup>lacZ/CyO Wg-lacZ* virgin females to generate *yw UAS-gbb<sup>9.7</sup> ; UAS-GFP/ dpp<sup>p10638</sup>lacZ ; dpp<sup>blk</sup>-Gal4/+* larvae

*ptc-Gal4 UAS-GFP ; +/T(2;3) SM5-TM6b* males to *yw UAS-gbb<sup>9.7</sup> ; dpp<sup>p10638</sup>lacZ/CyO Wg-lacZ* virgin females to generate *yw UAS-gbb<sup>9.7</sup> ; ptc-GAL4 UAS-GFP/ dpp<sup>p10638</sup>lacZ* larvae

*A9-Gal4/Y ; UAS-GFP/+* males to *yw UAS-gbb<sup>9.7</sup> ; dpp<sup>p10638</sup>lacZ/CyO Wg-lacZ* virgin females to generate *A9-GAL4/ yw UAS-gbb<sup>9.7</sup> ; UAS-GFP/ dpp<sup>p10638</sup>lacZ* larvae

Expression patterns of *dpp<sup>p10638</sup>lacZ* or pMad staining with over-expression of *UAS-dpp<sup>w</sup>* were examined by performing the following crosses:

*ptc-Gal4 UAS-GFP ; +/T(2;3) SM5-TM6b* males to *w ; dpp<sup>p10638</sup>lacZ ; UAS-dpp<sup>w</sup> /T(2;3) SM5-TM6b* virgin females to generate *ptc-GAL4 UAS-GFP/ dpp<sup>p10638</sup>lacZ ; UAS-dpp<sup>w</sup> /+* larvae, which did not survive to the third instar larval stage

*UAS-GFP ; dpp<sup>blk</sup>Gal4/T(2;3) SM5-TM6b* males to *w ; dpp<sup>p10638</sup>lacZ ; UAS-dpp<sup>w</sup> /T(2;3) SM5-TM6b* virgin females to generate *UAS-GFP/ dpp<sup>p10638</sup>lacZ ; dpp<sup>blk</sup>-Gal4/ UAS-dpp<sup>w</sup>* larvae

*A9-Gal4/Y ; UAS-GFP/+* males to *w ; dpp<sup>p10638</sup>lacZ ; UAS-dpp<sup>w</sup> /T(2;3) SM5-TM6b* virgin females to generate *A9-GAL4/+ ; UAS-GFP/ dpp<sup>p10638</sup>lacZ ; UAS-dpp<sup>w</sup> /+* larvae

Expression patterns of *dpp<sup>BS3.0</sup>lacZ* with over-expression of *UAS-dpp<sup>w</sup>* were examined by performing the following crosses:

*ptc-Gal4* to *UAS-dpp<sup>w</sup> dpp<sup>BS3.0</sup>lacZ/TM6b* to generate *ptc-Gal4/+ ; UAS-dpp<sup>w</sup> dpp<sup>BS3.0</sup>lacZ/+* larvae

*dpp<sup>blk</sup>-Gal4* to *UAS-dpp<sup>w</sup> dpp<sup>BS3.0</sup>lacZ/TM6b* to generate *dpp<sup>blk</sup>-Gal4/+ ; UAS-dpp<sup>w</sup> dpp<sup>BS3.0</sup>lacZ/+* larvae

*A9-Gal4* to *UAS-dpp<sup>w</sup> dpp<sup>BS3.0</sup>lacZ/TM6b* to generate *A9-Gal4/+ ; UAS-dpp<sup>w</sup> dpp<sup>BS3.0</sup>lacZ/+* larvae

Expression patterns of *dpp<sup>BS3.0</sup>lacZ* with over-expression of *UAS-gbb<sup>9.7</sup>* were examined by performing the following crosses:

*w/Y ; AyGal4 UAS-GFP; dpp<sup>BS3.0</sup>lacZ/T(2;3) SM5-TM6b* males to *yw UAS-gbb<sup>9.7</sup>* females to

generate *yw UAS-gbb<sup>9.7</sup> /+ ; AyGal4 UAS-GFP/+ ; dpp<sup>BS3.0</sup>lacZ/+* control larvae

*MS1096-Gal4 /Y ; AyGal4 UAS-GFP/+ ; dpp<sup>BS3.0</sup>lacZ/+* males to *yw UAS-gbb<sup>9.7</sup>* females to generate

*MS1096-Gal4 / yw UAS-gbb<sup>9.7</sup> ; AyGal4 UAS-GFP/+ ; dpp<sup>BS3.0</sup>lacZ/+* larvae

*A9-Gal4 /Y ; AyGal4 UAS-GFP/+ ; dpp<sup>BS3.0</sup>lacZ/+* males to *yw UAS-gbb<sup>9.7</sup>* females to generate *A9-*

*Gal4 / yw UAS-gbb<sup>9.7</sup> ; AyGal4 UAS-GFP/+ ; dpp<sup>BS3.0</sup>lacZ/+* larvae

*yw UAS-gbb<sup>9.7</sup> /Y ; AyGal4 UAS-GFP/+ ; dpp<sup>BS3.0</sup>lacZ/+* males to *w ; ptc-Gal4* females to generate

*yw UAS-gbb<sup>9.7</sup> /+ ; AyGal4 UAS-GFP/ptc-Gal4 ; dpp<sup>BS3.0</sup>lacZ/+* larvae

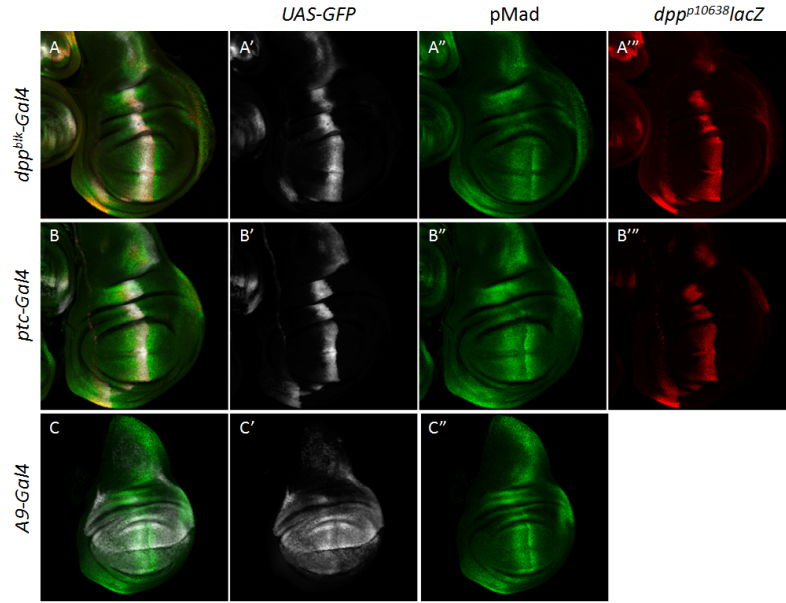
*yw UAS-gbb<sup>9.7</sup> /Y ; AyGal4 UAS-GFP/+ ; dpp<sup>BS3.0</sup>lacZ/+* males to *w ; sal-Gal4* females to generate

*yw UAS-gbb<sup>9.7</sup> /+ ; AyGal4 UAS-GFP/sal-Gal4 ; dpp<sup>BS3.0</sup>lacZ/+* larvae

## Results and Conclusions:

The expression patterns of the three *Gal4* drivers primarily used in these experiments is shown in figure 7.1 in white. To understand the expression of these drivers relative to *dpp* expression and with BMP signaling activity, the expression pattern of *dpp<sup>p10638</sup>lacZ* (in red) and the distribution of pMad (in green) are overlaid with *GFP. dpp<sup>blk</sup>Gal4*, which drives expression of *Gal4* in response to *dpp* cis-regulatory sequence, has an expression domain almost identical to the expression pattern of *dpp<sup>p10638</sup>lacZ* (Fig. 7.1A). *ptc-Gal4*, while also expressed along the anterior-posterior compartment boundary, is expressed in a narrower stripe of cells than *dpp* (Fig. 7.1B). *A9-Gal4* is broadly expressed across the wing imaginal disc, with slightly higher expression in the dorsal compartment than the ventral compartment (Fig. 7.1C).

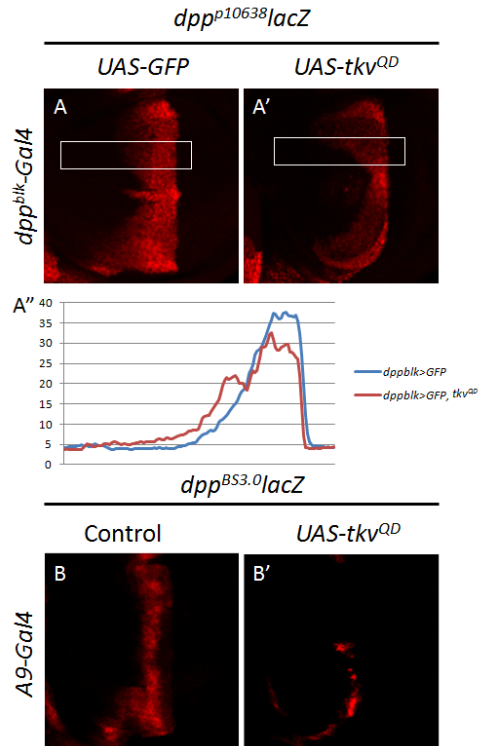
Using these *Gal4* drivers, we drove expression of activated type I receptor, *UAS-tkv<sup>QD</sup>*, and observed expression of *dpp* reporter lines. *dpp<sup>blk</sup>>tkv<sup>QD</sup>* (Fig. 7.2A) wing imaginal discs display a deep epithelial furrow along the anterior-posterior boundary, have altered epithelial morphology and folding in



**Figure 7.1: Expression patterns of several Gal4 drivers used to over-express components of the BMP signaling pathway.** *Gal4* expression domains are visualized by expression of *UAS-GFP*, displayed in white (second panel). The expression pattern of *dpp<sup>p10638</sup>lacZ* is shown in red (third panel) and pMad staining is shown in green (fourth panel). The overlay (left panel) demonstrates the relative overlap between each *Gal4* driver and the endogenous expression domain of *dpp* and activity level of BMP signaling. *Gal4* drivers used in our studies include: **A.** *dpp<sup>blk</sup>-Gal4*, which is expressed along the anterior-posterior compartment boundary of the wing imaginal disc, **B.** *ptc-Gal4*, which is expressed along the anterior-posterior compartment boundary of the wing imaginal disc in a slightly more narrow domain than *dpp<sup>blk</sup>-Gal4*, and **C.** *A9-Gal4*, which is expressed broadly across the wing pouch, with slightly higher expression in the dorsal compartment than the ventral compartment. All wing discs are oriented with anterior to the left, posterior to the right, dorsal to the top, and ventral to the bottom.

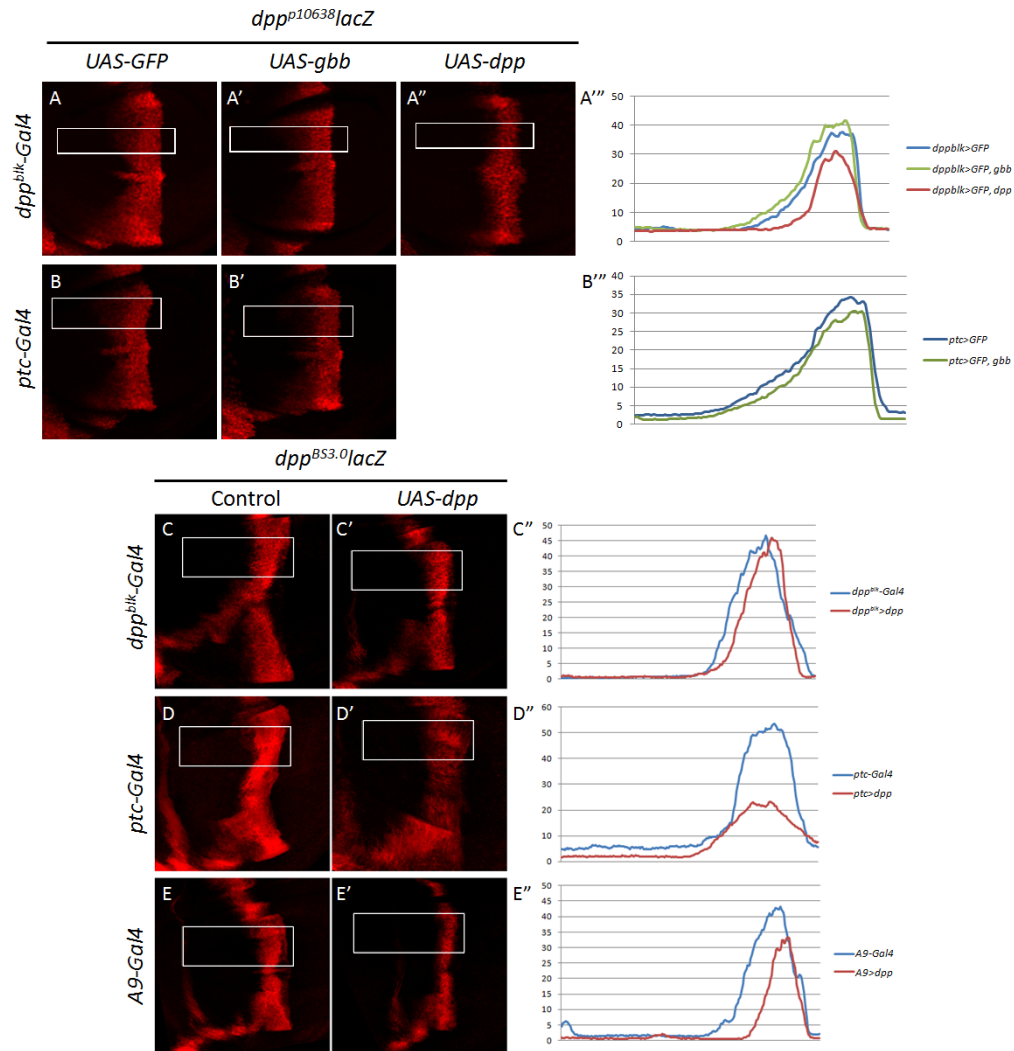
the anterior compartment, and are slightly smaller than their control counterparts. The result is an altered expression domain of *dpp<sup>p10638</sup>lacZ* that appears to extend further than wildtype *dpp<sup>p10638</sup>lacZ* (Fig. 7.2A'), apparent in the intensity profiles generated to compare *dpp<sup>p10638</sup>lacZ* expression in *dpp<sup>blk</sup>>GFP* and *dpp<sup>blk</sup>>GFP, tkv<sup>QD</sup>* wing pouches (Fig. 7.2A''). Despite this, expression levels of *dpp<sup>p10638</sup>lacZ* are reduced in response to increased BMP signaling. Attempts to observe *dpp<sup>p10638</sup>lacZ* expression in *ptc>tkv<sup>QD</sup>* wing imaginal discs were unsuccessful as these animals do not survive to larval stages. We did not observe viable larvae in either *ptc-Gal4 UAS-GFP/dpp<sup>p10638</sup>lacZ*; *UAS-tkv<sup>QD</sup>/+* or *ptc-Gal4 UAS-GFP/+*; *UAS-tkv<sup>QD</sup>/+* genotypes, possibly due to embryonic expression of the *ptc-Gal4* driver. As of this writing, we are still testing the effect on *dpp<sup>p10638</sup>lacZ* expression in *A9>tkv<sup>QD</sup>* animals. However, there is a strong reduction in expression of *dpp<sup>BS3.0</sup>lacZ* expression in *A9>tkv<sup>QD</sup>* animals (Fig. 7.2B).

To test the effect of over-expressing other components of the BMP signaling pathway, we used *dpp<sup>blk</sup>-Gal4*, *ptc-Gal4*, and *A9-Gal4* to express *UAS-gbb* and *UAS-dpp* in the wing discs and examined the



**Figure 7.2. Over-expression of activated Type I BMP receptor,  $tkv^{QD}$ , in the wing pouch leads to a significant reduction in  $dpp-lacZ$  expression.**  $UAS-tkv^{QD}$  was expressed in the wing imaginal disc and expression of  $dpp-lacZ$  was examined. For each  $Gal4$  and  $dpp-lacZ$  combination, control and  $tkv^{QD}$  over-expression images were taken at the same confocal settings to allow for comparison of levels between pouches. **A.**  $dpp^{blk}-Gal4$  was used to drive expression of  $UAS-GFP$  alone (**A**,  $n=3$ ) and with  $UAS-tkv^{QD}$  (**A'**,  $n=3$ ) along the anterior-posterior compartment of the wing pouch. Boxed areas in the wing pouches represent the areas used to generate intensity profiles of  $dpp-lacZ$  expression levels as shown in **A''**. Epithelial morphology is altered with  $tkv^{QD}$  expression and expression levels of  $dpp^{10638} lacZ$  are reduced. **B.**  $dpp^{BS3.0} lacZ$  expression was observed in  $A9-Gal4/+$ ;  $dpp^{BS3.0} lacZ/+$  (**B**) and  $A9-Gal4/+$ ;  $dpp^{BS3.0} lacZ/UAS-tkv^{QD}$  (**B'**) wing pouches, revealing a strong reduction in expression in response to BMP signaling.

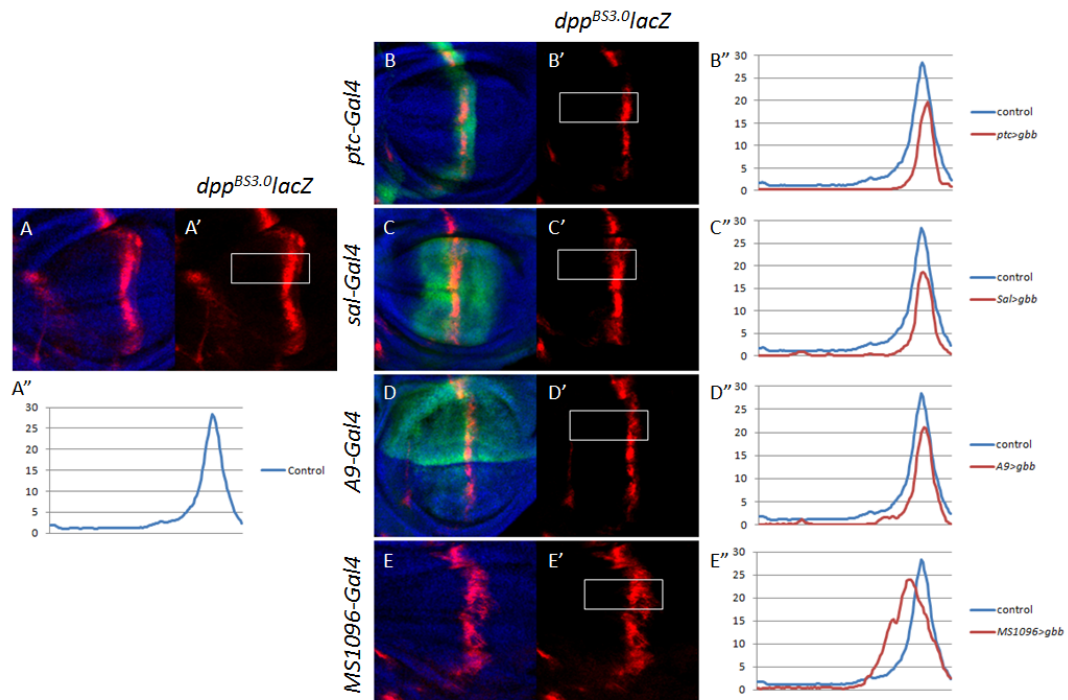
resulting expression of  $dpp^{10638} lacZ$  or  $dpp^{BS3.0} lacZ$ . Over-expression of  $gbb$  along the anterior-posterior boundary by either  $dpp^{blk}-Gal4$  or  $ptc-Gal4$  resulted in wing discs that appeared wildtype in both morphology and in expression pattern of  $dpp^{10638} lacZ$  (**Fig. 7.3A', A'', B', B''**). Expression of  $UAS-dpp$  by  $dpp^{blk}-Gal4$ , however, resulted in a repression of  $dpp^{10638} lacZ$  throughout its expression domain, giving a pattern that was both reduced and narrowed in comparison to controls (**Fig. 7.3A'', A'''**). As with our attempts to over-express  $UAS-tkv^{QD}$ , we could not recover  $ptc>dpp$  larvae in either  $ptc-Gal4$   $UAS-GFP/dpp^{10638} lacZ$ ;  $UAS-dpp/+$  or  $ptc-Gal4$   $UAS-GFP/+$ ;  $UAS-dpp/+$  genotypes, presumably due to embryonic expression of  $ptc-Gal4$ . We also examined the expression of  $dpp^{BS3.0} lacZ$  expression when  $UAS-dpp$  was driven in the wing disc and observed similar trends.  $dpp^{blk}>dpp$  animals display a  $dpp^{BS3.0} lacZ$  expression pattern is reduced in a subset of its expression domain, resulting in a stripe that looks narrowed compared to  $dpp^{BS3.0} lacZ$  expression in the  $dpp^{blk}-Gal4/+$  control (**Fig. 7.3C**). Surprisingly,  $ptc-Gal4/+$ ;  $UAS-dpp/dpp^{BS3.0} lacZ$  animals could be recovered, perhaps due to the absence of  $UAS-GFP$  in this experiment. Expression of  $dpp^{BS3.0} lacZ$  was repressed throughout its expression domain in  $ptc>dpp$  wing pouches (**Fig. 7.3D**).  $A9>dpp$  animals also showed reduced expression of  $dpp^{BS3.0} lacZ$ , consistent with repression of  $dpp$  expression in response to BMP signaling (**Fig. 7.3E**).



**Figure 7.3. Over-expression of the BMP ligands, *dpp* and *gbb*, have a variable effect on expression of *dpp-lacZ* reporters.** Ligands were over-expressed in the wing pouch using various *Gal4* drivers and the resulting expression patterns of *dpp<sup>p10638</sup>lacZ* (A-B) and *dpp<sup>BS3.0</sup>lacZ* (C-E) were examined. For each *Gal4* and *dpp-lacZ* combination, control and ligand over-expression images were taken at the same confocal settings to allow for comparison of levels between pouches. Boxed areas in the wing pouches represent the areas used to generate intensity profiles of *dpp-lacZ* expression levels as shown in the right panels. Intensity profiles displayed represent data from a single pouch or from an average of several pouches, as noted. **A.** *dpp<sup>blk</sup>-Gal4* was used to drive UAS-GFP alone (A, n=3) and in combination with UAS-*gbb* (A', n=3) or UAS-*dpp* (A'', n=3). **A'''**. Expression of *dpp<sup>p10638</sup>lacZ* was reduced in a subset of its expression domain in response to *dpp* over-expression but unchanged in response to *gbb* over-expression. **B.** *ptc-Gal4* was used to drive UAS-GFP alone (B, n=4) and in combination with UAS-*gbb* (B', n=4). *ptc>dpp* larvae were not recovered. **B'''**. Expression of *dpp<sup>p10638</sup>lacZ* was largely unchanged in response to *gbb* over-expression. **C.** Expression of *dpp<sup>BS3.0</sup>lacZ* was observed in *dpp<sup>blk</sup>-Gal4* (C, n=1) and *dpp<sup>blk</sup>>dpp* (C', n=1) wing imaginal pouches. **C''**. A slight change in expression of *dpp<sup>BS3.0</sup>lacZ* was observed with increased BMP signaling, seen as a narrowed expression domain of *dpp<sup>BS3.0</sup>lacZ*. **D.** Expression of *dpp<sup>BS3.0</sup>lacZ* was observed in *ptc-Gal4* (D, n=1) and *ptc>dpp* (D', n=1) wing imaginal pouches. **D''**. A reduction in expression of *dpp<sup>BS3.0</sup>lacZ* was observed with increased BMP signaling. **E.** Expression of *dpp<sup>BS3.0</sup>lacZ* is reduced in *A9>dpp* (E', n=1) wing pouches compared to *A9-Gal4* (E, n=1) wing pouches.



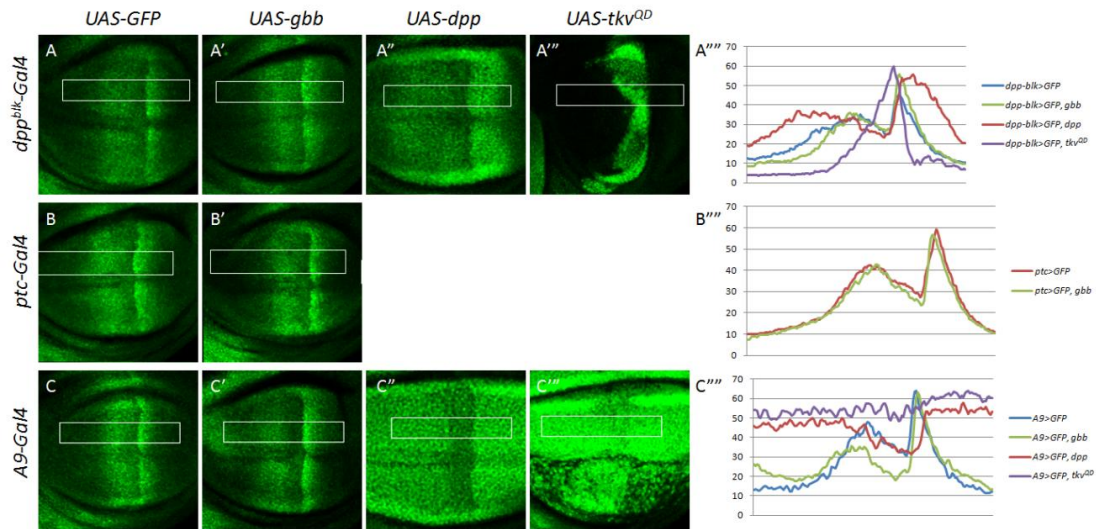
Despite wildtype expression patterns of *dpp*<sup>p10638</sup>*lacZ* with *gbb* expression under the control of *dpp*<sup>blk</sup>-*Gal4* or *ptc*-*Gal4*, the same experiments performed with *dpp*<sup>BS3.0</sup>*lacZ* resulted in a slight reduction in expression of the reporter. We drove expression of *UAS-gbb* by *ptc*-*Gal4* (Fig. 7.4B), *sal*-*Gal4* (Fig. 7.4C), *A9*-*Gal4* (Fig. 7.4D), and *MS1096*-*Gal4* (Fig. 7.4E). In each case, *dpp*<sup>BS3.0</sup>*lacZ* expression was slightly reduced compared to wildtype (Fig. 7.4A). *sal*-*Gal4*, a driver not previously described, is expressed in the medial wing imaginal pouch throughout most, but not all, of the wing pouch. The expression domain of this driver is shown in Fig. 7.4C in green.



**Figure 7.4: Over-expression of *gbb* in the wing imaginal disc has a variable effect on expression of *dpp*<sup>BS3.0</sup>*lacZ*.** *gbb* was over-expressed in the wing disc using various *Gal4* drivers and the resulting expression patterns of *dpp*<sup>BS3.0</sup>*lacZ* were examined. Red shows *dpp*<sup>BS3.0</sup>*lacZ* expression, blue is Hoechst to mark nuclei, and green is expression of *UAS-GFP*. In all cases tested, over-expression of *gbb* caused a slight reduction in expression of *dpp*<sup>BS3.0</sup>*lacZ*. **A.** Control expression of *dpp*<sup>BS3.0</sup>*lacZ* in red (n=3), to which all other wing pouches were compared. **B.** *dpp*<sup>BS3.0</sup>*lacZ* expression in *ptc>gbb*, *GFP* pouches (n=3). **C.** Expression of *dpp*<sup>BS3.0</sup>*lacZ* in *sal>gbb*, *GFP* pouches (n=3). *Sal-Gal4* is expressed in the medial region of the wing pouch, as shown in green. **D.** Expression of *dpp*<sup>BS3.0</sup>*lacZ* in *A9>gbb*, *GFP* pouches (n=3). **E.** Expression of *dpp*<sup>BS3.0</sup>*lacZ* in *MS1096>gbb* pouches (n=2). This wing disc did not have *UAS-GFP* in the background.

We next considered why over-expression of these three BMP pathway components, *gbb*, *dpp*, and *tkv*<sup>OD</sup> might have such a variable effect. In general, expression of *tkv*<sup>OD</sup> has a strong effect on expression of *dpp-lacZ*, *dpp* expression has a moderate effect on *dpp-lacZ* expression, and *gbb* has a very weak effect or no effect on *dpp-lacZ* expression. We hypothesized that the different response may be due

to variable effects on BMP signaling activity that expression of these components might elicit. If this is true, we expected that over-expression of *tkv<sup>QD</sup>* would cause a strong increase in pMad levels, *dpp* a moderate increase in pMad levels, and *gbb* a weak increase in pMad levels. Indeed, we did observe these trends in pMad levels. We examined pMad with over-expression of *UAS-gbb*, *UAS-dpp*, and *UAS-tkv<sup>QD</sup>* driven by *dpp<sup>blk</sup>-Gal4* and *A9-Gal4* and with over-expression of *UAS-gbb* driven by *ptc-Gal4*. For each *Gal4* tested, discs expressing *UAS-GFP* alone were used as a wildtype control and all pMad images were taken at the same settings to allow direct comparison between samples. Over-expression of *gbb* has a very mild effect on pMad levels. Compared to wildtype, *dpp<sup>blk</sup>>gbb* wing pouches have comparable pMad levels in the anterior compartment and in the lateral posterior compartment, but the posterior peak is slightly increased (**Fig 7.5A', A'''**). pMad levels in *ptc>GFP* and *ptc>gbb* pouches are virtually identical (**Fig 7.5B', B'''**). In *A9>gbb* animals, posterior pMad is identical to pMad in control pouches but the anterior peak and the trough are decreased. In addition, there is an increase in pMad in the lateral region of the anterior



**Figure 7.5. Over-expression of *gbb*, *dpp*, or *tkv<sup>QD</sup>* has a variable effect on BMP signaling levels in the wing imaginal pouch.** The BMP pathway components *gbb*, *dpp*, and *tkv<sup>QD</sup>* were over-expressed in the wing disc using various *Gal4* drivers and pMad levels were examined. Shown in green is pMad within wing pouches. **A.** *dpp<sup>blk</sup>-Gal4* was used to drive *UAS-GFP* alone (**A**, n=3) and in combination with *UAS-gbb* (**A'**, n=3), *UAS-dpp* (**A''**, n=3), or *UAS-tkv<sup>QD</sup>* (**A'''**, n=3). **B.** *ptc-Gal4* was used to drive *UAS-GFP* alone (**B**, n=4) and in combination with *UAS-gbb* (**B'**, n=4). **C.** *A9-Gal4* was used to drive *UAS-GFP* alone (**C**, n=1) and in combination with *UAS-gbb* (**C'**, n=3), *UAS-dpp* (**C''**, n=3), or *UAS-tkv<sup>QD</sup>* (**C'''**, n=2). **A'''-C'''**. Intensity profiles reveal that, in general, over-expression of *gbb* has only a mild effect on pMad levels, with a slight increase in the posterior peak of *dpp<sup>blk</sup>>gbb* animals and a reduced trough and anterior peak in *A9>gbb* animals. Over-expression of *dpp* has a more pronounced effect, with widened peaks in *dpp<sup>blk</sup>>gbb* animals and fairly uniform distribution in the anterior and posterior compartments of *A9>dpp* pouches. Over-expression of *tkv<sup>QD</sup>* results in elevated pMad throughout the expression domain of *dpp<sup>blk</sup>-Gal4* and *A9-Gal4*.

compartment (**Fig 7.5C', C'''**). Expression of *dpp* by both *dpp<sup>blk</sup>-Gal4* and *A9-Gal4* had a more pronounced effect on pMad levels. pMad in *dpp<sup>blk</sup>>dpp* pouches is similar to control in the trough and it reaches the same intensity in both peaks, but the peaks are widened, resulting in a broader pMad gradient (**Fig 7.5A'', A'''**). In *A9>dpp* pouches there is a striking effect on pMad, with pMad uniformly high in both the anterior and posterior compartments. The trough still remains at the same intensity as in the control pouches (**Fig 7.5C'', C'''**). Expression of *tkv<sup>QD</sup>* resulted in pMad levels at or above the highest levels seen in control pouches. In *dpp<sup>blk</sup>>tkv<sup>QD</sup>* pouches, a sharp peak of pMad is observed, corresponding to the expression domain of *dpp<sup>blk</sup>-Gal4* (**Fig 7.5A''', A'''**). *A9>tkv<sup>QD</sup>* pouches have high pMad across the dorsal compartment and in patches of the ventral compartment, consistent with *A9-Gal4* expression (**Fig 7.5C''', C'''**).

**Appendix 7.2 – Over-expression of *UAS-tkv<sup>QD</sup>* in the dorsal compartment by the *Gal4* driver *ap-Gal4* does not lead to a change in cell size or number.**

**Rationale:**

We hypothesized that the reduced expression of *dpp<sup>p10638</sup>lacZ* and *dpp<sup>BS3.0</sup>lacZ* observed in response to expression of *UAS-tkv<sup>QD</sup>* could be due to a change in the morphology of the cells expressing activated *tkv*. We were concerned that activating BMP signaling to this extent could alter cell size or number in such a way that made it appear that *dpp-lacZ* was reduced when it was not. This could happen if cell size were increased, effectively diluting the  $\beta$ Gal protein available for visualization, or if cell number were decreased, giving fewer cells to express *dpp*. After these experiments were performed, a report was published stating that increased BMP signaling does, indeed, affect cell size in the wing disc (Widmann and Dahmann, 2009).

**Approach:**

We over-expressed *UAS-tkv<sup>QD</sup>* in the dorsal compartment of the wing disc by *ap-Gal4* and examined expression of *dpp<sup>p10638</sup>lacZ* by performing the following crosses:

*dpp<sup>p10638</sup>lacZ/+ ; UAS-tkv<sup>QD</sup>/+* males to *w ; ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/dpp<sup>p10638</sup>lacZ ; UAS-tkv<sup>QD</sup>/+* and *ap-Gal4 UAS-GFP/dpp<sup>p10638</sup>lacZ* larvae.

*lacZ* was visualized by immunofluorescence using an antibody directed against  $\beta$ Gal (mouse monoclonal, 1:3000, Promega), nuclei were labeled with Hoechst-33342 (1:1000), and cell membranes were labeled with rhodamine-phalloidin (1:1000) to stain F-actin. This particular genotype was useful as it allowed us to compare cell number and shape between dorsal and ventral compartment of a single wing disc as well as to control discs. Samples were imaged at 63X with the Zeiss LSM 510 Meta Confocal Laser Scanning Microscope. Fiji was used to generate collapsed Z-sections throughout the entire disc.

To test whether cell size was altered with *tkv<sup>QD</sup>* expression, we measured the thickness of cells expressing *tkv<sup>QD</sup>* and compared this to ventral cells not expressing *tkv<sup>QD</sup>*. We then compared this to similar measurements in wing discs expressing *GFP* alone. Using the Zeiss LSM software, we created orthogonal

projections to observe the thickness of the disc through the z-plane. Using Fiji, we measured the thickness in the y-plane at three locations in the dorsal and three locations in the ventral compartment of the wing disc and averaged the values. We then determined the ratio of dorsal thickness to ventral thickness. This was repeated for three discs of each genotypes and the average ratio was determined. The students t-test was used to determine statistical significance of *ap>GFP*, *tkv<sup>QD</sup>* values compared to *ap>GFP* values.

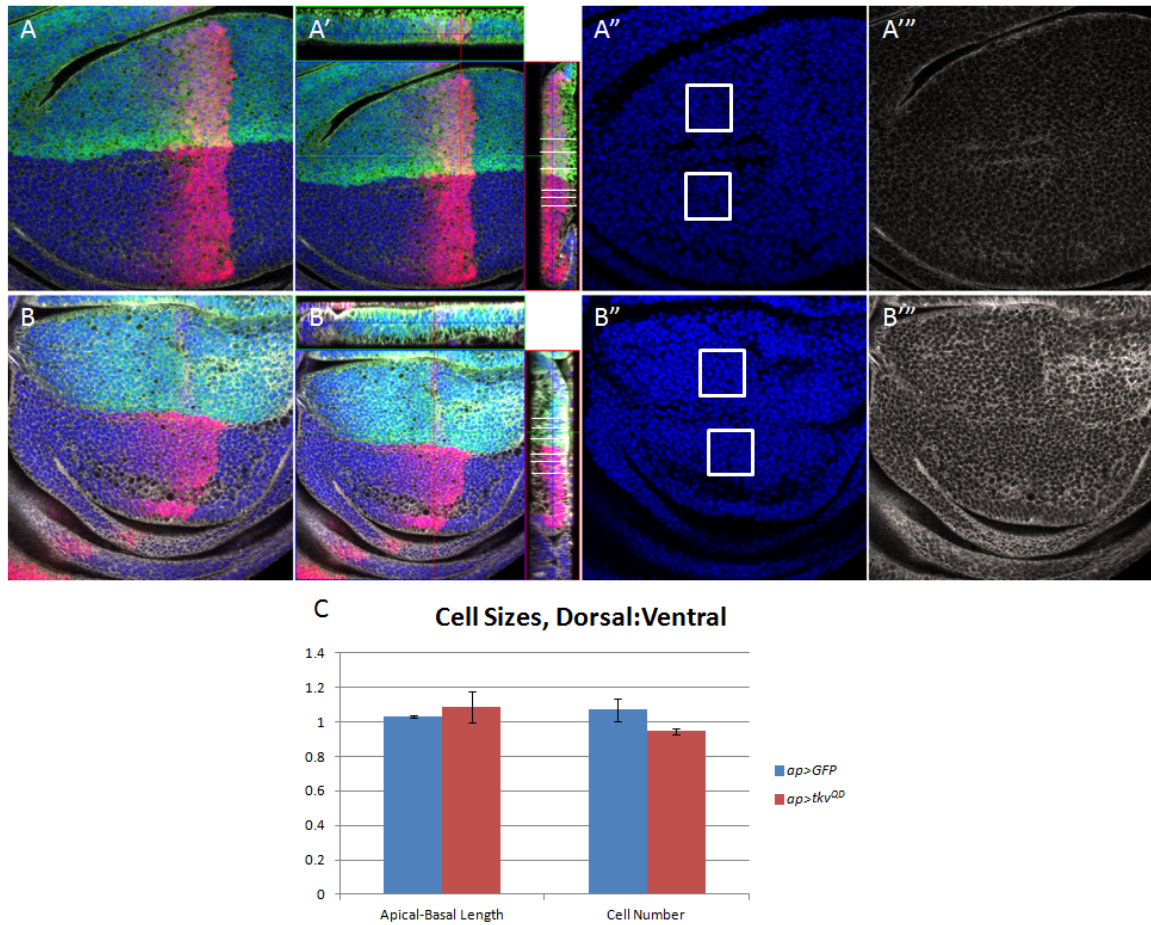
To test whether cell number is altered with *UAS-tkv<sup>QD</sup>* expression, we counted the number of cells within a specified region of dorsal cells compared to ventral cells in *ap>GFP*, *tkv<sup>QD</sup>* and *ap>GFP* wing discs. Using the Fiji software, we boxed regions of identical size in the anterior compartment within the *dpp<sup>p10638</sup>lacZ* expression domain of both the dorsal and ventral compartments. Using Hoechst and phalloidin staining to label individual cells, we manually counted the nuclei present in these boxes. We determined the ratio of dorsal cells to ventral cells in three discs of each genotype. These values were averaged and the students t-test was used to determine the statistical significance of values from *ap>GFP*, *tkv<sup>QD</sup>* compared to *ap>GFP* wing discs.

### Results and Conclusions:

We did not observe a statistically significant change in either cell size or number in *ap>GFP*, *tkv<sup>QD</sup>* wing pouches compared to *ap>GFP* wing pouches. There was a slight increase in the length of cells along the apical-basal axis of *ap>GFP*, *tkv<sup>QD</sup>* pouches, but this change was not significant ( $p=0.50$ ) (**Fig 7.5 A',B',C**). *ap>GFP*, *tkv<sup>QD</sup>* pouches also appeared to have a lower ratio of dorsal to ventral cells than *ap>GFP* pouches, suggesting a reduced cell number in the dorsal compartment of cells expressing *UAS-tkv<sup>QD</sup>*, but this result was also not significant ( $p=0.14$ ) (**Fig 7.5 A'',B'',C**). Phalloidin staining did not reveal disorganization of the epithelium in either dorsal or ventral compartments of either genotype observed (**Fig 7.5 A''', B'''**).

This is curious, considering the recent paper addressing the effect of BMP signaling on cell size (Widmann and Dahmann, 2009). In this paper, they performed similar measurements of cell size in anterior and dorsal compartment of *ap>tkv<sup>QD</sup>* wing pouches. In these experiments, an increase in dorsal to ventral cell length was observed from approximately 1 to 1.4 in early third instar and from approximately 1 to 1.2 in late third instar. This discrepancy from our observations could be due to the

specific age of the larvae we examined, particular staining and mounting techniques, or in the small number of wing discs that we observed. Despite this, we do not anticipate that the increase in cell length that was published could be responsible for the reduction in *dpp-lacZ* that we observed. In the event that it could play a slight role in our observations, we were careful to always image *dpp-lacZ* throughout the entire length of the apical-basal axis of the wing pouch.



**Figure 7.6. Expression of *UAS-tkv<sup>QD</sup>* in the dorsal wing pouch does not significantly alter cell size or cell number.** Activated *tkv* receptor, *tkv<sup>QD</sup>*, was expressed in the dorsal compartment of the wing disc using the *ap-Gal4* driver. *dpp<sup>p10638</sup>lacZ* is shown in red, confirming that repression of this reporter's expression is seen with increased BMP signaling. GFP in green marks the dorsal compartment. Nuclei are labeled with Hoechst and shown in green. F-actin, marking the cell membrane, was stained with phalloidin and is displayed in white. **A.** *ap>GFP* control pouch showing an overall of all components visualized. **B.** *ap>GFP, tkv<sup>QD</sup>* wing pouches showing an overlay of all components visualized. **A', B'.** Above the pouch is an x-axis projection generated from the horizontal green line within the pouch and to the right of the pouches is a y-axis projection generated from the vertical green line within the pouch in the *dpp<sup>p10638</sup>lacZ* expression domain. Cell length was measured along the apical-basal axis of the wing pouch using a y-axis orthogonal projection. Three measurements in the dorsal and the ventral compartments were taken, shown as white lines in the y-axis projections. **A'', B''.** Hoechst staining of nuclei was used to count nuclei in dorsal and ventral compartment of *ap>GFP* and *ap>GFP, tkv<sup>QD</sup>* wing pouches. The boxed regions, identical in size, were used to manually count cell number. **A''', B'''.** Phalloidin staining in *ap>GFP* and *ap>GFP, tkv<sup>QD</sup>* reveals no disorganization in either genotype. **C.** Average ratio of dorsal to ventral cell length and cell number. There is no significant difference in cell length ( $p=0.50$ ) or cell number ( $p=0.14$ ) between *ap>GFP* and *ap>GFP, tkv<sup>QD</sup>* wing pouches.

### Appendix 7.3 – *A9-Gal4* is expressed in the wing imaginal disc before third instar.

#### Rationale:

Our original experiments examining the effect of over-expressing *dpp* and *gbb* on expression of *dpp-lacZ* showed a fair amount of variability in the strength of the effect on *dpp-lacZ* expression depending on the method used to over-express ligand. In particular, over-expression of *gbb* by *A9-Gal4* had a milder effect on *dpp<sup>p10638</sup>-lacZ* expression than over-expression with *dpp<sup>blk</sup>-Gal4*, *ptc-Gal4*, or *MS1096-Gal4*. This prompted us to ask if the reason for the variable response with *A9-Gal4* was due to the timing of *A9-Gal4* expression. If *A9-Gal4* turns on late, in third instar or later, it may not be able to elicit a significant change in *dpp-lacZ* expression. To address this issue, we observed *A9-Gal4* in wing discs younger than wandering third instar.

#### Approach:

We generated larvae expressing *UAS-GFP* driven by *A9-Gal4* by crossing:

*UAS-GFP* males to *w A9-Gal4* females to generate *A9-Gal4/+ ; UAS-GFP/+* larvae.

Crosses were performed in cages on apple juice plates and examined *GFP* expression by direct immunofluorescence.

*GFP* was examined and imaged from mid 2<sup>nd</sup> instar (30-42 hours) and early 3<sup>rd</sup> instar (54-66 hours) larvae using a Zeiss Lumar V12 Fluorescence Stereomicroscope. Wandering third instar larvae (96-120 hours) were dissected and imaged by standard confocal microscopy procedures previous described.

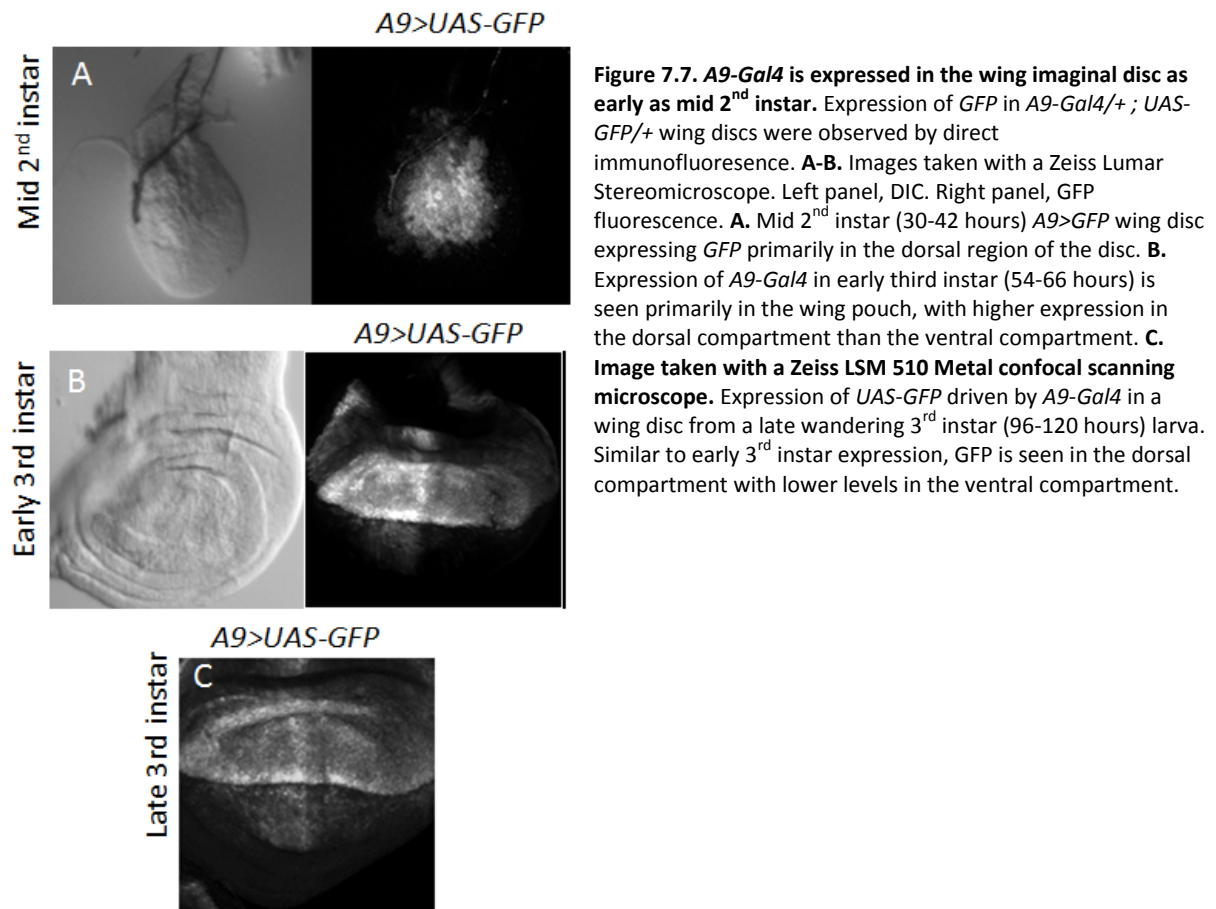
#### Results and Conclusions:

*A9-Gal4* is expressed as early as mid second instar. In the second instar stage examined, *GFP* driven by *A9-Gal4* is expressed throughout much of the dorsal region of the wing imaginal disc (**Fig 7.7A**). Without a marker for the dorsal or ventral compartments, it is hard to be sure exactly which compartment is expressing *A9-Gal4*. By early third instar, the expression domain of *A9-Gal4* is more refined to the dorsal compartment of the wing disc with lower levels observed in the ventral compartment (**Fig 7.7B**). Expression of *A9-Gal4* in late third instar larvae is similar to that of early third instar; it is largely confined to the dorsal wing pouch with low expression in the ventral compartment (**Fig 7.7C**). Attempts to observe *A9>GFP* in first instar were unsuccessful as we could not identify any *GFP*



expressing discs. This could be due to either experimental error (inability to locate the small first instar wing discs) or lack of *A9-Gal4* expression. These results suggest that *A9-Gal4* is likely turned on early mid second instar, at the latest. We have evidence indicating that mid second instar is early enough to elicit a transcriptional change in *dpp-lacZ*, as *tkv<sup>QD</sup>* clones generated at this time show a strong repression of *dpp<sup>p10638</sup>lacZ* expression. The timing of *A9-Gal4* expression should, therefore, not hinder its ability to affect transcription of *dpp*.

Since performing these experiments, we have now come to believe that the discrepancy that we had originally observed may have been due to low sample number or inconsistencies in confocal imaging. We had based our conclusions on images of *dpp<sup>p10638</sup>lacZ* expression in one or very few wing imaginal discs. Subsequent experiments have seen more consistent trends in the effect of over-expressing a particular BMP pathway component.





#### Appendix 7.4 – *dpp-lacZ* expression is increased in response to reduced BMP signaling.

##### Rationale:

We have strong evidence indicating that an increase in BMP signaling leads to a repression of *dpp* expression. If the converse is true then a reduction in BMP signaling should up-regulate *dpp* expression and, indeed, we have previously observed an up-regulation in expression of *dpp<sup>BS3.0</sup>lacZ* in *gbb<sup>2</sup>* loss-of-function clones. In these experiments, we ask if the same response will be observed with expression of the *dpp* reporter trap, *dpp<sup>p10638</sup>lacZ*.

##### Approach:

*gbb<sup>2</sup>* loss-of-function clones were generated and expression of *dpp<sup>p10638</sup>lacZ* was examined.

These were generated by crossing:

*y w f hfFLP122/Y ; FRT<sup>G13</sup> sha gbb<sup>2</sup>/CyO-GFP* males to *w ; dpp<sup>p10638</sup>lacZ FRT<sup>G13</sup> 2X-Ubi-nls-GFP/CyO-GFP* females to generate *y w f hfFLP122/+ ; FRT<sup>G13</sup> sha gbb<sup>2</sup>/dpp<sup>p10638</sup>lacZ FRT<sup>G13</sup> 2X-Ubi-nls-GFP* larvae.

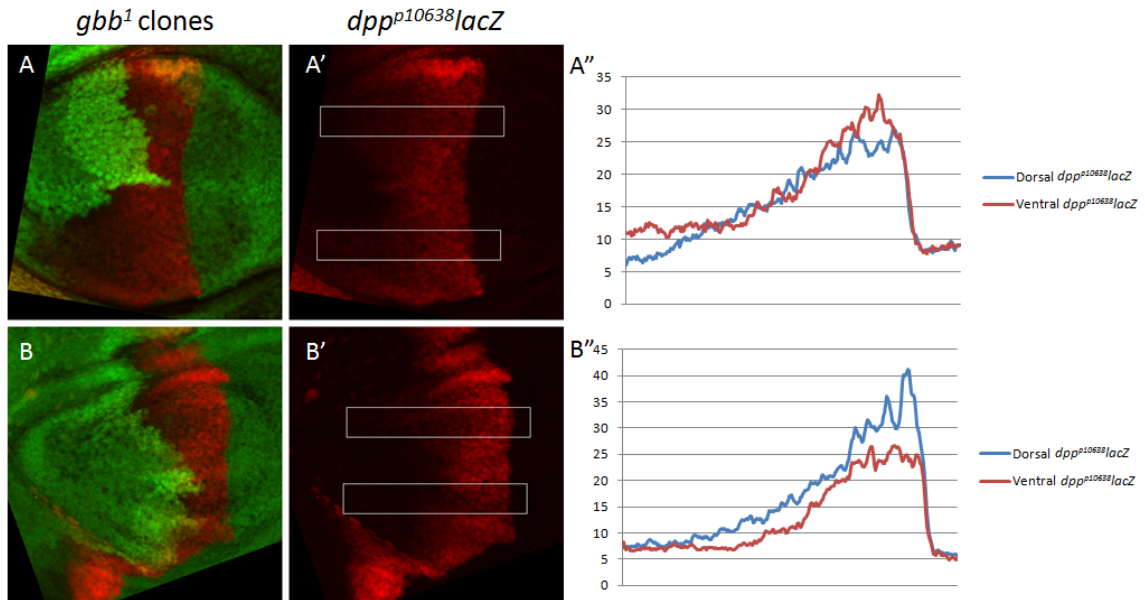
Crosses were pre-mated in glass vials and then moved to bottles and brooded every 24 hours. To induce the expression of FLP recombinase, bottles were heat shocked at 37°C by submersion in a water bath for 2 hours at 24-48 hours post lay to generate first instar clones. Larvae without *CyO-GFP* were selected and dissected as third instar crawlers. Clones were identified by the absence of GFP.

##### Results and Conclusions:

Clones of cells null for *gbb* lead to only a subtle change in *dpp<sup>p10638</sup>lacZ* expression. There does not appear to be a significant increase in expression levels of *dpp<sup>p10638</sup>lacZ* clones. However, large clones that begin at the anterior-posterior boundary and extend into the anterior compartment, past the endogenous expression domain of *dpp<sup>p10638</sup>lacZ*, may lead to a slight expansion of the *dpp<sup>p10638</sup>lacZ* domain. In figure 7.8 two pouches are shown with large clones that cover substantial areas of the anterior compartment. Intensity profiles, generated from the boxed regions in **A'** and **B'**, demonstrate a slight expansion of *dpp<sup>p10638</sup>lacZ* expression in the clones. The ventral clone in figure 7.8A results in *dpp<sup>p10638</sup>lacZ* further into the anterior compartment than in the dorsal compartment of the same pouch. A dorsal clone,

shown in figure 7.8B, has the opposite effect and results in expanded expression in the dorsal region within the clone compared to the ventral compartment of the same wing pouch.

These results conflict with the effect of *gbb* clones on expression of *dpp<sup>BS3.0</sup>lacZ*, where an obvious up-regulation of *lacZ* expression is observed within the *gbb* clone. We know that *dpp<sup>p10638</sup>lacZ* is sensitive to BMP signaling levels, as a decrease in *dpp<sup>p10638</sup>lacZ* expression has been observed in cells over-expressing *dpp* or *tkv<sup>QD</sup>*. This discrepancy suggests that *dpp<sup>p10638</sup>lacZ* is less sensitive to reduced BMP signaling levels than to increased BMP signaling, unlike *dpp<sup>BS3.0</sup>lacZ* which is sensitive to any changes in BMP signaling.



**Figure 7.8 *gbb* loss-of-function clones display an expansion of the *dpp<sup>p10638</sup>lacZ* expression domain.** *gbb<sup>2</sup>* loss-of-function clones were generated (marked by the absence of GFP, in green) in first instar and expression of *dpp<sup>p10638</sup>lacZ* was examined (in red) in third instar crawlers. An up-regulation of *dpp<sup>p10638</sup>lacZ* expression in these clones was not observed. We did observe a slight expansion in the *dpp<sup>p10638</sup>lacZ* expression domain of clones that are flush with the anterior-posterior boundary and extend far into the anterior compartment, encompassing the *dpp<sup>p10638</sup>lacZ* expression domain. The boxed regions in A' and B' were used to generate intensity profiles from the dorsal and ventral compartments. **A.** A large clone in the ventral compartment gives an intensity profile that is slightly higher in lateral regions of the wing pouch than a profile from the dorsal compartment of the same pouch. **B.** A clone the dorsal compartment gives a dorsal intensity profile that is slightly expanded compared to the ventral compartment.

## Appendix 7.5 – *gbb* positively regulates *dpp* expression in the eye imaginal disc.

### Rationale:

Given our findings that a reduction in *gbb* leads to an up-regulation of *dpp* in the wing imaginal disc, we asked if this process occurred in other imaginal discs. An understanding of when and where *dpp* is negatively regulated by BMP signaling may point to specific factors required for this process. It has been established that *dpp* is under positive regulation in the eye disc, such that a reduction in *Mad* or *dpp* function leads to a reduction in expression of a *dpp-lacZ* reporter line (Chanut and Heberlein, 1997; Lee et al., 2007; Pignoni and Zipursky, 1997; Wiersdorff et al., 1996). However, while *gbb* has been reported to express in the eye disc, a functional role for *gbb* in BMP signaling and in regulating *dpp* expression has not been established in this tissue.

### Approach:

*gbb*<sup>2</sup> loss-of-function clones were generated and expression of *dpp*<sup>BS3.0</sup>*lacZ* was examined in the eye-antennal imaginal disc. These were generated by crossing:

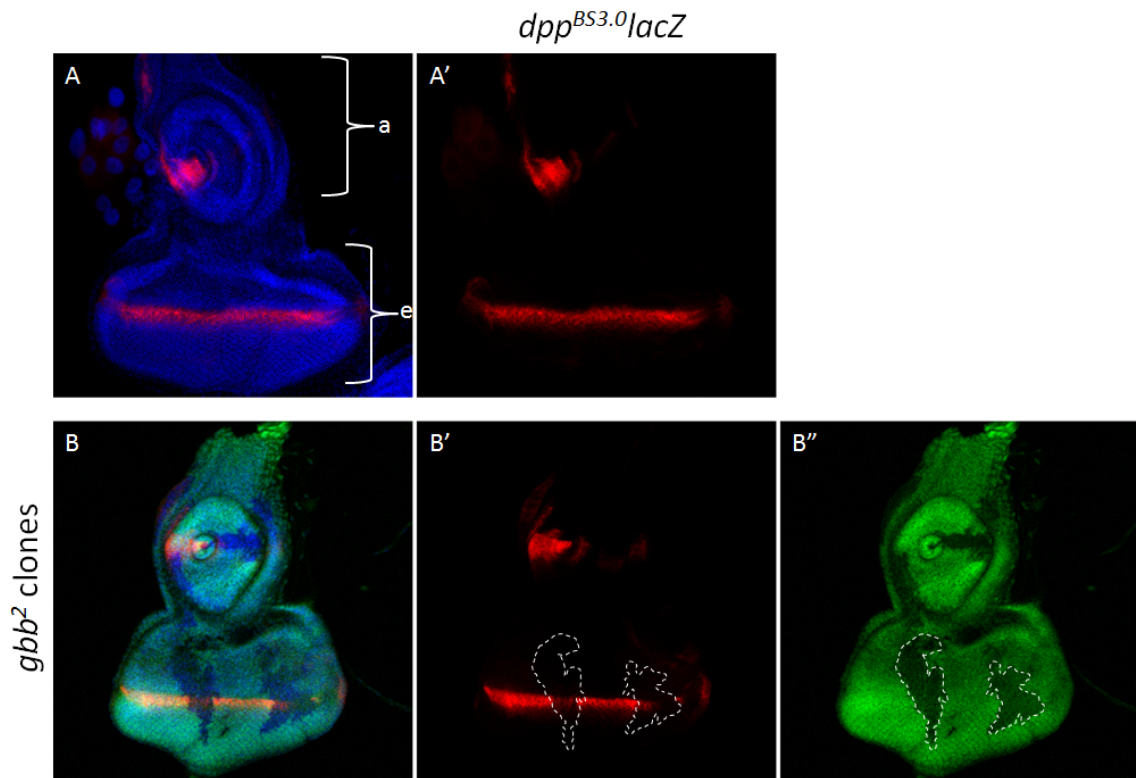
*w/Y; FRT<sup>G13</sup> gbb<sup>2</sup>; dpp<sup>BS3.0</sup>lacZ / T(2;3) SM5-TM6b* males to *hsFLP1; FRT<sup>G13</sup> GFP/CyO GFP* females to generate *hsFLP1; FRT<sup>G13</sup> gbb<sup>2</sup>/FRT<sup>G13</sup> GFP; dpp<sup>BS3.0</sup>lacZ /+* larvae.

Crosses were pre-mated in glass vials and then moved to bottles and brooded every 24 hours. To induce the expression of FLP recombinase, bottles were heat shocked at 37°C in a water bath for 2 hours at 24-48 hours post lay to generate first instar clones. Larvae without *CyO-GFP* were selected and dissected as third instar crawlers. Clones were identified by the absence of GFP.

### Results and Conclusions:

In the eye-antennal imaginal disc, *dpp*<sup>BS3.0</sup>*lacZ* is expressed in a ventral wedge in the antennal disc and in the morphogenetic furrow of the eye disc (Fig 7.9A). Consistent with previously published reports on auto regulation of *dpp* in the eye disc, clones of cells null for *gbb* display a cell-autonomous reduction in *dpp*<sup>BS3.0</sup>*lacZ* expression (Fig 7.9B). Based on this result, we conclude that *gbb* must contribute to BMP signaling in the eye disc and that a reduction in *gbb* levels, like a reduction in *Mad*, leads to a reduction in *dpp* expression. These results also indicate that *dpp* is positively regulated in the morphogenetic furrow, when previously only lateral regulation had been observed (Chanut and Heberlein, 1997; Wiersdorff et al.,

1996). These results indicate that regulation of *dpp* in the eye versus the wing imaginal disc is controlled by different mechanisms.



**Figure 7.9** Loss of *gbb* functions results in a cell-autonomous reduction in *dpp<sup>BS3.0</sup>lacZ* expression levels. *gbb<sup>2</sup>* loss-of-function clones were generated (marked by the absence of GFP, in green) in first instar and expression of *dpp<sup>BS3.0</sup>lacZ* was examined (in red) in third instar crawlers. Nuclei are labeled with Hoechst-33432 and shown in blue. **A.** Wildtype expression of *dpp<sup>BS3.0</sup>lacZ* (**A'**) is seen in a wedge on the ventral side of the antennal disc (a) and at the morphogenetic furrow of the eye disc (e). **B.** Clones of cells null for *gbb*, marked by the absence of GFP and outlined in **B'** and **B''**, display a cell autonomous reduction in *dpp<sup>BS3.0</sup>lacZ* expression (**B'**).

## Appendix 7.6 – Increased BMP signaling can partially rescue ectopic *dpp* associated with *hh<sup>MRT</sup>*.

### Rationale:

If altered BMP signaling levels can influence the expression of endogenous *dpp* in the wing pouch, we reasoned that BMP signaling may also influence the expression of ectopic *dpp* to either exacerbate or rescue phenotypes associated with ectopic *dpp* expression. To address this question, we made use of the dominant gain-of-function *hh* allele, *hh<sup>MRT</sup>*, that leads to ectopic Hh signaling and ectopic *dpp* expression in the anterior compartment of the wing pouch (Felsenfeld, 1995; Johnson et al., 1995). *hh<sup>MRT</sup>* adult wings display a variety of phenotypes, presumably, due to increased anterior Hh and BMP signaling, including an enlarged anterior wing compartment, loss of central vein pattern, and ectopic vein tissue in the anterior-most region of the wing. The *hh<sup>MRT</sup>* allele provides a sensitized system to monitor changes in *dpp* expression, as any rescue or exacerbation should give obvious and easily observable adult wing phenotypes. We asked whether *gbb* levels could interact with the *hh<sup>MRT</sup>* allele to enhance or rescue the phenotypes associated with this allele. If *dpp* is negatively regulated in this context, then we would expect that a reduction in *gbb* copy number would exacerbate *hh<sup>MRT</sup>* phenotypes and increase wing pouch expression of *dpp<sup>BS3.0</sup>lacZ*. Conversely, we would expect that an increase in *gbb* expression could rescue *hh<sup>MRT</sup>* phenotypes by reducing the amount of *dpp* expressed.

### Approach:

Analysis of *hh<sup>MRT</sup>/gbb<sup>1</sup>* interactions:

Adult wing phenotypes by performing the following crosses:

*b pr cn bw* males to *hh<sup>MRT</sup>/TM3 Sb* females to generate *b pr cn bw/+ ; hh<sup>MRT</sup>/+* adults  
*yw/Y ; gbb<sup>1</sup>/sp* males to *hh<sup>MRT</sup>/TM3 Sb* females to generate *gbb<sup>1</sup>/+ ; hh<sup>MRT</sup>/+* adults  
*yw/Y ; dpp<sup>12</sup>/sp* males to *hh<sup>MRT</sup>/TM3 Sb* females to generate *dpp<sup>d12</sup>/+ ; hh<sup>MRT</sup>/+* adults  
*yw/Y ; dpp<sup>d12</sup> bw gbb<sup>1</sup>/sp* males to *hh<sup>MRT</sup>/TM3 Sb* females to generate *dpp<sup>d12</sup> bw gbb<sup>1</sup>/+ ; hh<sup>MRT</sup>/+* adults

Expression of *dpp<sup>BS3.0</sup>lacZ* expression in larval wing pouches were observed by performing the following crosses:

$+/(\text{CyO } wg\text{-}lacZ) ; hh^{MRT} dpp^{BS3.0} lacZ/TM3$  *Sb* males to  $w^{1118}/Y$  ;  $dpp^{d12}/\text{CyO-GFP}$  females to generate  $dpp^{d12}/+ ; hh^{MRT} dpp^{BS3.0} lacZ/+$  larvae

$+/(\text{CyO } wg\text{-}lacZ) ; hh^{MRT} dpp^{BS3.0} lacZ/TM3$  *Sb* males to  $w^*$  ;  $bw\ gbb^1/\text{CyO-GFP}$  females to generate  $bw\ gbb^1/+ ; hh^{MRT} dpp^{BS3.0} lacZ/+$  larvae

$+/(\text{CyO } wg\text{-}lacZ) ; hh^{MRT} dpp^{BS3.0} lacZ/TM3$  *Sb* males to  $dpp^{d12}\ bw\ gbb^1/\text{CyO } wg\text{-}lacZ$  females to generate  $dpp^{d12}\ bw\ gbb^1/+ ; hh^{MRT} dpp^{BS3.0} lacZ/+$  larvae

$+/(\text{CyO } wg\text{-}lacZ) ; hh^{MRT} dpp^{BS3.0} lacZ/TM3$  *Sb* animals were observed as control larvae

Note: Lorena's notes sometimes indicated that the reciprocal cross was performed, in particular for the  $dpp^{BS3.0} lacZ$  experiments.

Analysis of  $hh^{MRT}$  with over-expression of *gbb* interactions:

We examined expression patterns of  $dpp^{BS3.0} lacZ$  in a  $hh^{MRT}$  mutant background with and without over-expression of *gbb* by crossing:

$hh^{MRT} dpp^{BS3.0} lacZ/TM3$  *Sb* males to  $y\ w\ UAS\text{-}gbb^{9.7}$  virgin females to generate  $y\ w\ UAS\text{-}gbb^{9.7}/+ ;$

$hh^{MRT} dpp^{BS3.0} lacZ/+$  control larvae

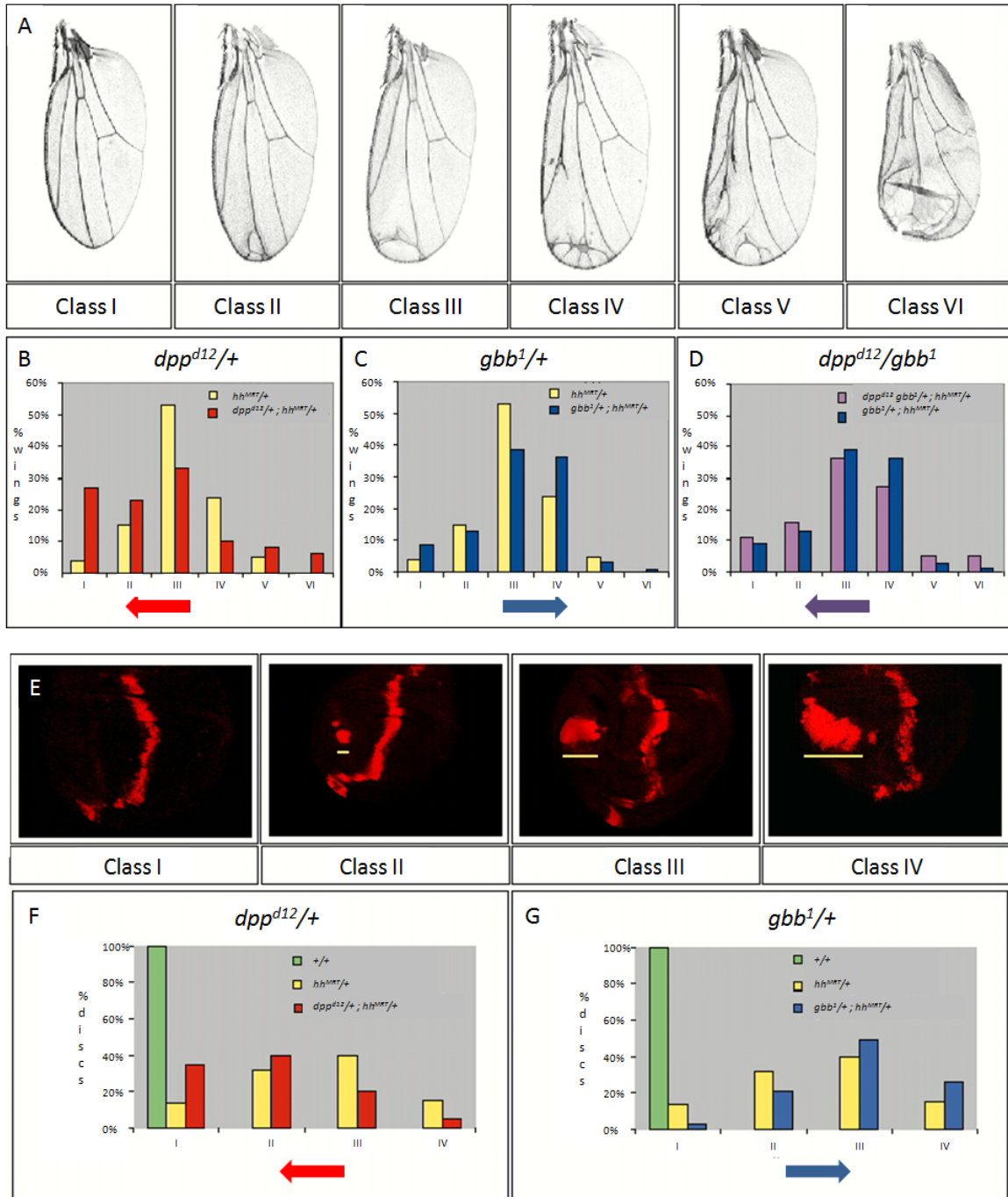
$w\ A9\text{-}Gal4/Y ; hh^{MRT} dpp^{BS3.0} lacZ/+$  males to  $y\ w\ UAS\text{-}gbb^{9.7}$  virgin females to generate  $y\ w\ UAS\text{-}gbb^{9.7}/w\ A9\text{-}Gal4 ; hh^{MRT} dpp^{BS3.0} lacZ/+$  larvae

$w\ MS1096\text{-}Gal4/Y ; hh^{MRT} dpp^{BS3.0} lacZ/+$  males to  $y\ w\ UAS\text{-}gbb^{9.7}$  virgin females to generate  $y\ w\ UAS\text{-}gbb^{9.7}/w\ MS1096\text{-}Gal4 ; hh^{MRT} dpp^{BS3.0} lacZ/+$  larvae

Statistical significance of these results was tested by  $\chi^2$  analysis.

## Results and Conclusions:

We first examined the effect of decreasing *gbb* copy number in a  $hh^{MRT}$  background and found that this did, indeed, cause an exacerbation of the  $hh^{MRT}$  phenotypes.  $hh^{MRT}$  animals display variable wing phenotypes designated into classes I to VI, from least to most severe, that vary in the degree of anterior overgrowth and in the degree of vein defects (Fig 7.10A). Class I appears mostly wildtype. A mild class, Class II, displays minor ectopic vein tissue in the distal-most region of L3 with little to no change in the size of the anterior compartment. A more severe class, Class V, displays much more ectopic vein tissue between L2 and L3 and has a large anterior compartment, expanded primarily in the L2/L3 intervein



**Figure 7.10: The  $hh^{MRT}$  phenotype is enhanced by decreasing  $gbb$ .** Adult wing and wing imaginal disc phenotypes of  $hh^{MRT}$ , a dominant allele of  $hh$ , are enhanced with a reduction in  $gbb$  copy number. **A-D.** Adult wings of animals in a  $hh^{MRT}/+$  background were examined and classified based on severity of wing phenotype. Over 200 wings were examined for each genotype **A**. Classification of wing phenotypes in  $hh^{MRT}/+$  animals, shown from least severe (Class I), looking mostly wildtype, to most severe (class VI), showing a greatly expanded anterior compartment, ectopic vein tissue, and blistering. **B.** Distribution of wing classes in  $hh^{MRT}/+$  (yellow) and  $dpp^{d12}/+; hh^{MRT}/+$  (red), demonstrating a highly significant ( $p < .001$ ) shift in the distribution of wing phenotypes to less severe classes in the presence of one copy of the  $dpp^{d12}$  allele. **C.** Distribution of wing classes in  $hh^{MRT}/+$  (yellow) and  $gbb^1/+; hh^{MRT}/+$  (blue) animals. A highly significant ( $p < .001$ ) shift in phenotype distribution to more severe classes in the presence of one copy of  $gbb^1$  was observed. **D.** Distribution of wing phenotypes in  $dpp^{d12} gbb^1/+; hh^{MRT}/+$  compared to  $gbb^1/+; hh^{MRT}/+$ ;

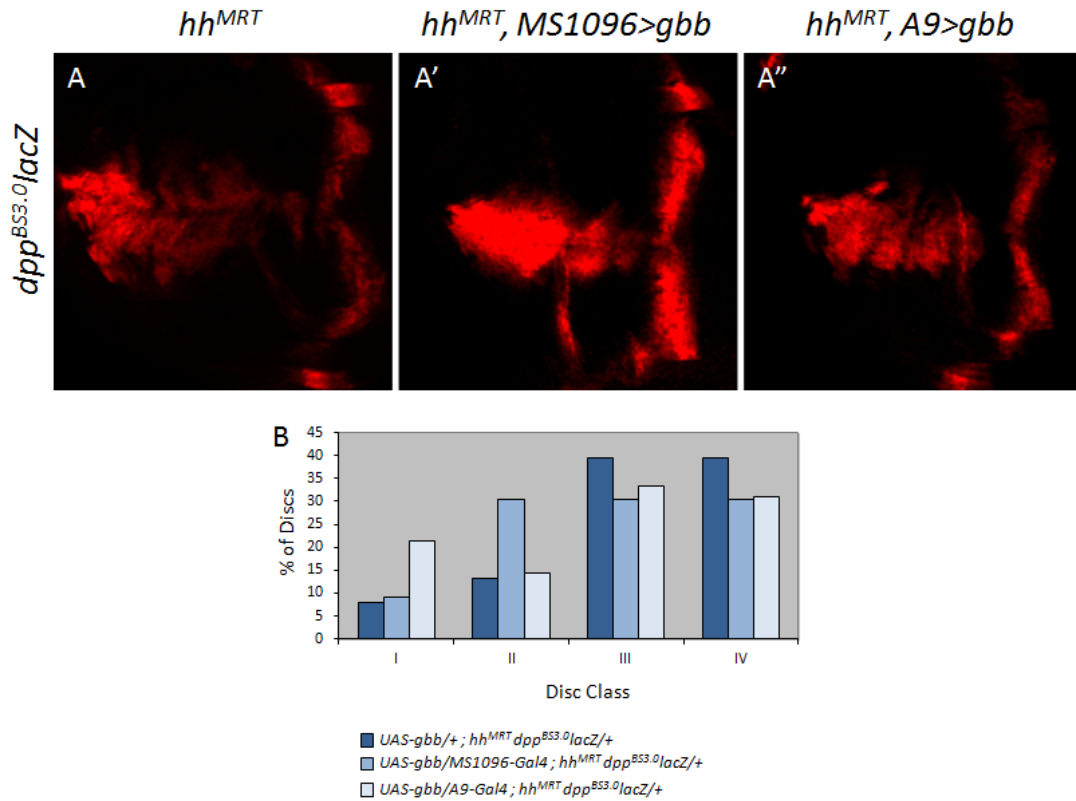
*hh<sup>MRT</sup>/+*. A reduction in *dpp* causes a slight but significant ( $p < .05$ ) rescue in wing phenotypes. **E-G.** Third instar wing pouch expression of *dpp<sup>BS3.0</sup>lacZ* in a *hh<sup>MRT</sup>/+* background was examined and classified based on the amount of ectopic *dpp* observed in the anterior compartment. **E.** Classification of *dpp<sup>BS3.0</sup>lacZ* expression in *hh<sup>MRT</sup>/+* animals. Discs are classified based on the amount of ectopic *dpp* seen, with no ectopic *dpp* designated as Class I and the most ectopic *dpp* designated as Class IV. **F.** Distribution of wing pouch classes in *+/+* (green), *hh<sup>MRT</sup>/+* (yellow), and *dpp<sup>d12</sup>/+ ; hh<sup>MRT</sup>/+* (red) wing pouches reveals a shift in the distribution of the classes to the less severe in *dpp<sup>d12</sup>/+ ; hh<sup>MRT</sup>/+* wing pouches compared to *hh<sup>MRT</sup>/+* wing pouches. **G.** Distribution of wing pouch classes in *+/+* (green), *hh<sup>MRT</sup>/+* (yellow), and *gbb<sup>1</sup>/+ ; hh<sup>MRT</sup>/+* (blue) pouches. An exacerbation of wing pouch *hh<sup>MRT</sup>/+* phenotypes was observed with a reduction in *gbb* copy number.

region. Class VI wings contain large blisters on the wing, making vein pattern difficult to interpret. Within a sample of *hh<sup>MRT</sup>/+* adults, all of these classes will be represented due to variability in phenotype, with the distribution of wing phenotypes from *hh<sup>MRT</sup>/+* adults centering at class III (Fig 7.10B,C, in yellow). The wing class distribution of *dpp<sup>d12</sup>/+ ; hh<sup>MRT</sup>/+* animals (red bars) is shifted toward less severe classes, indicating the *dpp* expression in the wing pouch is responsible for many of the *hh<sup>MRT</sup>* phenotypes observed (Fig 7.10B). *dpp<sup>d12</sup>* is a strong *dpp<sup>d</sup>* allele that results from a chromosomal rearrangement that breaks within the class V region of the *dpp<sup>disk</sup>* sequence, removing most of the *cis*- regulatory sequences required for wing imaginal disc expression and leading to reduced *dpp* expression in the wing disc (St Johnston et al., 1990).

We hypothesize that a reduction in *gbb* copy number could increase wing pouch expression of *dpp<sup>BS3.0</sup>lacZ* and exacerbate *hh<sup>MRT</sup>* phenotypes. To test this, we examined *hh<sup>MRT</sup>* adult wing phenotypes in a *gbb* heterozygous background. The distribution of wing phenotypes in *gbb<sup>1</sup>/+ ; hh<sup>MRT</sup>/+* animals was shifted towards more severe classes compared to control *hh<sup>MRT</sup>/+* animals, suggesting increased BMP signaling in the wing pouches (Fig 7.10C). These results are consistent with reduced *gbb* levels leading to a further up-regulation of *dpp* expression in the anterior compartment. Furthermore, reducing *dpp* can rescue the exacerbation associated with *gbb* reduction as *dpp<sup>d12</sup> gbb<sup>1</sup>/+ ; hh<sup>MRT</sup>/+* animals display a less severe phenotypic distribution compared to *gbb<sup>1</sup>/+ ; hh<sup>MRT</sup>/+* animals (Fig 7.10D). This result further supports our hypothesis that *gbb<sup>1</sup>* exacerbates the *hh<sup>MRT</sup>* phenotype by increasing *dpp* levels.

Our results thus far are consistent with reduced *gbb* copy number leading to increased *dpp* expression in *hh<sup>MRT</sup>* animals, but to directly test this we examined expression of *dpp<sup>BS3.0</sup>lacZ* in *hh<sup>MRT</sup>* wing pouches. Consistent with the variability observed in adult wing phenotypes, *hh<sup>MRT</sup> dpp<sup>BS3.0</sup>lacZ/+* pouches





**Figure 7.11. Increased *gbb* expression in the wing disc can partially rescue ectopic *dpp* associated with *hh<sup>MRT</sup>*.** **A.** *UAS-gbb* was expressed in the wing disc using *MS1096-Gal4* (A') and *A9-Gal4* (A'') in a *hh<sup>MRT</sup>/+* background and the resulting expression patterns of *dpp<sup>BS3.0</sup>lacZ* (in red) was examined. In the control *hh<sup>MRT</sup> dpp<sup>BS3.0</sup>lacZ/+* wing pouch, *dpp<sup>BS3.0</sup>lacZ* expression is observed along the anterior-posterior boundary and in the anterior compartment along the dorsal-ventral boundary (A). Over-expression of *gbb* in the wing pouch did not result in a noticeable change in *dpp<sup>BS3.0</sup>lacZ* expression (A'-A''). **B.** The severity of the *hh<sup>MRT</sup>* phenotype in each particular pouch examined was classified based on the amount of ectopic *dpp<sup>BS3.0</sup>lacZ* observed, with class I indicating no ectopic *dpp<sup>BS3.0</sup>lacZ* expression and class IV indicating extensive ectopic *dpp<sup>BS3.0</sup>lacZ* expression (for a better description of the classification system, refer to figure 7.10E). The distribution of classes in *UAS-gbb/+; hh<sup>MRT</sup> dpp<sup>BS3.0</sup>lacZ/+* (n=38), *UAS-gbb/MS1096; hh<sup>MRT</sup> dpp<sup>BS3.0</sup>lacZ/+* (n=33), and *UAS-gbb/A9; hh<sup>MRT</sup> dpp<sup>BS3.0</sup>lacZ/+* (n=42) was graphed and a  $\chi^2$  analysis was performed. Despite no obvious change in *dpp<sup>BS3.0</sup>lacZ* expression with *gbb* over-expression, there is a statistically significant ( $p < 0.001$ ) shift in the distribution of wing pouch phenotypes to less severe classes in both *MS1096>gbb* and *A9>gbb* wing pouches.

display variable levels of ectopic *lacZ* expression in the anterior wing pouch, designed classes I to IV from least to most ectopic expression of *lacZ* (Fig 7.10E). *dpp<sup>d12</sup>/+; hh<sup>MRT</sup> dpp<sup>BS3.0</sup>lacZ/+* animals exhibit a partial rescue of the amount of ectopic *dpp* expression compared to *hh<sup>MRT</sup> dpp<sup>BS3.0</sup>lacZ/+*, shown by a shift in the class distribution to less severe classes (Fig 7.10F). Conversely, *gbb<sup>1</sup>/+; hh<sup>MRT</sup>/+* animals, with a reduction in *gbb* copy number, show an enhancement of the amount of ectopic anterior *dpp* expression demonstrated by a shift in the distribution of ectopic *dpp<sup>BS3.0</sup>lacZ* expression to more severe classes (Fig

**7.10G).** These trends are the same as seen with adult wings, supporting our conclusion that changes in *dpp* expression are responsible for changes in wing phenotypes.

Next, we examined wings and wing pouches of animals over-expressing *gbb* by *MS1096-Gal4* and *A9-Gal4* in a *hh<sup>MRT</sup>* background. We hypothesized that increased *gbb* levels will decrease ectopic expression of *dpp* in the *hh<sup>MRT</sup>* background, leading to a partial rescue of *hh<sup>MRT</sup>* phenotypes. We attempted to observe adult phenotypes in animals over-expressing *gbb* but blistering of the wing tissue with over-expression of *gbb* made adult pattern and phenotypes impossible to discern (data not shown). Instead, we observed expression of *dpp<sup>BS3.0</sup>lacZ* in the wing pouches of these animals.

Over-expression in the wing pouch did not result in a noticeable change in *dpp<sup>BS3.0</sup>lacZ* expression in either the endogenous expression domain of *dpp* or in the ectopically expressed *dpp<sup>BS3.0</sup>lacZ* in the anterior compartment (Fig **7.11A**). When *UAS-gbb* was driven either throughout the wing pouch by *MS1096-Gal4* (Fig **7.11A'**) or predominantly in the dorsal compartment of the wing pouch (Fig **7.11A''**) by *A9-Gal4*, *dpp<sup>BS3.0</sup>lacZ* expression remained at or near wildtype levels, consistent with our previous observations that over-expression of *gbb* does not strongly influence *dpp-lacZ* expression (Fig **2.5**). However, when we performed a more rigorous analysis of *dpp<sup>BS3.0</sup>lacZ* expression in these three genotypes, we did observe a change in expression of *dpp<sup>BS3.0</sup>lacZ* in response to *gbb* over-expression. We classified discs based on the amount of ectopic *dpp<sup>BS3.0</sup>lacZ* observed from the mild class, class I, that had no ectopic *dpp<sup>BS3.0</sup>lacZ* expression to the severe class, class IV, that had extensive ectopic *dpp<sup>BS3.0</sup>lacZ* expression in the anterior compartment (Fig **7.11B**, classification displayed in Fig **7.10E**). There is a statistically significant ( $p < 0.001$ ) shift in the distribution of wing pouch phenotypes to less severe classes in both *MS1096>gbb* and *A9>gbb* wing pouches. We conclude that increased *gbb* levels can partially rescue *hh<sup>MRT</sup>/+* wing pouch phenotypes.

## Appendix 7.7 – *gbb* copy number influences viability of *dpp<sup>d</sup>/dpp<sup>hr</sup>* animals.

### Rationale:

Both Dpp and Gbb are required for proper patterning of the wing imaginal disc (Bangi and Wharton, 2006; Khalsa et al., 1998; Ray and Wharton, 2001). To test if cooperatively between Dpp and Gbb occurs in other developmental contexts, *gbb* copy number was altered in animals with compromised *dpp* expression level. Our original experiments (described in **Table 2.1**) concluded that an increase in *gbb* copy number can exacerbate viability defects observed in various *dpp<sup>d</sup>/dpp<sup>hr</sup>* allelic combinations. To test if the converse is true, we tested the effect of decreasing *gbb* copy number on the viability of *dpp<sup>d</sup>/dpp<sup>hr</sup>* mutant backgrounds.

### Approach:

For the experiments in table 7.1:

Crosses were performed between either *dpp<sup>hr92</sup>cn bw/Sm6a*, *dpp<sup>hr27</sup>cn bw/Sm6a*, *dpp<sup>hr4</sup>cn bw/Sm6a*, or *dpp<sup>hr56</sup>cn bw/Sm6a* males and *dpp<sup>d6</sup>/CyO* or *dpp<sup>d5</sup>/CyO* virgin females to generate the following progeny classes:

$$dpp^d/dpp^{hr}cn\ bw \qquad dpp^d/Sm6a \qquad dpp^{hr}cn\ bw/CyO$$

Crosses were performed between either *dpp<sup>hr92</sup>cn bw/Sm6a*, *dpp<sup>hr27</sup>cn bw/Sm6a*, *dpp<sup>hr4</sup>cn bw/Sm6a*, or *dpp<sup>hr56</sup>cn bw/Sm6a* males and *dpp<sup>d6</sup>bw gbb<sup>1</sup>/Sm6a* or *dpp<sup>d5</sup>bw gbb<sup>1</sup>/SM6a* virgin females to generate the following progeny classes:

$$dpp^d\ bw\ gbb^1/dpp^{hr}cn\ bw \qquad dpp^d\ bw\ gbb^1/Sm6a \qquad dpp^{hr}cn\ bw/SM6a$$

For the experiments in table 7.2:

Crosses were performed between either *dpp<sup>d6</sup>/CyO* or *dpp<sup>d5</sup>/CyO* males and either *dpp<sup>hr27</sup>cn bw/Sm6a*, *dpp<sup>hr4</sup>cn bw/Sm6a*, or *dpp<sup>hr56</sup>cn bw/Sm6a* virgin females to generate the following progeny classes:

$$dpp^d/dpp^{hr}cn\ bw \qquad dpp^d/Sm6a \qquad dpp^{hr}cn\ bw/CyO$$

Crosses were performed between either *dpp<sup>d6</sup>bw gbb<sup>1</sup>/Sm6a* or *dpp<sup>d5</sup>bw gbb<sup>1</sup>/SM6a* males and either *dpp<sup>hr27</sup>cn bw/Sm6a*, *dpp<sup>hr4</sup>cn bw/Sm6a*, or *dpp<sup>hr56</sup>cn bw/Sm6a* virgin females to generate:

$$dpp^d\ bw\ gbb^1/dpp^{hr}cn\ bw \qquad dpp^d\ bw\ gbb^1/Sm6a \qquad dpp^{hr}cn\ bw/SM6a$$

For every cross, all progeny were scored. Viability was determined as:

the number of *dpp<sup>d</sup>/dpp<sup>hr</sup>* as a percentage of expected, which was the number of *dpp<sup>d</sup>/CyO*

the number of *dpp<sup>d</sup>gbb<sup>1</sup>/dpp<sup>hr</sup>* as a percentage of expected, which was the number of *dpp<sup>d</sup>gbb<sup>1</sup>/Sm6a*

## Results and Conclusions:

Preliminary viability experiments testing the effect of one copy of *gbb*<sup>1</sup> on viability of *dpp*<sup>d</sup>/*dpp*<sup>hr</sup> animals were performed by Erdem Bangi. He tested the following *dpp* allelic combinations with and without one copy of *gbb*<sup>1</sup>: *dpp*<sup>d5</sup>/*dpp*<sup>hr92</sup>, *dpp*<sup>d5</sup>/*dpp*<sup>hr27</sup>, *dpp*<sup>d5</sup>/*dpp*<sup>hr4</sup>, *dpp*<sup>d5</sup>/*dpp*<sup>hr56</sup>, and *dpp*<sup>d6</sup>/*dpp*<sup>hr4</sup> (Table 7.1). In each trial where the *dpp*<sup>d5</sup> allele was used, introduction of one copy of *gbb*<sup>1</sup> led to increased viability of *dpp*<sup>d</sup>/*dpp*<sup>hr</sup> progeny. In essence, viability of these animals was partially rescued. The degree of rescue and the specific viability percentages varied between genotypes, presumably due to variable severity of the particular *dpp*<sup>hr</sup> alleles employed, but the trend was consistent. The 0% viability in *dpp*<sup>d5</sup>/*dpp*<sup>hr92</sup> and *dpp*<sup>d5</sup>/*dpp*<sup>hr27</sup> progeny was increased to 24% and 40% with one copy of *gbb*<sup>1</sup>, respectively. *dpp*<sup>d5</sup>/*dpp*<sup>hr4</sup> also displayed a large increase in viability with the introduction of *gbb*<sup>1</sup>, from 10% to 79%. The change in *dpp*<sup>d5</sup>/*dpp*<sup>hr56</sup> animals was more subtle with *gbb*<sup>1</sup>, increasing from 64% to 80%, but viability was increased. In contrast, the viability of *dpp*<sup>d6</sup>/*dpp*<sup>hr4</sup> was unchanged compared to *dpp*<sup>d6</sup> *gbb*<sup>1</sup>/*dpp*<sup>hr4</sup> animals with both displaying 43% viability, suggesting that a *dpp*<sup>d6</sup> background is less sensitive to changes in *gbb* level than a *dpp*<sup>d5</sup> background.

Based on the results from this first round of viability experiments, we hypothesized that *dpp*<sup>d5</sup> and *dpp*<sup>d6</sup> may differ in their sensitivities to altered *gbb* level. As the initial experiments only tested *dpp*<sup>d6</sup> in combination with *dpp*<sup>hr4</sup>, we decided to repeat several of these viability experiments, including additional genomic combinations containing the *dpp*<sup>d6</sup> allele. We tested the following *dpp* allelic combinations with and without one copy of *gbb*<sup>1</sup>: *dpp*<sup>d5</sup>/*dpp*<sup>hr27</sup>, *dpp*<sup>d5</sup>/*dpp*<sup>hr4</sup>, *dpp*<sup>d5</sup>/*dpp*<sup>hr56</sup>, *dpp*<sup>d6</sup>/*dpp*<sup>hr27</sup>, *dpp*<sup>d6</sup>/*dpp*<sup>hr4</sup>, and *dpp*<sup>d6</sup>/*dpp*<sup>hr56</sup> (Table 7.2). For all crosses, the *dpp*<sup>hr</sup> allele was paternal. Unlike the previous experiment performed by Erdem, we did not observe any rescue in

| Genotype                                                                      | Viability | N    |
|-------------------------------------------------------------------------------|-----------|------|
| <i>dpp</i> <sup>d5</sup> / <i>dpp</i> <sup>hr92</sup>                         | 0%        | 694  |
| <i>dpp</i> <sup>d5</sup> <i>gbb</i> <sup>1</sup> / <i>dpp</i> <sup>hr92</sup> | 24%       | 635  |
| <i>dpp</i> <sup>d5</sup> / <i>dpp</i> <sup>hr27</sup>                         | 0%        | 315  |
| <i>dpp</i> <sup>d5</sup> <i>gbb</i> <sup>1</sup> / <i>dpp</i> <sup>hr27</sup> | 40%       | 941  |
| <i>dpp</i> <sup>d5</sup> / <i>dpp</i> <sup>hr4</sup>                          | 10%       | 532  |
| <i>dpp</i> <sup>d5</sup> <i>gbb</i> <sup>1</sup> / <i>dpp</i> <sup>hr4</sup>  | 79%       | 1654 |
| <i>dpp</i> <sup>d5</sup> / <i>dpp</i> <sup>hr56</sup>                         | 64%       | 1139 |
| <i>dpp</i> <sup>d5</sup> <i>gbb</i> <sup>1</sup> / <i>dpp</i> <sup>hr56</sup> | 80%       | 1926 |
| <i>dpp</i> <sup>d6</sup> / <i>dpp</i> <sup>hr4</sup>                          | 43%       | 1161 |
| <i>dpp</i> <sup>d6</sup> <i>gbb</i> <sup>1</sup> / <i>dpp</i> <sup>hr4</sup>  | 43%       | 1478 |

**Table 7.1: A decrease in *gbb* copy number partially rescues viability defects in *dpp* compromised animals.**

Shown is a series of viability experiments testing the effect of a reduction in *gbb* copy number, by introduction of one copy of *gbb*<sup>1</sup>, on the viability of *dpp*<sup>d5</sup>/*dpp*<sup>hr</sup> (**top four rows**) and *dpp*<sup>d6</sup>/*dpp*<sup>hr</sup> (**bottom row**) animals. Crosses were designed such that the *dpp*<sup>hr</sup> allele was supplied paternally. For each cross, all progeny were scored. The viability of *dpp*<sup>d</sup>/*dpp*<sup>hr</sup> and *dpp*<sup>d</sup> *gbb*<sup>1</sup>/*dpp*<sup>hr</sup> animals was calculated as a percentage of expected, either *dpp*<sup>d</sup>/*SM6a* or *dpp*<sup>d</sup> *gbb*<sup>1</sup>/*SM6a*, respectively. N represents the total number of animals counted for each experiment in all genotypes. **Top three rows:** In all cases tested, animals with one copy of *dpp*<sup>d5</sup> display a partial rescue in the percent viable progeny with the introduction of one copy of *gbb*<sup>1</sup>. While the absolute values varied between individual experiments, the trend was consistent. **Bottom row:** A reduction in *gbb* copy number does not affect the viability of *dpp*<sup>d6</sup>/*dpp*<sup>hr4</sup> animals and each genotype displayed 43% viability. This experiment was performed by Erdem Bangi.

viability of  $dpp^{d5}/dpp^{hr27}$  or  $dpp^{d5}/dpp^{hr4}$  animals when one copy of  $gbb^1$  was introduced but, instead, we observed a reduction in viability. The viability of  $dpp^{d5}/dpp^{hr27}$  animals was decreased from 116% to 87% after  $gbb$  was reduced and the viability of  $dpp^{d5}/dpp^{hr4}$  was reduced from 77% to 2% with  $gbb$  reduction. We did, however, observe increased viability of  $dpp^{d5} gbb^1/dpp^{hr56}$  animals compared to  $dpp^{d5}/dpp^{hr56}$ . In this genotype, viability was increased from 65% to 76% in  $gbb^1/+$  animals.

Despite the inconsistencies observed for  $dpp^{d5}$  containing progeny, our results for animals containing  $dpp^{d6}$  were more consistent with Erdem's initial observations. In all genetic combinations examined, the addition of one copy of  $gbb^1$  led to a reduction in the viability of  $dpp^{d6}/dpp^{hr}$  animals. The viability of  $dpp^{d6}/dpp^{hr27}$  animals was decreased from 107% to 44% with reduced  $gbb$  levels. In a  $dpp^{d6}/dpp^{hr4}$  background, reduced  $gbb$  decreased viability from 64% to 0%.  $dpp^{d6}/dpp^{hr56}$  animals displayed 16% viability which was decreased to 4% in  $dpp^{d6} gbb^1/dpp^{hr56}$  animals. In these experiments, a  $dpp^{d6}$  background is not insensitive to a reduction in  $gbb$  as Erdem observed but, rather, displays an exacerbation of the viability defect with reduced  $gbb$  levels.

Our initial observations concerning viability changes in  $dpp^{d5}$  compared to  $dpp^{d6}$  animals led us to speculate that the different  $dpp$  cis-regulatory regions remaining in these alleles may have a differential ability to negatively regulate  $dpp$  expression. While we still believe this to be true, we observed several inconsistencies between experiments that have led us to conclude that this particular viability assay may not be reliable. First, we noted inconsistencies between the two experiments described above on the effect that one copy of  $gbb^1$  has on  $dpp^{d5}/dpp^{hr}$  viability. One major difference in how these experiments were performed is the genotype of the parents. These experiments represent reciprocal crosses, so the different effects observed may be a paternal effect. We have since

| Genotype                    | Viability | N    |
|-----------------------------|-----------|------|
| $dpp^{d5}/dpp^{hr27}$       | 116%      | 263  |
| $dpp^{d5} gbb^1/dpp^{hr27}$ | 87%       | 387  |
| $dpp^{d5}/dpp^{hr4}$        | 77%       | 546  |
| $dpp^{d5} gbb^1/dpp^{hr4}$  | 2%        | 217  |
| $dpp^{d5}/dpp^{hr56}$       | 65%       | 1408 |
| $dpp^{d5} gbb^1/dpp^{hr56}$ | 76%       | 2128 |
| $dpp^{d6}/dpp^{hr27}$       | 107%      | 459  |
| $dpp^{d6} gbb^1/dpp^{hr27}$ | 44%       | 310  |
| $dpp^{d6}/dpp^{hr4}$        | 64%       | 1151 |
| $dpp^{d6} gbb^1/dpp^{hr4}$  | 0%        | 124  |
| $dpp^{d6}/dpp^{hr56}$       | 16%       | 1705 |
| $dpp^{d6} gbb^1/dpp^{hr56}$ | 4%        | 1570 |

**Table 7.2: A decrease in  $gbb$  copy number has a variable effect on the viability of  $dpp$  compromised animals.** Shown is a series of viability experiments testing the effect of a reduction in  $gbb$  copy number, by introduction of one copy of  $gbb^1$ , on the viability of  $dpp^{d5}/dpp^{hr}$  (top three rows) and  $dpp^{d6}/dpp^{hr}$  (bottom three rows) animals. Crosses were designed such that the  $dpp^{hr}$  allele was supplied maternally. For each cross, all progeny were scored and the viability of  $dpp^d/dpp^{hr}$  and  $dpp^d gbb^1/dpp^{hr}$  animals was calculated as a percentage of expected, either  $dpp^d/SM6a$  or  $dpp^d gbb^1/SM6a$ . N represents the total number of animals counted for each experiment in all genotypes. **Top three rows:** In  $dpp^{d5}/dpp^{hr27}$  and  $dpp^{d5}/dpp^{hr4}$  combinations, addition of one copy of  $gbb$  reduces viability of these animals. A slight rescue in viability is observed for  $dpp^{d5}/dpp^{hr56}$  animals with the introduction of one copy of  $gbb$ , from 65% to 76%. **Bottom three rows:** A reduction in  $gbb$  copy number exacerbates defects in viability of  $dpp^{d6}/dpp^{hr4}$  animals. For all  $dpp^{d6}$  allelic combinations examined, this trend was consistent.

performed a series of reciprocal crosses within a single experiment to test this (table **2.2**) which revealed that the parental genotypes do matter.

We also noted that the absolute viability values calculated vary widely between experiments. This is particularly apparent in the viability of  $dpp^{d5}/dpp^{hr27}$  and  $dpp^{d5}/dpp^{hr4}$  animals in the experiments described above. The initial experiment found that both of these allelic combinations have 0% viability, but when these crosses were repeated we observed 116% viability of  $dpp^{d5}/dpp^{hr27}$  animals and 77% viability of  $dpp^{d5}/dpp^{hr4}$  animals. While this may also reflect a paternal effect, there are several other factors that may influence our results including the particular genetic background used in these experiments, experimental error in scoring subtle eye phenotypes, and slight fluctuations in temperature. Many  $dpp^{hr}$  alleles are temperature sensitive (Wharton et al., 1996), so slight alterations in the temperature of the incubators used or of the fly room may influence our results. Despite inconsistencies, we do believe that *gbb* copy number can influence the viability of  $dpp^d/dpp^{hr}$  allelic combinations and that these observations represent a bona fide example of *dpp* negative feedback regulation. However, while our viability observations are intriguing and, this experimental system has proved to be an unreliable system in which to test *dpp* feedback regulation.

**Appendix 7.8 – The *dpp* cis-regulatory sequence contains many highly conserved binding sites for factors associated with BMP signaling and *dpp* regulation.**

**Rationale:**

Based on our observations that both *dpp*<sup>p10638</sup>*lacZ* and *dpp*<sup>BS3.0</sup>*lacZ* reporters display a transcriptional response to altered BMP signaling, we hypothesized that we could determine particular regions of the *dpp*<sup>BS3.0</sup>*lacZ* cis-regulatory sequence that are competent to mediate negative feedback regulation of *dpp*. As functionally relevant sequences are often highly conserved, we examined the *dpp* cis-regulatory sequence for consensus transcription factor binding sites and determined the conservation at each site.

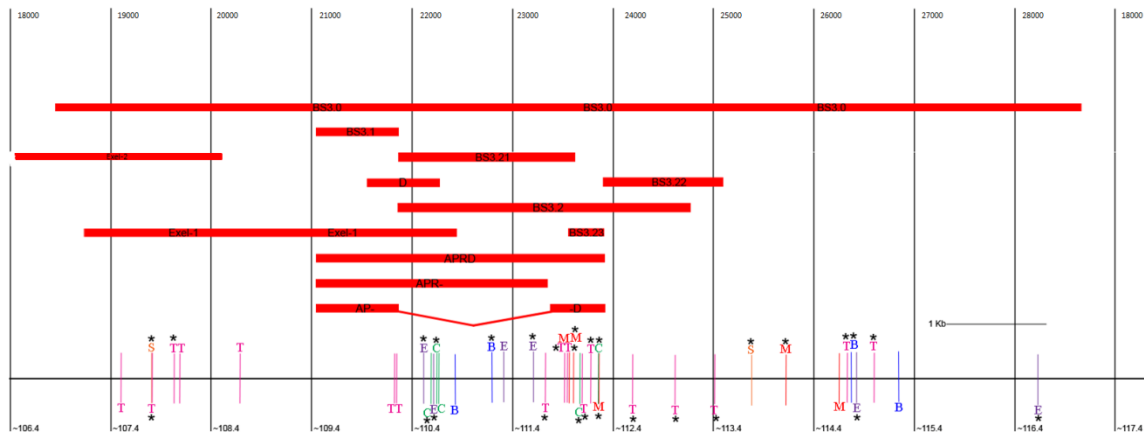
**Approach:**

*dpp* cis-regulatory sequence was examined for consensus binding sites for transcription factors known to be associated with BMP signaling (methods described in chapter three). Conservation at each site was determined using the UCSC genome browser, comparing sequences in this region between twelve *Drosophila* species, the mosquito (*A. gambiae*), the honeybee (*A. mellifera*), and the beetle (*A. castaneum*). Peaks represent conservation score, with high peaks representing areas of high conservation.

**Results and Conclusions:**

Examination of the *dpp*<sup>disk</sup> regulatory sequences revealed the presence of consensus binding sites for several transcription factors associated with BMP signaling, including En, Ci, Mad, Shn/pMad/Med, Brk, and dTCF (Fig 7.12, for details see chapter 2). Since regulatory sequences that control key developmental genes are often conserved, we examined the conservation of sites within *dpp*<sup>BS3.0</sup>*lacZ*. Using the UCSC genome browser (<http://genome.ucsc.edu/>), we examined the conservation of each of these potential binding sites (Kent et al., 2002). The peaks in each conservation graph represent the conservation score, with high peaks representing areas of high conservation. Conservation at each of these sites is displayed in figure 7.13-7.17, with the exception of the En sites, as these will not be addressed in this work. The Ci binding sites display high conservation at four of the five binding sites, with only the third binding site displaying low conservation (Fig 7.13). Of the five Mad binding sites identified, sites two, three, and four are highly conserved (Fig 7.14). Site three is of special concern as this site

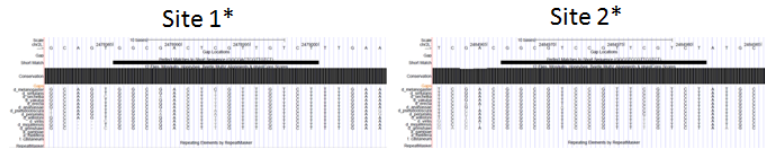
overlaps a Ci binding site and was directly tested by mutational analysis. Due to gaps in the genomes of several species at this location, the conservation of Mad site three appears to be lower than it actually is. Two Shn/pMad/Med consensus binding sites were identified, both of which display high conservation (Fig 7.15). Half of the Brk sites display high conservation (Fig 7.16). Finally, of the seventeen dTCF sites identified, all but four are highly conserved (Fig 7.17). The high conservation observed at most of these binding sites suggests that they may play important roles in regulating *dpp*.



**Figure 7.12. Map depicting *cis*-regulatory sequences contained within *dpp-lacZ* reporters tested and consensus binding sites for several transcription factors.** Shown is approximately 11kb of *dpp<sup>disk</sup>* regulatory region, 10.2kb of which is located within the *dpp<sup>BS3.0</sup>lacZ* reporter construct (Blackman et al., 1991). Locations of consensus binding sites for several transcriptional factors known to be associated with BMP signaling are marked: B is Brinker in blue (Sivasankaran et al., 2000; Zhang et al., 2001); C is Cubitus Interruptus in green (Kinzler and Vogelstein, 1990); T is *Drosophila* TCF/Pangolin in pink (Tetsu and McCormick, 1999; van de Wetering et al., 1997); E is Engrailed in purple (Desplan et al., 1988; Kissinger et al., 1990); M is Mad in red (Kim et al., 1997); and S is Shn/pMad/Med complex in orange (Gao et al., 2005; Pyrowolakis et al., 2004). Asterisks mark highly conserved binding sites (Figures 7.13-7.17). Above the map, the regions of the regulatory sequence contained within various reporter constructs tested for their expression response to increased BMP signaling. These reporter constructs include: *dpp<sup>BS3.1</sup>lacZ* (0.8kb), *dpp<sup>BS3.21</sup>lacZ* (1.8kb), *dpp<sup>BS3.22</sup>lacZ* (1.2kb), *dpp<sup>BS3.2</sup>lacZ* (2.9kb), *dpp<sup>BS3.23</sup>lacZ* (360bp) (Blackman et al., 1991; Hepker et al., 1999; Kopp et al., 1999), *dpp<sup>Exel-2</sup>lacZ* (approximately 4kb, precise endpoints unknown), *dpp<sup>exel1</sup>lacZ* (3.7kb) (Parks, 2004), *dpp<sup>D</sup>GFP* (0.7kb) (Johnson et al., 2008; Parker et al., 2011), *dpp<sup>APRD</sup>lacZ* (2.9kb, see materials and methods), *dpp<sup>APR</sup>lacZ* (2.3kb, see materials and methods), and *dpp<sup>AP-D</sup>lacZ* (815bp) (Theisen et al., 2007). The numbers at the bottom of the map denote the map locations based on a chromosomal walk performed on the *dpp* region (St Johnston et al., 1990).

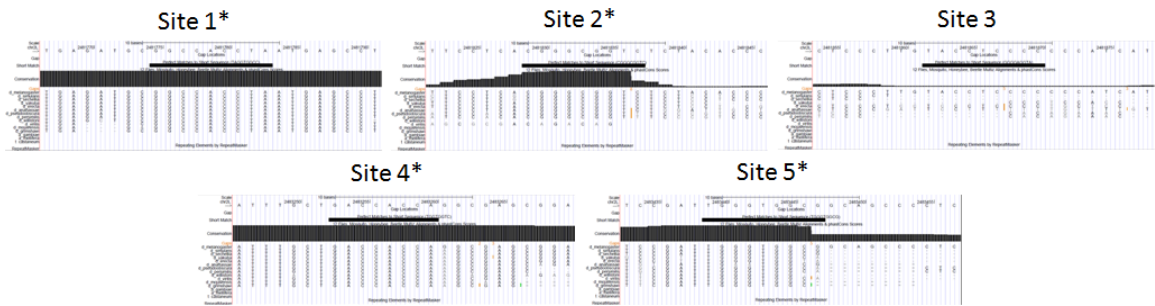


## Mad/Med/Shn binding sites



**Figure 7.13. Conservation of consensus Shn/pMad/Med binding sites within the *dpp*<sup>BS3.0</sup> *lacZ* control sequences.** Both sites are highly conserved. The sequences and locations of these sites are: site one, GGCGACTCGTTGTCT, found at 19403 of the *dpp*<sup>disk</sup> sequence in *cis*; site two, GGCGTCCGTTCGTCT, found at 25384 of the *dpp*<sup>disk</sup> sequence in *cis*. Locations within the *dpp* *cis*-regulatory sequences described in figures 7.13-7.16 are based on a sequence file called dppdisc.seq that contains 30,060bp of *dpp*<sup>disk</sup> regulatory sequence. This genomic sequence was obtained from the NCBI database, entry AE003583, in 2002. For reference, the first 20bp of this sequence are ATGTTGGATAAATTAATAGA and the last 20bp are AAAGATGGGCTTTCAGGGAT. Location numbers given in this work refer to the specific base pair location within this 30,060 base pair sequence.

## Ci binding sites



**Figure 7.14. Conservation of consensus Ci binding sites within the *dpp*<sup>BS3.0</sup> *lacZ* control sequences.** All but site three are highly conserved. The sequences and locations of these sites are: site one, GGCCACCTA, found at 22192 of the *dpp*<sup>disk</sup> sequence in *trans*; site two, CGGGCGGTC, found at 22246 of the *dpp*<sup>disk</sup> sequence in *cis*; site three, TACCTCCCC, found at 22279 of the *dpp*<sup>disk</sup> sequence in *trans*; site four, GACCACCA, found at 23670 of the *dpp*<sup>disk</sup> sequence in *trans*; site five, TGGGTGGCG, found at 23866 of the *dpp*<sup>disk</sup> sequence in *cis*.

## Mad binding sites



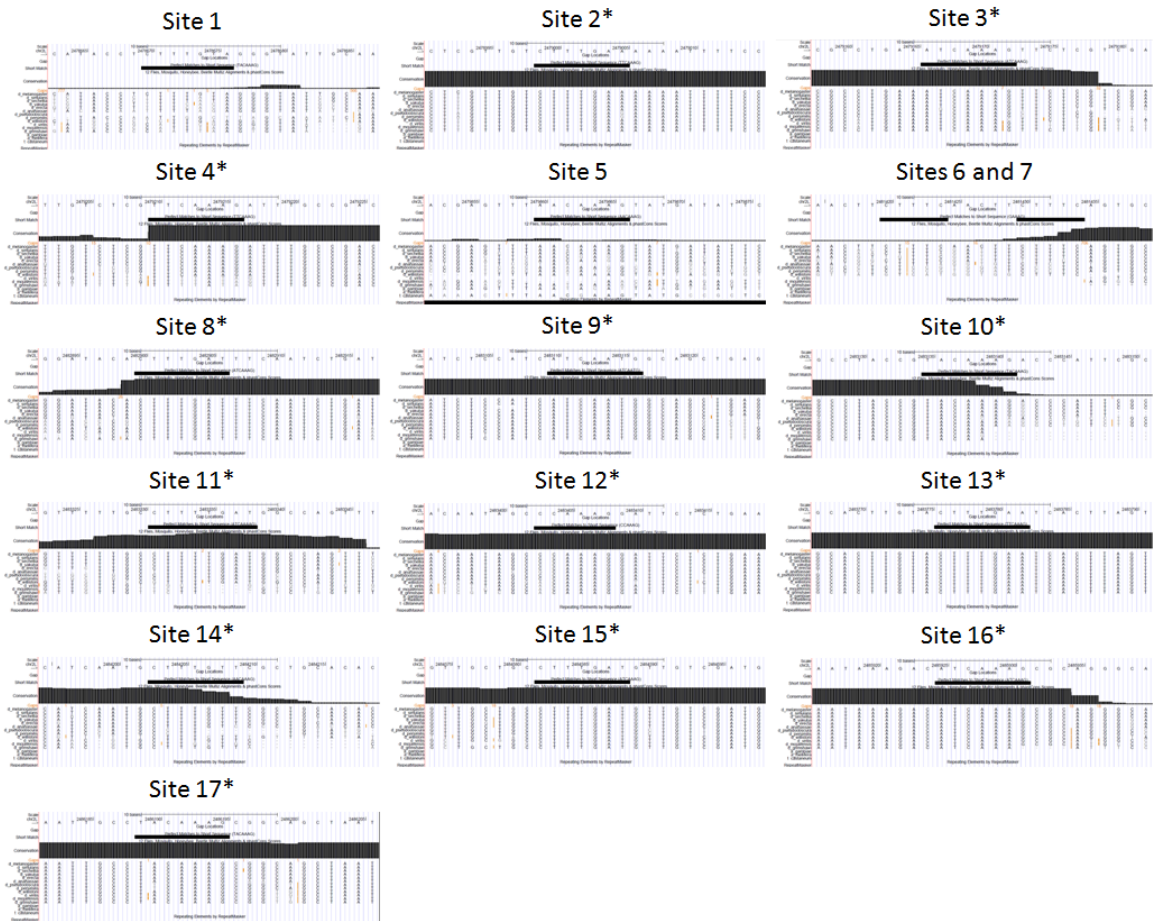
**Figure 7.15. Conservation of consensus Mad binding sites within the  $dpp^{BS3.0}lacZ$  control sequences.** All but sites one and five are highly conserved. The sequences and locations of these sites are: site one, GCCGTCT, found at 23571 of the  $dpp^{disk}$  sequence in *cis*; site two, GCCGTG, found at 23613 of the  $dpp^{disk}$  sequence in *cis*; site three, TGGCGGC, found at 23860 of the  $dpp^{disk}$  sequence in *trans*; site four, GCCCGCG, found at 25726 of the  $dpp^{disk}$  sequence in *cis*; site five, GCGGCGGC, found at 26248 of the  $dpp^{disk}$  sequence in *trans*.

## Brk binding sites



**Figure 7.16. Conservation of consensus Brk binding sites within the  $dpp^{BS3.0}lacZ$  control sequences.** Sites two and three are highly conserved. The sequences and locations of these sites are: site one, AACGCCA, found at 22440 of the  $dpp^{disk}$  sequence in *trans*; site two, AGGCGTC, found at 22808 of the  $dpp^{disk}$  sequence in *cis*; site three, TGGCGCT, found at 26369 of the  $dpp^{disk}$  sequence in *cis*; site four, AACGCCA, found at 26843 of the  $dpp^{disk}$  sequence in *trans*.

## dTCF binding sites



**Figure 7.17. Conservation of consensus dTCF binding sites within the *dpp*<sup>BS3.0</sup>*lacZ* control sequences.** All except sites one, five, six, and seven are highly conserved. The sequences and locations of these sites are: site one, TACAAAG, found at 19087 of the *dpp*<sup>disk</sup> sequence in *trans*; site two, TTCAAAG, found at 19416 of the *dpp*<sup>disk</sup> sequence in *trans*; site three, ATCAAAG, found at 19583 of the *dpp*<sup>disk</sup> sequence in *cis*; site four, TTCAAAG, found at 19627 of the *dpp*<sup>disk</sup> sequence in *cis*; site five, AACAAAG, found at 20277 of the *dpp*<sup>disk</sup> sequence in *cis*; site six, TTGAAAG, found at 21837 of the *dpp*<sup>disk</sup> sequence in *trans*; site seven, GACAAAG, found at 21847 of the *dpp*<sup>disk</sup> sequence in *trans*; site eight, ATCAAAG, found at 23317 of the *dpp*<sup>disk</sup> sequence in *trans*; site nine, ATCAATG, found at 23527 of the *dpp*<sup>disk</sup> sequence in *cis*; site ten, TACAAAG, found at 23552 of the *dpp*<sup>disk</sup> sequence in *cis*; site eleven, ATCAAAAG, found at 23748 of the *dpp*<sup>disk</sup> sequence in *trans*; site twelve, CCCAAAG, found at 23819 of the *dpp*<sup>disk</sup> sequence in *cis*; site thirteen, TTCAAAG, found at 24193 of the *dpp*<sup>disk</sup> sequence in *trans*; site fourteen, AACAAAG, found at 24620 of the *dpp*<sup>disk</sup> sequence in *trans*; site fifteen, ATCAAAG, found at 24999 of the *dpp*<sup>disk</sup> sequence in *trans*; site sixteen, ATCAAAG, found at 26342 of the *dpp*<sup>disk</sup> sequence in *cis*; site seventeen, TACAAAG, found at 26606 of the *dpp*<sup>disk</sup> sequence in *cis*.

## Appendix 7.9 – Many regions of the *dpp cis*-regulatory sequences play a role in negative feedback regulation of *dpp*

### Rationale:

Based on our observations that both *dpp<sup>p10638</sup>lacZ* and *dpp<sup>BS3.0</sup>lacZ* reporters display a transcriptional response to altered BMP signaling, we hypothesized that we could determine a smaller region of the *dpp cis*-regulatory sequence that is competent to mediate negative feedback regulation of *dpp*. To map these sequences to particular *dpp* control regions, we observed expression of *dpp* reporters constructed of smaller regions of *dpp cis*-regulatory sequence in wing imaginal pouches with wildtype and elevated levels of BMP signaling. The experiments presented in this section serve as supplementary information to chapter three.

### Approach:

Expression of *dpp-lacZ* reporters was examined in wildtype wing pouches and wing pouches with increased BMP signaling. BMP signaling was increased by expressing *UAS-tnv<sup>QD</sup>* under the control of *A9-Gal4* (expression pattern displayed in figure 7.1) or *ap-Gal4* (expression pattern displayed in figure 2.1).

The following crosses were performed to examine expression of the *dpp-lacZ* reporter lines noted:

*dpp<sup>BS3.22</sup>lacZ*: *A9-Gal4/Y*; *dpp<sup>BS3.22</sup>lacZ/+* males to *OR* wildtype females to generate *A9-Gal4/+*; *dpp<sup>BS3.22</sup>lacZ/+* larvae.

*dpp<sup>BS3.1</sup>lacZ* *ap-Gal4 UAS-GFP/+*; *dpp<sup>BS3.1</sup>/+* males to *UAS-tnv<sup>QD</sup>* females to generate *ap-Gal4 UAS-GFP/+*; *dpp<sup>BS3.1</sup>lacZ / UAS-tnv<sup>QD</sup>* larvae.

The following crosses were performed to examine expression of the *dpp-lacZ* reporter lines noted with increased BMP signaling:

*dpp<sup>BS3.21</sup>lacZ*: *A9-Gal4/+*; *dpp<sup>BS3.21</sup>lacZ/+* males to *OR* wildtype and *UAS-tnv<sup>QD</sup>* females to generate *A9-Gal4/+*; *dpp<sup>BS3.21</sup>lacZ / UAS-tnv<sup>QD</sup>* and *dpp<sup>BS3.21</sup>lacZ / UAS-tnv<sup>QD</sup>* larvae.

*dpp<sup>Exel-2-2</sup>lacZ*: *A9-Gal4/+*; *dpp<sup>Exel-2</sup>lacZ/+* males to *OR* wildtype and *UAS-tnv<sup>QD</sup>* females to generate *A9-Gal4/+*; *dpp<sup>Exel-2</sup>lacZ/+*; *UAS-tnv<sup>QD</sup> /+* and *dpp<sup>Exel-1</sup>lacZ/+*; *UAS-tnv<sup>QD</sup> /+* larvae.

*dpp<sup>Exel-2-3</sup>lacZ*: *A9-Gal4/+*; *dpp<sup>Exel-2</sup>lacZ/+* males to *OR* wildtype and *UAS-tnv<sup>QD</sup>* females to generate *A9-Gal4/+*; *dpp<sup>Exel-2</sup>lacZ / UAS-tnv<sup>QD</sup>* and *dpp<sup>Exel-1</sup>lacZ / UAS-tnv<sup>QD</sup>* larvae.

- dpp<sup>Exel-1</sup>lacZ*: A9-*Gal4/+* ; *dpp<sup>Exel-1</sup>lacZ/+* males to OR wildtype and *UAS-tkv<sup>QD</sup>* females to generate A9-*Gal4/+* ; *dpp<sup>Exel-1</sup>lacZ/+* ; *UAS-tkv<sup>QD</sup> /+* and *dpp<sup>Exel-1</sup>lacZ/+* ; *UAS-tkv<sup>QD</sup> /+* larvae.
- dpp<sup>BS3.23</sup>lacZ*: A9-*Gal4/+* ; *dpp<sup>BS3.23</sup>lacZ /+* males to OR wildtype and *UAS-tkv<sup>QD</sup>* females to generate A9-*Gal4/+* ; *dpp<sup>BS3.23</sup>lacZ /UAS-tkv<sup>QD</sup>* and *dpp<sup>BS3.23</sup>lacZ /UAS-tkv<sup>QD</sup>* larvae.
- dpp<sup>BS3.2</sup>lacZ*: A9-*Gal4/+* ; *dpp<sup>BS3.2</sup>lacZ /+* males to OR wildtype and *UAS-tkv<sup>QD</sup>* females to generate A9-*Gal4/+* ; *dpp<sup>BS3.2</sup>lacZ /UAS-tkv<sup>QD</sup>* and *dpp<sup>BS3.2</sup>lacZ /UAS-tkv<sup>QD</sup>* larvae.
- ap-Gal4 UAS-GFP/+* ; *dpp<sup>BS3.2</sup>lacZ /+* males to OR wildtype and *UAS-tkv<sup>QD</sup>* females to generate *ap-Gal4 UAS-GFP /+* ; *dpp<sup>BS3.2</sup>lacZ /UAS-tkv<sup>QD</sup>* and *dpp<sup>BS3.2</sup>lacZ /UAS-tkv<sup>QD</sup>* larvae.
- dpp<sup>APR</sup>lacZ*: *dpp<sup>APR</sup>lacZ/+* ; *UAS-tkv<sup>QD</sup> /+* males to *ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/ dpp<sup>APR</sup>lacZ ; UAS-tkv<sup>QD</sup> /+* and *ap-Gal4 UAS-GFP/ dpp<sup>APR</sup>lacZ* larvae.
- dpp<sup>AP-D</sup>lacZ*: *dpp<sup>AP-D</sup>lacZ /+* ; *UAS-tkv<sup>QD</sup> /+* males to *ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/ dpp<sup>AP-D</sup>lacZ ; UAS-tkv<sup>QD</sup> /+* and *ap-Gal4 UAS-GFP/ dpp<sup>AP-D</sup>lacZ* larvae.

The following crosses were performed to examine expression of *dpp<sup>BS3.2</sup>lacZ* in *tkv<sup>QD</sup>* over-expression FLP-out clones:

*y w/Y ; AyGal4 UAS-GFP/+* ; *dpp<sup>BS3.2</sup>lacZ* males to *y w f hsFLP122 ; UAS-tkv<sup>QD</sup>* and OR wildtype females to generate *y w f hs-FLP122/+* ; *AyGal4 UAS-GFP /+* ; *dpp<sup>BS3.2</sup>lacZ /UAS-tkv<sup>QD</sup>* and *AyGal4 UAS-GFP /+* ; *dpp<sup>BS3.2</sup>lacZ /+* larvae.

Crosses were pre-mated in glass vials and then moved to apple juice plates with yeast paste and brooded every 24 hours. To induce the expression of FLP recombinase, apple juice plates containing larvae were sealed and heat shocked at 37°C by submersion in a water bath for 10-15 minutes at either 48-72 or 54-76 hours post lay to generate early or mid second instar clones. Clones were identified by expression of GFP.

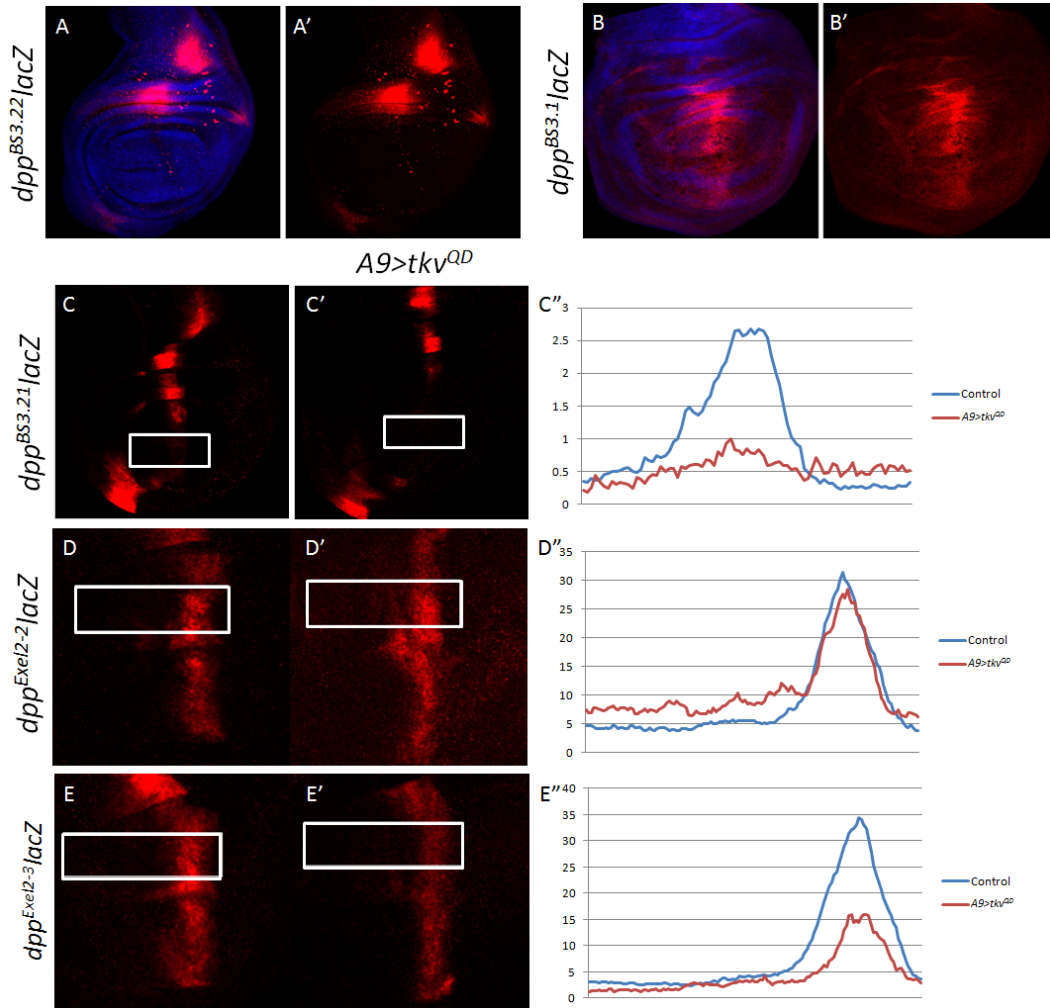
## Results and Conclusions:

To test particular regions of the *dpp* cis-regulatory sequence for a role in mediating negative feedback regulation of *dpp*, we obtained *dpp* reporter lines created with smaller pieces of control region than what is contained within *dpp*<sup>BS3.0</sup>*lacZ*. We examined their expression patterns in pouches with endogenous and elevated levels of BMP signaling. In all, twelve different regions of the *dpp* regulatory sequence were tested for their expression or response to increased BMP signaling. The sequences present within each of these reporters are displayed in figure 7.12 and these reporters include: *dpp*<sup>BS3.1</sup>*lacZ* (0.8kb), *dpp*<sup>BS3.21</sup>*lacZ* (1.8kb), *dpp*<sup>BS3.22</sup>*lacZ* (1.2kb), *dpp*<sup>BS3.2</sup>*lacZ* (2.9kb), *dpp*<sup>BS3.23</sup>*lacZ* (360bp) (Blackman et al., 1991; Hepker et al., 1999; Kopp et al., 1999), *dpp*<sup>Exel-2</sup>*lacZ* (approximately 4kb, precise endpoints unknown), *dpp*<sup>exel1</sup>*lacZ* (3.7kb) (Parks, 2004), *dpp*<sup>D</sup>*GFP* (0.7kb) (Johnson et al., 2008; Parker et al., 2011), *dpp*<sup>APRD</sup>*lacZ* (2.9kb, see materials and methods), *dpp*<sup>APR</sup>*lacZ* (2.3kb, see materials and methods), and *dpp*<sup>AP-D</sup>*lacZ* (815bp) (Theisen et al., 2007). In the below experiments, we present data and conclusions from each of the *dpp* reporter lines tested.

We were able to exclude several reporters from further study due to low expression in the wing pouch. These include *dpp*<sup>BS3.22</sup>*lacZ*, which is expressed in two bright spots in the notum but excluded from the wing pouch (Fig 7.18A), and *dpp*<sup>BS3.1</sup>*lacZ*, which displays a diffuse expression pattern at low levels in the wing pouch (Fig 7.18B). *dpp*<sup>BS3.21</sup>*lacZ* (Fig 7.18C), *dpp*<sup>Exel2-2</sup>*lacZ* (Fig 7.18D), and *dpp*<sup>Exel2-3</sup>*lacZ* (Fig 7.18E) all display weak expression in the wing pouch but since a weak stripe of expression is visible for all three of these, we tested them for their response to increased BMP signaling. We observed expression of these three reporter lines in wildtype wing pouches compared to *A9>tkv*<sup>QD</sup> wing pouches and, with the exception of *dpp*<sup>Exel2-2</sup>*lacZ*, a reduction in βGal levels was observed in response to increased BMP signaling (Fig 7.18C-E). The low wing pouch expression of these reporters can largely be explained by the absence of any Ci binding sites, as Ci plays an important role in regulating expression of *dpp* in the wing pouch. Only *dpp*<sup>BS3.21</sup>*lacZ* contains Ci binding sites.

*dpp*<sup>BS3.2</sup>*lacZ* contains all Ci binding sites but expression of this reporter was inconsistent and often weak. We also observed high background staining in several trials. Despite this, we tested the response of this reporter to increased BMP signaling in *A9>tkv*<sup>QD</sup> (Fig 7.19A) and *ap>tkv*<sup>QD</sup> (Fig 7.19B) wing pouches, as

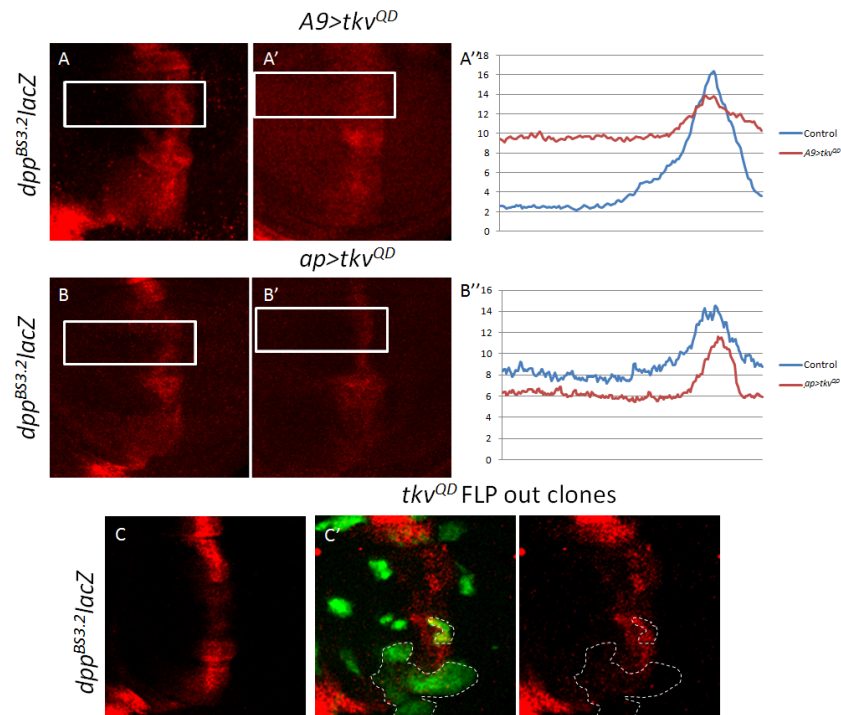
well as within  $tkv^{QD}$  FLP-out over-expression clones (Fig 7.19C). In comparison to control pouches, expression of  $dpp^{BS3.2}lacZ$  was reduced in response to  $tkv^{QD}$  controlled by  $A9-Gal4$  and  $ap-Gal4$ . FLP-out clones over-expressing  $tkv^{QD}$  also resulted in a reduction in  $dpp^{BS3.2}lacZ$  expression, but this effect was excluded from the cells at the dorsal-ventral boundary where  $dpp^{BS3.2}lacZ$  expression remained high.



**Figure 7.18. Several  $dpp-lacZ$  reporters express poorly in the wing imaginal disc.** Expression patterns of  $dpp^{BS3.22}lacZ$ ,  $dpp^{BS3.1}lacZ$ ,  $dpp^{BS3.21}lacZ$  and  $dpp^{Exel-2}lacZ$  were examined in the wing imaginal disc. Due to poor expression, these reporters were not used for further study. Third instar larvae were dissected and expression of reporters was visualized by anti- $\beta$ Gal antibody (in red). Nuclei are stained with Hoechst (in blue). **A.**  $dpp^{BS3.22}lacZ$  is expressed in two bright, diffuse spots in the notum but is not expressed at all in the wing pouch. **B.** Low and diffuse levels of  $dpp^{BS3.1}lacZ$  expression is seen in the wing pouch and notum. **C-D.** Expression of reporters in wildtype (left panel) and  $A9>tkv^{QD}$  (center panel) wing discs. **C.** Expression of  $dpp^{BS3.21}lacZ$  in wildtype (**C**, n=6) and  $A9>tkv^{QD}$  (**C'**, n=4) wing pouches. **C''.** Expression of  $dpp^{BS3.21}lacZ$  is observed in a stripe along the anterior-posterior boundary of the wing pouch, with lower levels in the wing pouch. Despite low initial expression, expression is further reduced with expression of  $UAS-tkv^{QD}$ . **D-E.** Expression patterns of two  $dpp^{Exel-2}lacZ$  lines. The exact 3' endpoint of the sequences used in the construction of this reporter is unknown. The 5' breakpoint is not shown on the map in figure 7.18. For both of these reporters, expression is weak and inconsistent along the anterior-posterior axis. **D.** Expression of  $dpp^{Exel-2-2}lacZ$  in wildtype (**D**, n=3) and  $A9>tkv^{QD}$  (**D'**, n=2) wing discs. Expression levels are unchanged with increased BMP signaling (**D''**). **E.** Expression of  $dpp^{Exel-2-3}lacZ$  in wildtype (**E**, n=4) and  $A9>tkv^{QD}$  (**E'**, n=2) wing discs. Expression levels are reduced with increased BMP signaling (**E''**).

The remainder of the *dpp-lacZ* reporter lines displayed stronger and more reliable expression patterns in the wing pouch. The behavior of *dpp<sup>Exel1</sup>lacZ* and *dpp<sup>BS3.23</sup>lacZ* was previously described in chapter two in *ap>tkv<sup>QD</sup>* wing pouches, but we also examined their expression in *A9>tkv<sup>QD</sup>* wing pouches. In response to *UAS-tkv<sup>QD</sup>* under control of *A9-Gal4*, expression of *dpp<sup>Exel1</sup>lacZ* expression is reduced throughout its expression domain (Fig 7.20A). Expression of *dpp<sup>BS3.23</sup>lacZ*, on the other hand, is reduced in all cells except at the intersection of the anterior-posterior and dorsal-ventral boundaries where a bright spot of expression persists (Fig 7.20B).

Finally, we examined two reporters received from the Marsh lab (Theisen et al., 2007), *dpp<sup>APR</sup>lacZ* and *dpp<sup>AP-D</sup>lacZ*, for their expression patterns in wildtype and *ap>tkv<sup>QD</sup>* wing pouches. This

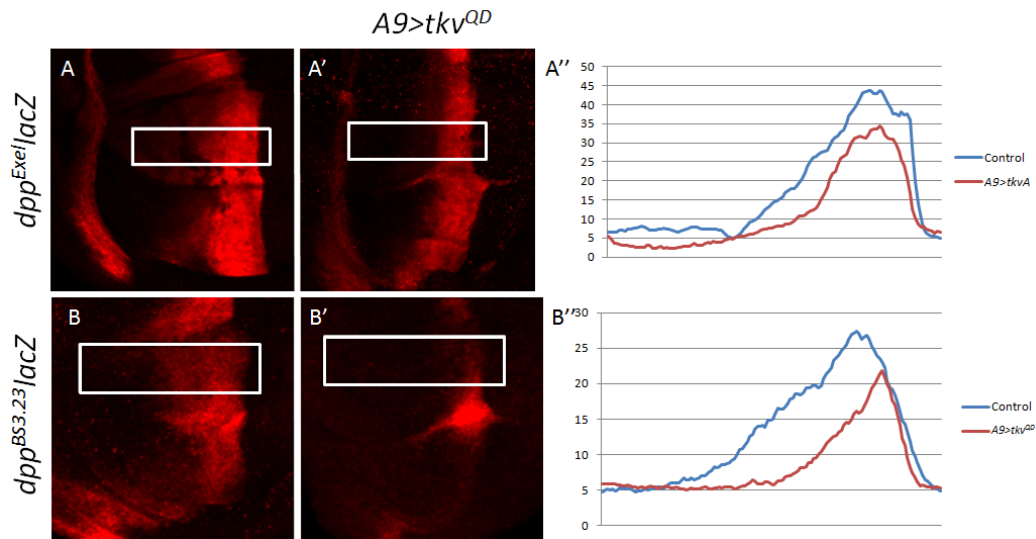


**Figure 7.19 Expression of *dpp<sup>BS3.2</sup>lacZ* is slightly reduced in response to increased BMP signaling.** Expression strength of this reporter was inconsistent, but expression was reduced in response to increased BMP signaling. **A-B.** Activated type I receptor, *tkv<sup>QD</sup>*, was over-expressed in the wing pouch using two *Gal4* drivers and expression of *dpp<sup>BS3.2</sup>lacZ* (in red) was examined. **A.** Reporter expression was observed in control (**A**, n=4) and *A9>tkv<sup>QD</sup>* (**A'**, n=4) wing pouches. **A''.** Expression of the reporter is reduced in response to increased BMP signaling, but high background staining made results difficult to interpret. **B.** Reporter expression was observed in control (**B**, n=2) and *ap>tkv<sup>QD</sup>* (**B'**, n=2) wing pouches. **B''.** Expression levels are decreased in response to BMP signaling, but high background staining made results difficult to interpret. **C.** FLP-out clones were generated during first larval instar to over-express *tkv<sup>QD</sup>* and *lacZ* expression was examined in third larval instar. *GFP*, in green, marks clone location. Wildtype expression of the reporter is shown in a pouch without clones (**C**). **C'.** Clones over-expressing both *UAS-GFP* and *UAS-tkv<sup>QD</sup>* display a decrease in *dpp<sup>BS3.2</sup>lacZ* expression away from the dorsal-ventral boundary.



$dpp^{APR}$ - $lacZ$  reporter is the original version we received and all other reference to  $dpp^{APR}$ - $lacZ$  in chapter three is a re-creation of this reporter that we constructed in the lab. Expression of  $dpp^{APR}$ - $lacZ$  is reduced throughout its expression domain in the dorsal compartment of  $ap>tkv^{QD}$  wing pouches (Fig 7.21A), resulting in expression within only a narrow stripe of cells along the anterior-posterior boundary. This reporter also displays positive regulation in the lateral regions of the wing pouch but, unlike others, this effect is mostly non cell-autonomous and observed in the cells contacting the dorsal compartment. Only a small patch of cell-autonomous up-regulation was observed in the posterior dorsal compartment (Fig 7.21B).  $dpp^{AP-D}$ - $lacZ$  also displays an altered expression pattern with increased BMP signaling (Fig 7.22). In  $ap>tkv^{QD}$  wing pouches, expression of  $dpp^{AP-D}$ - $lacZ$  is reduced in a subset of its expression domain (Fig 7.22A) and cell autonomously up-regulated at the dorsal-ventral boundary in both the anterior and posterior compartments (Fig 7.22B).

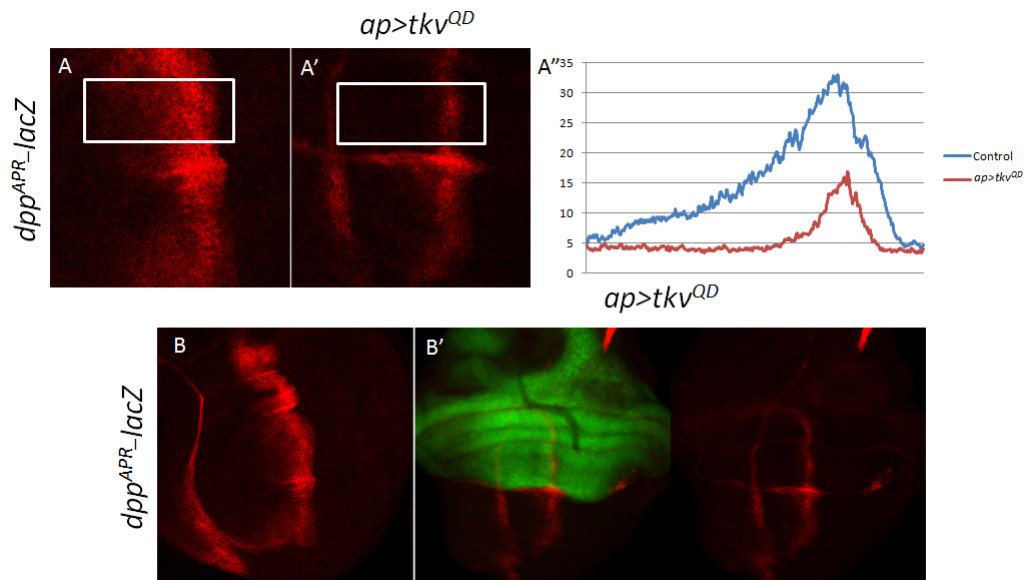
Through our examination of the reporters presented here and in chapter three, we observed a change in expression in each of the reporters tested. This change manifested itself as a reduction in expression of  $dpp$ - $lacZ$  within its endogenous expression domain, either throughout this domain or within



**Figure 7.20. Expression of  $dpp^{Exel}/lacZ$  and  $dpp^{BS3.23}/lacZ$  is reduced in response to increased BMP signaling.** Expression of  $dpp$ - $lacZ$  reporters (in red) were examined in control (left) and  $A9>tkv^{QD}$  (center) wing pouches. **A.** Expression of  $dpp^{Exel}/lacZ$  in control (A, n=2) and  $A9>tkv^{QD}$  (A', n=2) wing pouches. Expression of  $dpp^{Exel}/lacZ$  was reduced throughout its expression domain (A''). **B.** Expression of  $dpp^{BS3.23}/lacZ$  in control (B, n=5) and  $A9>tkv^{QD}$  (B', n=5) wing pouches. Expression of  $dpp^{Exel}/lacZ$  was reduced throughout its expression domain (A''), excluding the bright spot of expression at the dorsal-ventral boundary.

only a subset of it, and an up-regulation of *dpp-lacZ* in the lateral regions of the wing pouch, mostly confined to the cells closest to the dorsal-ventral boundary. As many of these reporters contain control sequences that are non-overlapping we conclude that multiple regions within the *dpp cis*-regulatory sequence can mediate negative feedback regulation of *dpp*.

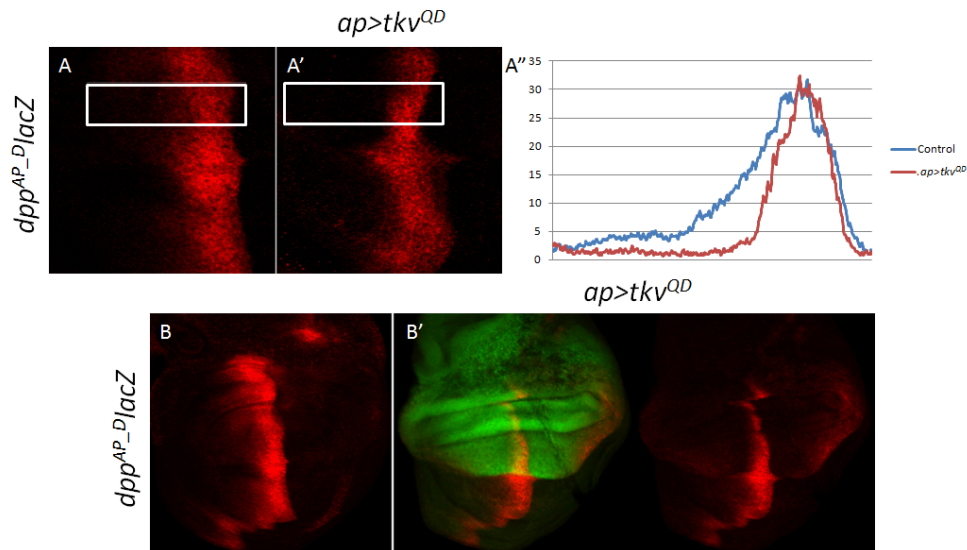
It is worth noting that our conclusions concerning negative regulation of *dpp<sup>BS3.23</sup>-lacZ* and *dpp<sup>BS3.2</sup>-lacZ* are in conflict to previously published results (Hepker et al., 1999). In the previous study, the authors examined the wildtype expression of each reporter and noted that *dpp<sup>BS3.23</sup>-lacZ* expression will fade towards the end of the third instar stage but *dpp<sup>BS3.2</sup>-lacZ* expression will remain strong. They then examined expression of both reporters in a *dpp<sup>d12</sup>/+* background and observed that expression of *dpp<sup>BS3.23</sup>-lacZ* proceeded normally and faded while expression of *dpp<sup>BS3.2</sup>-lacZ* now faded over time as well. In addition, they examined expression in wing discs expressing *UAS-dpp* under the control of *30A-Gal4*, a *Gal4* driver expressed predominately in the hinge region of the wing disc, and found that expression of *dpp<sup>BS3.23</sup>-lacZ* was unchanged while expression of *dpp<sup>BS3.2</sup>-lacZ* was increased. They concluded that



**Figure 7.21. Expression of *dpp<sup>APR</sup>-lacZ* is reduced in response to increased BMP signaling.** This reporter is the original *dpp<sup>APR</sup>-lacZ* received from H. Theisen (Theisen et al., 2007). All other reference to *dpp<sup>APR</sup>-lacZ* is a re-creation of this reporter that we constructed in the lab. Expression of this reporter was examined in control (A,B, n=1) and *ap>tkv<sup>QD</sup>* (A',B', n=1) wing pouches. In response to BMP signaling, expression of *dpp<sup>APR</sup>-lacZ* is reduced throughout its expression domain, resulting in a narrow stripe of cells expressing it. B. Expression of this reporter is up-regulated at the dorsal-ventral boundary in both the anterior and posterior compartments. In the anterior compartment, up-regulation is non-cell autonomous (B'), but in the posterior compartment there is a patch of cell autonomous up-regulation (B').

$dpp^{BS3.23}lacZ$  was insensitive to autoregulation and expression fades as development proceeds because of this.  $dpp^{BS3.2}lacZ$ , on the other hand, was concluded to be positively autoregulated by BMP signaling.

However, our results clearly indicate that expression of both  $dpp^{BS3.23}lacZ$  and  $dpp^{BS3.2}lacZ$  can be repressed in response to BMP signaling and they are, thus, subject to negative autoregulation. The discrepancies between the previous study and ours could be due to the methods used in these experiments. We used immunofluorescence and confocal microscopy to assay for expression of the reporter lines, a method that gives higher resolution than the X-Gal staining used in the published report. We have found that over-expression of  $dpp$  leads to only subtle change in  $dpp-lacZ$  expression (see chapter 2), which may explain why the previous study did not observe negative regulation of the reporters. Finally, the previous study did not take into account any spatial aspects of regulation. We have, indeed, noted that expression of  $dpp^{BS3.23}lacZ$  is insensitive to increased BMP signaling at the dorsal-ventral axis, but it clearly behaves quite differently in other regions of the wing disc. In addition, the previous study used the  $30A-Gal4$  driver in their experiments which expresses in the hinge. Given the domain-specific regulation of  $dpp-lacZ$  that we have observed, it is possible that  $dpp^{BS3.23}lacZ$  and  $dpp^{BS3.2}lacZ$  may be regulated differently in the hinge than in other regions of the wing disc.



**Figure 7.22 Expression of  $dpp^{AP-D}lacZ$  is reduced in response to increased BMP signaling.** Expression of this reporter was examined in control (A, B, n=1) and  $ap>tkv^{QD}$  (A', B', n=1) wing pouches. Intensity profiles in A'' were generated from the boxed regions in A and A'. In response to BMP signaling, expression of  $dpp^{AP-D}lacZ$  is reduced in a subset of its expression domain. B. Expression of this reporter is cell autonomously up-regulated at the dorsal-ventral boundary in both the anterior and posterior compartments in response to increased BMP signaling.

### Appendix 7.10 – Expression of *dpp<sup>BS3.23</sup>lacZ* and *dpp<sup>BS3.2</sup>lacZ* is expanded in *gbb* loss-of-function clones.

#### Rationale:

We know that expression of both *dpp<sup>BS3.23</sup>lacZ* and *dpp<sup>BS3.2</sup>lacZ* is decreased in response to increased BMP signaling. We hypothesized that, conversely, they might display an increase in expression in cells with reduced BMP function. Data from experiments with *dpp<sup>BS3.0</sup>lacZ* and *dpp<sup>p10638</sup>lacZ* indicated that a reduction in *gbb* levels could lead to increased expression of *dpp* reporters, but we had not yet tested reporters containing smaller regions of *dpp* control sequence.

#### Approach:

*gbb<sup>2</sup>* loss-of-function clones were generated and expression of *dpp-lacZ* was examined. These were generated by crossing:

*y w/Y ; FRT<sup>G13</sup>sha bw gbb<sup>2</sup>/+ ; dpp<sup>BS3.2</sup>lacZ/+* males to *y w f hfFLP122 ; FRT<sup>G13</sup> 2X-Ubi-nls-GFP/CyO-GFP* females to generate *y w f hfFLP122 ; FRT<sup>G13</sup>sha bw gbb<sup>2</sup>/FRT<sup>G13</sup> 2X-Ubi-nls-GFP ; dpp<sup>BS3.2</sup>lacZ* larvae

*y w/Y ; FRT<sup>G13</sup>sha bw gbb<sup>2</sup>/+ ; dpp<sup>BS3.23</sup>lacZ/+* males to *y w f hfFLP122 ; FRT<sup>G13</sup> 2X-Ubi-nls-GFP/CyO-GFP* females to generate *y w f hfFLP122 ; FRT<sup>G13</sup>sha bw gbb<sup>2</sup>/FRT<sup>G13</sup> 2X-Ubi-nls-GFP ; dpp<sup>BS3.23</sup>lacZ* larvae

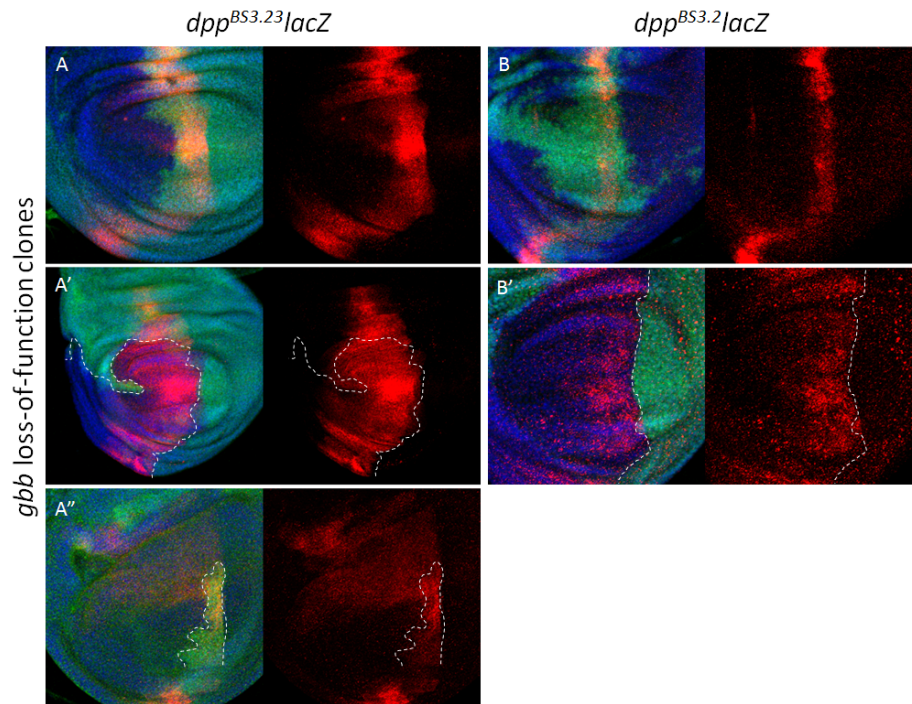
Crosses were pre-mated in glass vials and then moved to bottles and brooded every 24 hours. To induce the expression of FLP recombinase, bottles were heat shocked at 37°C by submersion in a water bath for 2 hours at 24-48 hours post lay to generate first instar clones. Larvae without *CyO-GFP* were selected and dissected as third instar crawlers. Clones were identified by the absence of GFP.

#### Results and conclusions:

Expression of both reporters was altered, but neither displayed an increase in expression higher than the peak of wildtype expression. When clones were large and covered the expression domain of the reporters and extended into the anterior compartment, the expression domain of each reporter was expanded into that clone (Fig 7.23A', A'', B''). This was not observed in wing pouches with clones located further into the anterior compartment (Fig 7.23A, B). In a clone that encompasses most of the anterior compartment with the exception of a thin stripe of cells along the anterior-posterior boundary of the

ventral compartment, expression of  $dpp^{BS3.23}lacZ$  is only expanded in the dorsal compartment (Fig 7.23A"). We conclude that in response to decrease BMP signaling, expression of  $dpp^{BS3.23}lacZ$  and  $dpp^{BS3.2}lacZ$  can be up-regulated in some cells of the wing pouch but this effect requires a large clone that both covers much of the anterior compartment and encompasses the width of the endogenous expression domain of the reporter.

This is in contrast to our observations with  $dpp^{BS3.0}lacZ$ , where a distinct cell autonomous up-regulation of reporter expression was observed in *gbb* loss-of-function clones. However, we did observe what appeared to be a slight expansion of the expression domains of  $dpp^{BS3.0}lacZ$  and  $dpp^{p10638}lacZ$ . We



**Figure 7.23. Decreased BMP signaling results in an expanded expression domain of  $dpp^{BS3.23}lacZ$  and  $dpp^{BS3.2}lacZ$ .** *gbb*<sup>2</sup> loss-of-function clones were generated (marked by the absence of GFP, in green) in first instar and expression of  $dpp^{BS3.23}lacZ$  (A) and  $dpp^{BS3.2}lacZ$  (B) was examined (in red) in third instar crawlers. Nuclei are marked by Hoechst and are displayed in blue. While none of these clones display an increase in expression level of either reporter, there is an expansion of the expression domain of the reporters in clones that are flush with the anterior-posterior boundary and encompass the *dpp-lacZ* expression domain, extending far into the anterior compartment. Clones are outlined in white. **A.** Expression of  $dpp^{BS3.23}lacZ$  in a clone that does not cover its endogenous expression domain (A), resulting in a wildtype expression pattern. In clones that cover most of the anterior compartment (A') or most of the wing pouch (A''), expression of  $dpp^{BS3.23}lacZ$  occurs in more cells than in wildtype. **B.** Expression of  $dpp^{BS3.2}lacZ$  is normal when clones do not cover its endogenous expression domain. A large clone that covers most of the anterior compartment (B') displays an expanded expression pattern of  $dpp^{BS3.2}lacZ$ . As previously mentioned, this reporter displays weak expression with high background.

were uncertain of this observation at the time, but in light of these observations of *dpp*<sup>BS3.23</sup>*lacZ* and *dpp*<sup>BS3.2</sup>*lacZ* it seems that a genuine, albeit slight, expansion of *dpp*<sup>BS3.0</sup>*lacZ* and *dpp*<sup>p10638</sup>*lacZ* occurs with decreased *gbb* levels.

### Appendix 7.11 – Expression of *dpp<sup>blk</sup>Gal4* is not reduced in response to increased BMP signaling

#### Rationale:

The same 3.7kb bamHI fragment used to create *dpp<sup>Exel-1</sup>lacZ* was used to create the *dpp<sup>blk</sup>Gal4* driver that was used in several of our ligand over-expression studies (Figures 7.1-7.3,7.5) (Staepling-hampton et al., 1994). In its role directing expression as a *Gal4* driver, this region of the *dpp<sup>disk</sup>* sequence is accepted to recapitulate the endogenous expression domain of *dpp*. Since, based on our observations of *dpp<sup>Exel-1</sup>lacZ*, these sequences do seem to respond to altered BMP levels, use of this driver in over-expression studies could give unexpected results from negative feedback regulation of the *Gal4* driver. We decided to test this *Gal4* driver to see if expression of it was altered with increased BMP signaling by observing GFP in *dpp<sup>blk</sup>>GFP* and *dpp<sup>blk</sup>>GFP, tkv<sup>QD</sup>* wing pouches.

#### Approach:

We observed GFP by direct immunofluoresence with and without increased BMP signaling by performing the following crosses:

*UAS-GFP* males to *dpp<sup>blk</sup>Gal4* females to generate *UAS-GFP/+ ; dpp<sup>blk</sup>Gal4/+* larvae

*UAS-GFP ; UAS-tkv<sup>QD</sup>* males to *dpp<sup>blk</sup>Gal4* females to generate *UAS-GFP/+ ; dpp<sup>blk</sup>Gal4/ UAS-tkv<sup>QD</sup>* larvae

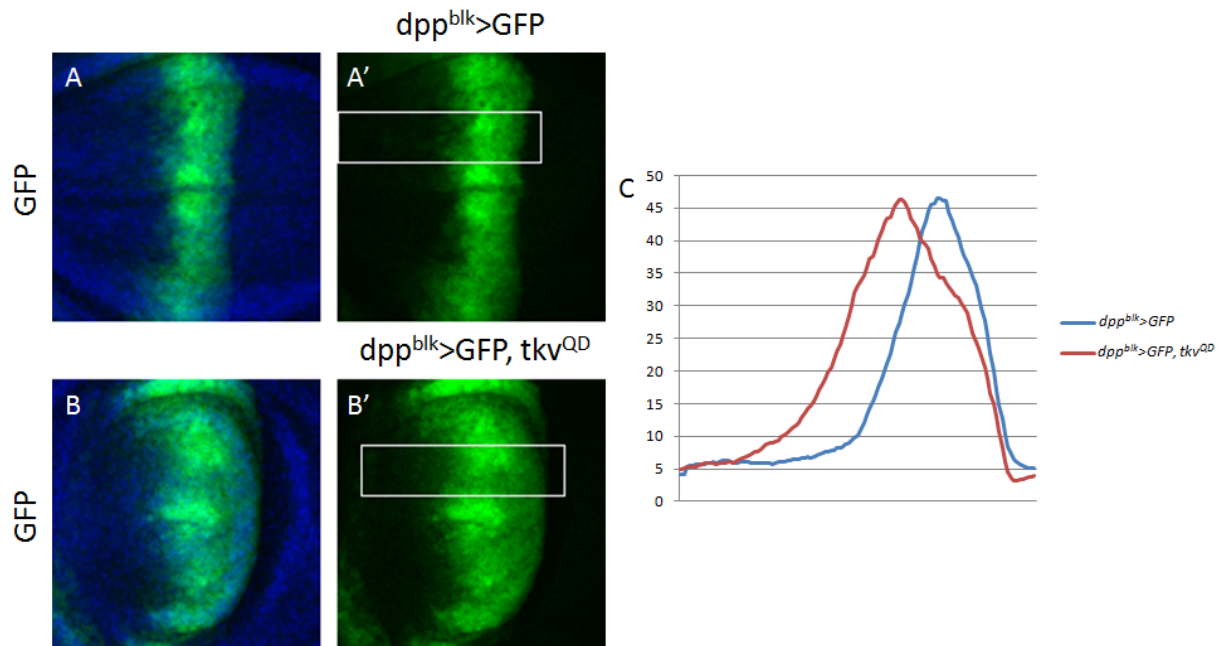
We observed GFP expression by direct immunofluoresence.

#### Results and conclusions:

Unlike *dpp<sup>Exel-1</sup>lacZ*, the expression level of *dpp<sup>blk</sup>Gal4* expression is not reduced with over-expression of *UAS-tkv<sup>QD</sup>* but instead displays an expansion of its expression domain. This observation of an expanded domain is consistent with our observations of *dpp<sup>p10638</sup>lacZ* in *dpp<sup>blk</sup>>tkv<sup>QD</sup>* wing pouches where we did see a similar results but also observed a reduction in the level of *dpp<sup>p10638</sup>lacZ* expression. It should be noted that *dpp<sup>Exel-1</sup>lacZ* expression has not been tested in *dpp<sup>blk</sup>>tkv<sup>QD</sup>* wing imaginal pouches. Based on GFP levels, we conclude that expression of *dpp<sup>blk</sup>-Gal4* is not being reduced in response to increased BMP signaling.

One reason we may not see a change in the expression of *GFP* could be due to cycling between episodes of expression of *dpp<sup>blk</sup>Gal4* and *UAS-tkv<sup>QD</sup>* followed by episodes of repression of *dpp<sup>blk</sup>Gal4* and

*UAS-tkv<sup>QD</sup>*. This feedback loop could make it difficult to observe a change in the expression of *UAS-GFP*. A second possibility could be due to differential stability of GFP compared to  $\beta$ Gal, the detection system used to observe expression of *dpp<sup>Exel-1</sup>lacZ*.



**Figure 7.24 Expression of the *dpp-Gal4*, *dpp<sup>blk</sup>Gal4* is not reduced in response to BMP signaling.** Expression of *UAS-GFP* driven by *dpp<sup>blk</sup>Gal4* was examined in wing pouches with and without over-expression of *UAS-tkv<sup>QD</sup>*. GFP is shown in green and Hoechst staining nuclei is shown in blue. Images were taken at the same confocal settings to allow for comparison of expression levels between pouches. Boxed areas in the wing pouches represent the areas used to generate intensity profiles as shown in the right panels. Intensity profiles displayed represent data an average of several pouches, as noted. **A.** GFP expression in a *dpp<sup>blk</sup>>GFP* wing pouch (n=4). **B.** GFP expression in a *dpp<sup>blk</sup>>GFP, tkv<sup>QD</sup>* (n=5) wing pouch. Hoechst staining reveals a deep posterior furrow along the anterior-posterior border. **C.** Intensity profiles reveal an expansion of the *GFP* expression domain with *tkv<sup>QD</sup>* over-expression without a reduction in expression levels.



## Appendix 7.12 – Ci binding sites are not required for negative feedback of *dpp* expression

### Rationale:

Considering the dual role that Ci plays in regulating *dpp*, we hypothesized that Ci could mediate negative transcriptional regulation of *dpp*. To test this, we obtained and generated *dpp-lacZ* reporter lines with mutated Ci binding sites that alter binding of Ci to the *cis*-regulatory sequences. The experiments presented in this section serve as supplementary information to experiments contained within chapter three.

### Approach:

*dpp* reporter lines analyzed:

We obtained three versions of the *dpp<sup>D</sup>GFP* reporters from S Barolo (Johnson et al., 2008; Parker et al., 2011) : *dpp<sup>D-WT</sup>GFP* with wildtype Ci binding sites (designated as *dpp<sup>D</sup>GFP* in chapter three), *dpp<sup>D-CiKO</sup>GFP* with mutant Ci binding sites that disrupt binding of Ci (designated as *dpp<sup>D-Ci3</sup>GFP* in chapter three), and *dpp<sup>D-CiHIAf</sup>GFP* with mutant Ci binding sites that create high affinity Ci binding sites. For each, we have two separate lines that were tested. We examined expression of all reporters in wildtype wing pouches and wing pouches with *UAS-tkv<sup>QD</sup>* expression driven by *MS1096-Gal4*, *A9-Gal4*, and *ap-Gal4*. The experiments for these reporters not presented in chapter three are shown here.

We generated *dpp cis*-regulatory sequences with wildtype and mutant Ci binding sites within the context of the *dpp<sup>APRD</sup>lacZ* and *dpp<sup>APR</sup>lacZ* sequences. Presented in this section is data for a reporter denoted as *dpp<sup>APRD-Ci3</sup>lacZ* which has only the first three Ci binding sites mutated. Construction of this reporter was created as described in the materials and methods in chapter three, with the exception of the step in which sites four and five were cloned into *dpp<sup>APRD-Ci3</sup>lacZ*. This step was not performed and *dpp* control sequence with the first three Ci binding sites mutated was cloned into *pHSG398-hsp-lacZ* and *pAttB*. Also included in this section is additional data for the reporters *dpp<sup>APRD</sup>lacZ*, *dpp<sup>APRD-Ci5</sup>lacZ*, *dpp<sup>APRD-Ci4-M</sup>lacZ*, *dpp<sup>APRD-Ci5-M</sup>lacZ*, *dpp<sup>APR</sup>lacZ*, and *dpp<sup>APR-Ci3</sup>lacZ* not included in chapter three.

Crossing schemes performed:

To increase BMP signaling in the presence of *dpp<sup>D</sup>GFP* reporters, we performed the following crosses:

Expression in *A9>tkv<sup>QD</sup>* wing pouches:

*A9-Gal4/Y ; dpp<sup>D-CiWT10</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate A9-Gal4/+ ; dpp<sup>D-</sup>*

*CiWT10*GFP/+ ; UAS-tkv<sup>QD</sup>/+ and *dpp<sup>D-CiWT10</sup>GFP/+ ; UAS-tkv<sup>QD</sup>/+ larvae*

*A9-Gal4/Y ; dpp<sup>WT30</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate A9-Gal4/+ ; dpp<sup>WT30</sup>GFP /UAS-*

*tkv<sup>QD</sup> and dpp<sup>D-CiWT10</sup>GFP / UAS-tkv<sup>QD</sup> larvae*

*A9-Gal4/Y ; dpp<sup>D-CiKO19</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate A9-Gal4/+ ; dpp<sup>D-CiKO19</sup>GFP*

*/+ ; UAS-tkv<sup>QD</sup>/+ and dpp<sup>D-CiWT10</sup>GFP/+ ; UAS-tkv<sup>QD</sup>/+ larvae*

*A9-Gal4/Y ; dpp<sup>D-CiKO59</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate A9-Gal4/+ ; dpp<sup>D-CiKO59</sup>GFP*

*/UAS-tkv<sup>QD</sup> and dpp<sup>D-CiKO59</sup>GFP / UAS-tkv<sup>QD</sup> larvae*

*A9-Gal4/Y ; dpp<sup>D-CiHIAf3</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate A9>tkv<sup>QD</sup> with dpp<sup>D-</sup>*

*CiHIAf3*GFP and UAS-tkv<sup>QD</sup> with *dpp<sup>D-CiHIAf3</sup>GFP* (note: insertion site of *dpp<sup>D-CiHIAf3</sup>GFP* is unknown, but

it is on the 2<sup>nd</sup> or 3<sup>rd</sup> chromosome)

*A9-Gal4/Y ; dpp<sup>D-CiHIAf50</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate A9>tkv<sup>QD</sup> with dpp<sup>D-</sup>*

*CiHIAf3*GFP and UAS-tkv<sup>QD</sup> with *dpp<sup>D-CiHIAf50</sup>GFP* (note: insertion site of *dpp<sup>D-CiHIAf50</sup>GFP* is unknown,

but it is on the 2<sup>nd</sup> or 3<sup>rd</sup> chromosome)

Expression in *MS1096>tkv<sup>QD</sup>* wing pouches:

*MS1096-Gal4/Y ; dpp<sup>D-CiWT10</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate MS1096-Gal4/+ ;*

*dpp<sup>D-CiWT10</sup>GFP/+ ; UAS-tkv<sup>QD</sup>/+ and dpp<sup>D-CiWT10</sup>GFP/+ ; UAS-tkv<sup>QD</sup>/+ larvae*

*MS1096-Gal4/Y ; dpp<sup>WT30</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate MS1096-Gal4/+ ;*

*dpp<sup>WT30</sup>GFP /UAS-tkv<sup>QD</sup> and dpp<sup>D-CiWT10</sup>GFP / UAS-tkv<sup>QD</sup> larvae*

*MS1096-Gal4/Y ; dpp<sup>D-CiKO19</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate MS1096-Gal4/+ ;*

*dpp<sup>D-CiKO19</sup>GFP /+ ; UAS-tkv<sup>QD</sup>/+ and dpp<sup>D-CiWT10</sup>GFP/+ ; UAS-tkv<sup>QD</sup>/+ larvae*

*MS1096-Gal4/Y ; dpp<sup>D-CiKO59</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate MS1096-Gal4/+ ;*

*dpp<sup>WT30</sup>GFP /UAS-tkv<sup>QD</sup> and dpp<sup>D-CiKO59</sup>GFP / UAS-tkv<sup>QD</sup> larvae*

*MS1096-Gal4/Y ; dpp<sup>D-CiHIAf3</sup>GFP /+ males to UAS-tkv<sup>QD</sup> females to generate MS1096>tkv<sup>QD</sup> with*

*dpp<sup>D-CiHIAf3</sup>GFP and UAS-tkv<sup>QD</sup> with dpp<sup>D-CiHIAf3</sup>GFP* (note: insertion site of *dpp<sup>D-CiHIAf3</sup>GFP* is

unknown, but it is on the 2<sup>nd</sup> or 3<sup>rd</sup> chromosome)

*MS1096-Gal4/Y ; dpp<sup>D-CiHIAf50</sup>GFP/+* males to *UAS-tkv<sup>QD</sup>* females to generate *MS1096>tkv<sup>QD</sup>* with *dpp<sup>D-CiHIAf3</sup>GFP* and *UAS-tkv<sup>QD</sup>* with *dpp<sup>D-CiHIAf50</sup>GFP* (note: insertion site of *dpp<sup>D-CiHIAf50</sup>GFP* is unknown, but it is on the 2<sup>nd</sup> or 3<sup>rd</sup> chromosome)

Expression in *ap>tkv<sup>QD</sup>* wing pouches

*dpp<sup>D-CiWT10</sup>GFP/+ ; UAS-tkv<sup>QD</sup>/+* males to *ap-Gal4/CyO-GFP* females to generate *ap-Gal4/ dpp<sup>D-CiWT10</sup>GFP ; UAS-tkv<sup>QD</sup>/+* and *ap-Gal4/ dpp<sup>D-CiWT10</sup>GFP* larvae.

*w/Y ; ap-Gal4/+ ; dpp<sup>D-CiWT30</sup>GFP/+* to *UAS-tkv<sup>QD</sup>* females to generate *ap-Gal4/+ ; dpp<sup>D-CiWT30</sup>GFP / UAS-tkv<sup>QD</sup>* and *dpp<sup>WT30</sup>GFP / UAS-tkv<sup>QD</sup>* larvae

*dpp<sup>D-CiKO19</sup>GFP/+ ; UAS-tkv<sup>QD</sup>/+* males to *ap-Gal4/CyO-GFP* females to generate *ap-Gal4/ dpp<sup>D-CiKO19</sup>GFP ; UAS-tkv<sup>QD</sup>/+* and *ap-Gal4/ dpp<sup>D-CiWT10</sup>GFP* larvae.

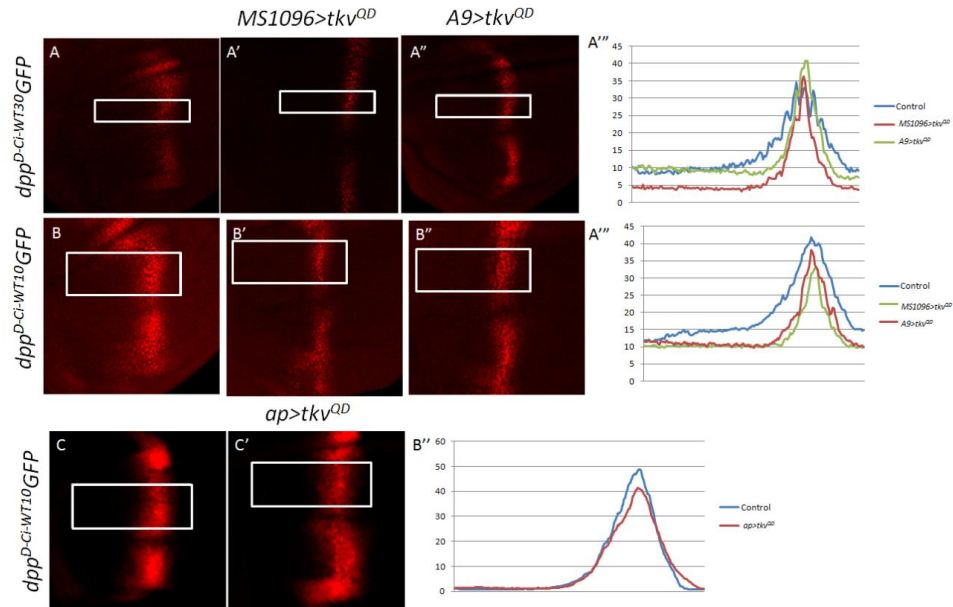
*w/Y ; ap-Gal4/+ ; dpp<sup>D-CiKO59</sup>GFP/+* to *UAS-tkv<sup>QD</sup>* females to generate *ap-Gal4/+ ; dpp<sup>D-CiKO59</sup>GFP / UAS-tkv<sup>QD</sup>* and *dpp<sup>WT30</sup>GFP / UAS-tkv<sup>QD</sup>* larvae

To increase BMP signaling in the presence of *dpp<sup>APRD</sup>lacZ* reporters, we performed the following crosses:

*w/Y ; ap-Gal4, UAS-GFP/+ ; dpp<sup>APRD</sup>lacZ/+* males to *UAS-tkv<sup>QD</sup>* and to *w* females to generate *ap-Gal4, UAS-GFP/+ ; dpp<sup>APRD</sup>lacZ / UAS-tkv<sup>QD</sup>* and *ap-Gal4, UAS-GFP/+ ; dpp<sup>APRD</sup>lacZ/+* larvae, respectively.

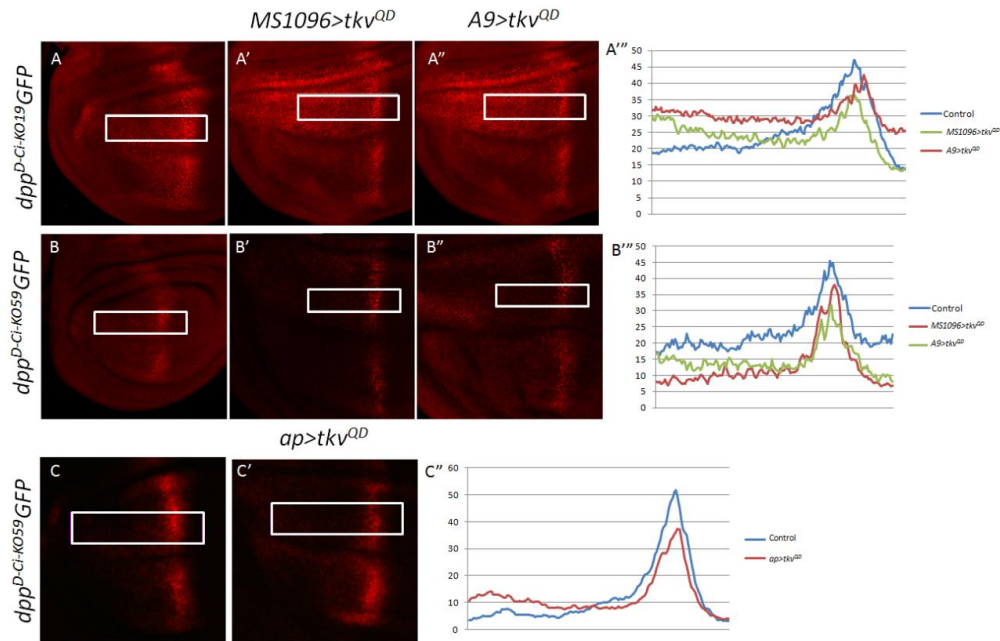
## Results and Conclusions:

To test a requirement for Ci binding in negative transcriptional feedback regulation of *dpp*, we examined the behavior of *dpp-lacZ* reporter lines with and without mutant Ci binding sites. This was performed within the context of three different *dpp* reporter lines containing different regions of the *dpp* cis-regulatory sequence. *dpp<sup>D</sup>GFP* contains 700bp of control sequence, within which is found the left cluster of Ci binding sites. *dpp<sup>APR</sup>lacZ* also contains the left cluster of Ci binding sites but this cluster is located within 2.3kb piece of control sequence. *dpp<sup>APRD</sup>lacZ* contains all five Ci binding sites within a 2.9kb section of the *dpp* cis-regulatory sequence. Many of our results with these reporters were described in chapter three but the material presented here contains additional experiments and versions of these reporters not included in the main text.



**Figure 7.25. Expression of  $dpp^{D-CiWT}$  GFP reporters is reduced in response to increased BMP signaling.** We observed expression of two transgenic lines of this reporter,  $dpp^{D-CiWT30}$  GFP (A) and  $dpp^{D-CiWT10}$  GFP (B-C), in pouches with and without increased BMP signaling. To increase BMP signaling, we over-expressed  $UAS-tkv^{QD}$  by  $MS1096-Gal4$  (A',B'),  $A9-Gal4$  (A'',B''), or  $ap-Gal4$  (C). Expression was observed by direct immunofluorescence of GFP (in red). A.  $dpp^{D-CiWT30}$  GFP expression was observed in control (A, n=1),  $MS1096>tkv^{QD}$  (A', n=1) and  $A9>tkv^{QD}$  (A'', n=1) wing pouches. A'''. Expression of the reporter is repressed in a subset of its expression domain in response to BMP signaling. B.  $dpp^{D-CiWT10}$  GFP expression was observed in control (B, n=3),  $MS1096>tkv^{QD}$  (B', n=1) and  $A9>tkv^{QD}$  (B'', n=1) wing pouches. B'''. Expression of the reporter is repressed in a subset of its expression domain in response to BMP signaling. C.  $dpp^{D-CiWT10}$  GFP expression was observed in control (C, n=1) and  $ap>tkv^{QD}$  (C', n=1) wing pouches. C''. Expression of the reporter is slightly repressed in response to BMP signaling.

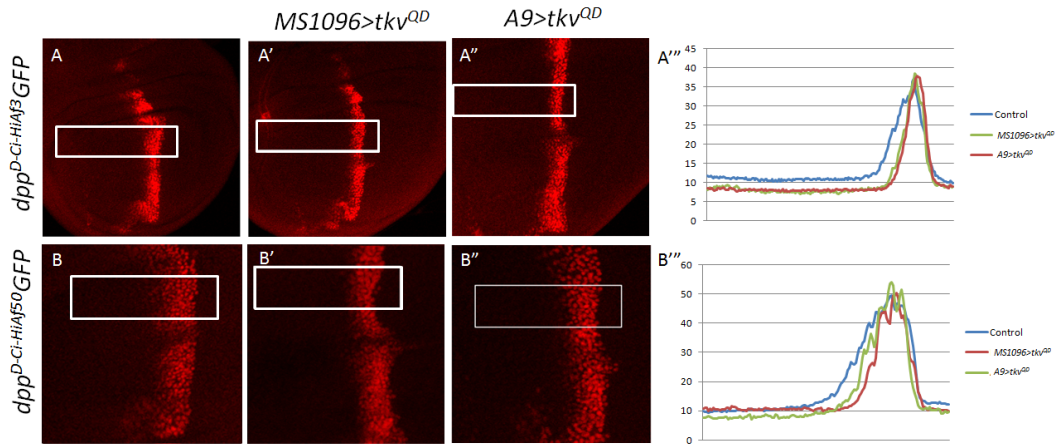
We received three versions of the  $dpp^D$  GFP  $dpp$  reporter construct from the Barolo lab with wildtype and altered Ci binding sites:  $dpp^{D-CiWT}$  GFP,  $dpp^{D-CiKO}$  GFP, and  $dpp^{D-CiH1A}$  GFP.  $dpp^{D-CiWT}$  GFP, referred to as simply  $dpp^D$  GFP in chapter three, contains three wildtype and functional Ci binding sites (Johnson et al., 2008; Parker et al., 2011). We examined expression of two independent insertions of this line, one called  $dpp^{D-CiWT10}$  GFP and one called  $dpp^{D-CiWT30}$  GFP. Both displayed similar expression patterns in the wildtype wing imaginal pouch of a bright and fairly narrow stripe of expression along the anterior-posterior axis of the wing pouch with a disruption in expression at the dorsal-ventral boundary (Fig 7.25A,B,C). In response to increased BMP signaling, accomplished by expressing  $UAS-tkv^{QD}$  under the control of  $MS1096-Gal4$  (Fig 7.25A',B'),  $A9-Gal4$  (Fig 7.25A'',B''), or  $ap-Gal4$  (Fig 7.25C), expression of both lines of  $dpp^{D-CiWT}$  GFP is reduced. In  $MS1096>tkv^{QD}$  and  $A9>tkv^{QD}$  animals, expression is reduced in all cells expressing the  $dpp^D$  GFP reporters, resulting in a narrowed expression domain (Fig 7.25A,B). Expression of  $tkv^{QD}$  by  $ap-Gal4$  resulted in a more subtle change in the expression of  $dpp^{D-CiWT10}$  GFP, but a reduction was



**Figure 7.26. Expression of  $dpp^{D-CiKO}$  GFP reporters along the anterior-posterior boundary is reduced in response to increased BMP signaling but increased in the lateral wing disc.** We observed expression of two transgenic lines of this reporter,  $dpp^{D-CiKO19}$  GFP (A) and  $dpp^{D-CiKO59}$  GFP (B,C), in pouches with and without increased BMP signaling. To increase BMP signaling, we over-expressed  $UAS-tkv^{QD}$  by  $MS1096-Gal4$  (A',B'),  $A9-Gal4$  (A'',B''), or  $ap-Gal4$  (C). Expression was observed by direct immunofluorescence of GFP (in red). **A.**  $dpp^{D-CiKO19}$  GFP expression was observed in control (A, n=3),  $MS1096>tkv^{QD}$  (A', n=3) and  $A9>tkv^{QD}$  (A'', n=3) wing pouches. **A'''**. Expression of the reporter is repressed in the medial wing pouch but up-regulated in the lateral regions. **B.**  $dpp^{D-CiKO59}$  GFP expression was observed in control (B, n=1),  $MS1096>tkv^{QD}$  (B', n=1) and  $A9>tkv^{QD}$  (B'', n=1) wing pouches. **B'''**. Expression of the reporter is repressed throughout its expression domain in response to BMP signaling. **C.**  $dpp^{D-CiKO59}$  GFP expression was observed in control (C, n=1) and  $ap>tkv^{QD}$  (C', n=2) wing pouches. **C'''**. Expression of the reporter is repressed in the medial wing pouch but up-regulated in the lateral regions.

observed (Fig 7.25C).  $dpp^{D-CiWT30}$  GFP expression in  $ap>tkv^{QD}$  is reduced compared to controls, as shown in figure 3.3A.

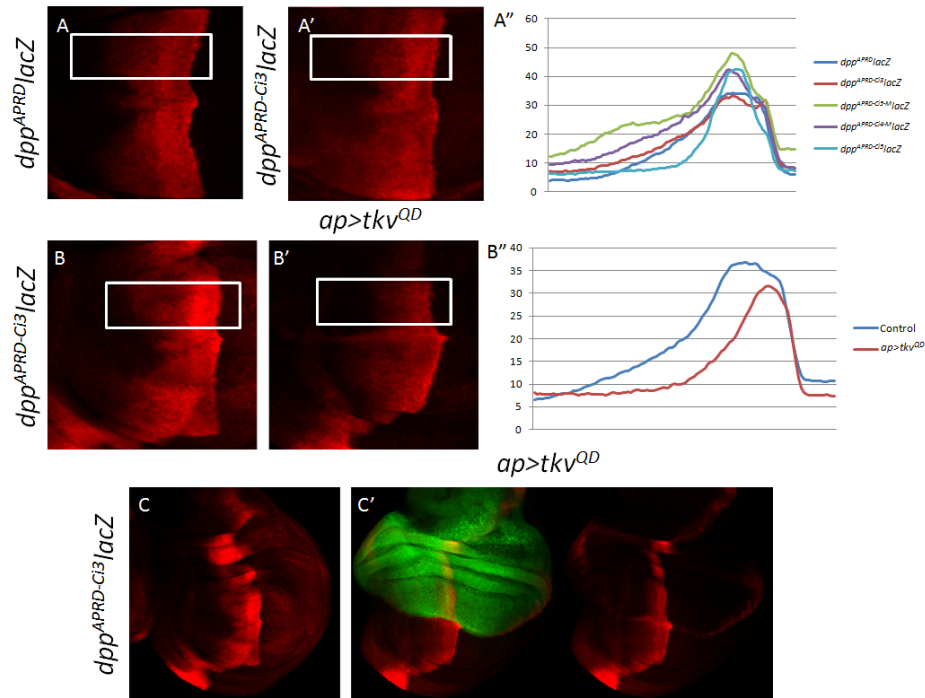
Consistent with published observations, mutation of Ci binding sites to non-functional sites within this stretch of *dpp* regulatory sequences gives an expression pattern that is expanded into the anterior compartment but expressed at lower levels than wildtype (Fig 7.26A,B,C). We observed this pattern in two lines of the reporter with non-functional Ci binding sites,  $dpp^{D-CiKO19}$  GFP (Fig 7.26A) and  $dpp^{D-CiKO59}$  GFP (Fig 7.26B,C). In wing pouches with increased BMP signaling, both of these reporters displayed an altered expression pattern. When  $tkv^{QD}$  was expressed under the control of  $MS1096-Gal4$  (Fig 7.26A',B'),  $A9-Gal4$  (Fig 7.26A'',B'') or  $ap-Gal4$  (Fig 7.26C') expression of either  $dpp^{D-CiKO}$  GFP reporter was reduced along and close to the anterior-posterior boundary. However, further from the anterior-posterior boundary we observed an up-regulation in reporter expression. Intensity profiles depicting GFP



**Figure 7.27. Expression of  $dpp^{D-CiHIAf}$  GFP reporters is slightly repressed in response to BMP signaling.** We observed expression of two transgenic lines of this reporter,  $dpp^{D-CiHIAf3}$  GFP (A) and  $dpp^{D-CiHIAf50}$  GFP (B), in pouches with and without increased BMP signaling. To increase BMP signaling, we over-expressed  $UAS-tkv^{QD}$  by  $MS1096-Gal4$  (A', B'), or  $A9-Gal4$  (A'', B''). Expression was observed by direct immunofluorescence of GFP (in red). **A.**  $dpp^{D-CiHIAf3}$  GFP expression was observed in control (A, n=3),  $MS1096>tkv^{QD}$  (A', n=2) and  $A9>tkv^{QD}$  (A'', n=2) wing pouches. **A'''**. Expression of the reporter is repressed in a subset of its expression domain in response to BMP signaling, generating an expression pattern that appears narrowed compared to control. **B.**  $dpp^{D-CiHIAf50}$  GFP expression was observed in control (B, n=3),  $MS1096>tkv^{QD}$  (B', n=1) and  $A9>tkv^{QD}$  (B'', n=1) wing pouches. **B'''**. Expression of the reporter is slightly repressed in a subset of its expression domain in response to BMP signaling.

levels throughout the anterior compartment reveal the increase in GFP expression further from the anterior-posterior boundary (Fig 7.26A''', B''', C'''). This is in stark contrast to either  $dpp^{D-CiWT}$  GFP reporter where an up-regulation in the lateral wing pouch was never observed. As these reporter lines display an altered expression pattern in response to BMP signaling, binding of Ci at these sequences must not be required for negative feedback regulation of *dpp*.

The third version of the  $dpp^D$  GFP reporter that we examined was  $dpp^{D-CiHIAf} lacZ$ , a version in which the three Ci binding sites have been mutated to be higher-affinity perfect matches to the consensus Gli binding site. These mutant sites were specifically designed to match the Ci binding sites found within the regulatory sequence for *ptc*, a higher threshold target of Ci than *dpp*. We examined two lines of this reporter,  $dpp^{D-CiHIAf3} lacZ$  and  $dpp^{D-CiHIAf50} lacZ$ , both of which are expressed in a narrower stripe of cells along the anterior-posterior boundary than the wildtype version of the reporter (Fig 7.27A, B). In response to increased BMP signaling in  $MS1096>tkv^{QD}$  (Fig 7.27A', B') and  $A9>tkv^{QD}$  (Fig 7.27A'', B'') wing pouches, there is a subtle change in the expression domain of  $dpp^{D-CiHIAf} lacZ$ , observed as a slightly narrowed pattern without a significant reduction in the expression level. This indicates that expression of these reporters does respond to negative feedback regulators of *dpp*, but they display reduced sensitivity



**Figure 7.28. Expression of  $dpp^{APRD-Ci3}lacZ$  is reduced in response to increased BMP signaling.** **A.** We examined wildtype expression of  $dpp^{APRD-Ci3}lacZ$  (**A'**, n=6, in red) compared to  $dpp^{APRD}lacZ$  wildtype reporter (**A**, n=5) and to our other reporter lines with mutated Ci and Mad binding sites (**A''**, refer to figure 3.2). Expression of  $dpp^{APRD-Ci3}lacZ$  is observed in a similar expression pattern as  $dpp^{APRD}lacZ$ . **B-C.** We observed expression of  $dpp^{APRD-Ci3}lacZ$  in wildtype and  $ap>tkv^{QD}$  wing pouches. **B.**  $dpp^{APRD-Ci3}lacZ$  expression was observed in control (**B**, n=10) and  $ap>tkv^{QD}$  (**B'**, n=6) wing pouches. **B''.** Expression of the reporter is repressed throughout its expression domain. **C.** Expression of this reporter is cell autonomously up-regulated at the dorsal-ventral boundary in both the anterior and posterior compartments in response to increased BMP signaling.

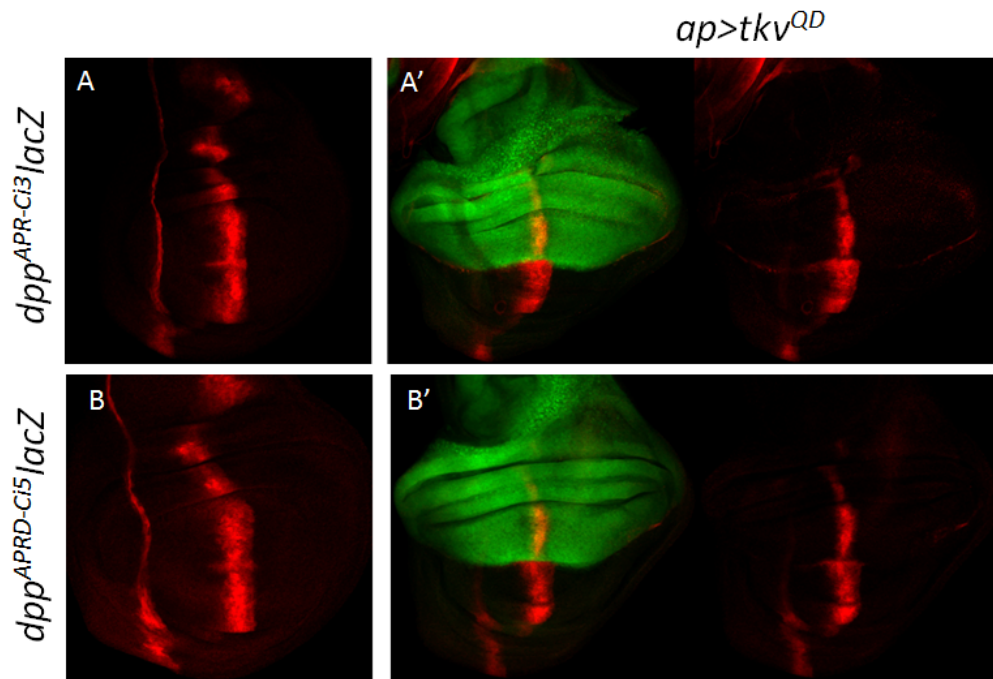
compared to the two other versions. This is most likely due to a higher sensitivity to the levels of  $Ci^A$  and  $Ci^R$  in the wing pouch than to the factor that alters expression of  $dpp$  in response to BMP signaling.

There are two other functional Ci binding sites within the  $dpp$  cis-regulatory region that we wanted to examine in regards to their potential involvement in negative feedback regulation of  $dpp$ . In addition, our sequence analysis had revealed that the intervening sequences between the Ci binding site clusters may play an important role in  $dpp$  regulation, so we wanted to specifically test those as well. To do this, we generated non-functional binding sites within  $dpp^{APRD}lacZ$  and  $dpp^{APR}lacZ$  reporter lines (see chapter three materials and methods). In addition to the reporter lines already described in chapter three, we also created a construct called  $dpp^{APRD-Ci3}lacZ$ , in which the first three Ci binding sites were mutant and the last two were wild type. We compared the expression pattern of this reporter to wildtype  $dpp^{APRD}lacZ$  and to the other versions of this reporter and observed it to be almost identical to the completely



wildtype version (Fig 7.28A), indicating that the two remaining Ci sites are sufficient to give wildtype expression of this reporter. In response to increased BMP signaling in  $ap>tkv^{QD}$  wing pouches, expression of  $dpp^{APR-Ci3}lacZ$  responds similarly as  $dpp^{APRD}lacZ$  as well. It is reduced throughout its expression domain (Fig 7.28B) and slightly up-regulated along the dorsal-ventral compartment boundary (Fig 7.28C).

In chapter three, we reported on positive regulation of several *dpp* reporter lines in response to increased BMP signaling. Of our new  $dpp^{APRD}lacZ$  and  $dpp^{APR}lacZ$  reporter series, most did display an up-regulation in  $ap>tkv^{QD}$  wing pouches (see figure 3.4). However, neither  $dpp^{APRD-Ci5}lacZ$  or  $dpp^{APR-Ci3}lacZ$  exhibited this behavior. Both of these reporter lines are expressed in a narrow stripe of cells along the anterior-posterior axis and do not display the graded expression pattern into the anterior compartment that the other versions of these reporters displayed. With increased BMP signaling, expression of both  $dpp^{APRD-Ci5}lacZ$  and  $dpp^{APR-Ci3}lacZ$  is reduced in its expression domain but there is only very faint or no up-regulation along the dorsal-ventral boundary (Fig 7.29).



**Figure 7.29.  $dpp^{APR-Ci3}lacZ$  and  $dpp^{APRD-Ci5}lacZ$  only display weak up-regulation in the lateral wing pouch in response to increased BMP signaling.** We observed expression of  $dpp^{APR-Ci3}lacZ$  (A) and  $dpp^{APRD-Ci5}lacZ$  (B) (in red) in wildtype and  $ap>tkv^{QD}$  wing pouches. GFP marking the *ap-Gal4* expression along domain is displayed in green. **A.** Expression of  $dpp^{APR-Ci3}lacZ$  is slightly up-regulated in a one-cell stripe along the dorsal-ventral compartment boundary in response to  $tkv^{QD}$  expression. **B.** Expression of  $dpp^{APRD-Ci5}lacZ$  is mostly wildtype in the lateral wing pouch in response to increased BMP signaling. A small spot of up-regulation is observed in the posterior compartment at the dorsal-ventral boundary.



### Appendix 7.13 – Ci protein is not required for negative feedback regulation of *dpp*

#### Rationale:

Our experiments testing the expression of *dpp* reporter lines with mutant Ci binding sites under endogenous and altered BMP signaling conditions suggested that binding of Ci to the *dpp* control regions is not necessary for negative feedback of *dpp* expression to occur. However, these experiments do not indicate that Ci is altogether not required. If Ci is playing a role, the distribution of Ci protein may change with altered BMP signaling. To test this, we observed Ci<sup>A</sup> protein in cells with decreased BMP signaling. To ask if Ci protein is absolutely required for negative feedback regulation of *dpp*, we removed Ci through use of a *UAS-ci-RNAi* construct and asked whether *dpp* expression would be repressed as BMP signaling levels were increased.

#### Approach:

Several approaches were taken to test a role for Ci in regulating *dpp* negative feedback regulation. To examine the distribution of Ci<sup>A</sup> protein in cells with reduced BMP signaling, we created *gbb* loss-of-function clones by crossing:

*y w f hs-FLP122/Y ; FRT<sup>G13</sup> sha gbb<sup>1</sup>/CyO-GFP* males to *w ; dpp<sup>p10638</sup> lacZ FRT<sup>G13</sup> 2X-GFP/CyO-GFP* virgin females to generate *y w f hs-FLP122/+ ; FRT<sup>G13</sup> sha gbb<sup>1</sup>/dpp<sup>p10638</sup> lacZ FRT<sup>G13</sup> 2X-GFP* larvae

Several *ci-RNAi* lines were tested for their efficiency in Ci knock-down by expressing the *RNAi* line and examining the resulting Ci<sup>A</sup> staining pattern. We tested: *UAS-ci-TRiP*, *UAS-ci-RNAi-V1* (from VDRC, transformant ID 105620), and *UAS-ci-RNAi-V2* (from VDRC, transformant ID 51479).

This was achieved by performing the following crosses to test the noted *UAS-ci-RNAi* lines:

*UAS-ci-RNAi-TRiP:* *UAS-ci-RNAi-TRiP* males to *w ; ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/+ ; UAS-ci-RNAi-TRiP/+* larvae

*UAS-ci-RNAi-V1:* *UAS-ci-RNAi-V1* males to *w ; ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/UAS-ci-RNAi-V1*

*UAS-ci-RNAi-V2:* *UAS-ci-RNAi-V2* males to *w ; ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/+ ; UAS-ci-RNAi-V2/+* larvae

To test the effect of loss of Ci on negative feedback of *dpp*, the following crosses were performed to examine expression of *dpp*<sup>BS3.0</sup>*lacZ*, *dpp*<sup>APRD</sup>*lacZ*, *dpp*<sup>APRD-Ci4-M</sup>*lacZ*, *dpp*<sup>APR</sup>*lacZ*, and *dpp*<sup>APR-Ci3</sup>*lacZ*:

*ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* males were crossed to *w* females to generate *ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* larvae

*ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* males were crossed to *w ; UAS-tnv<sup>QD</sup>* females to generate *ap-Gal4, UAS-GFP/+ ; dpp-lacZ/ UAS-tnv<sup>QD</sup>* larvae

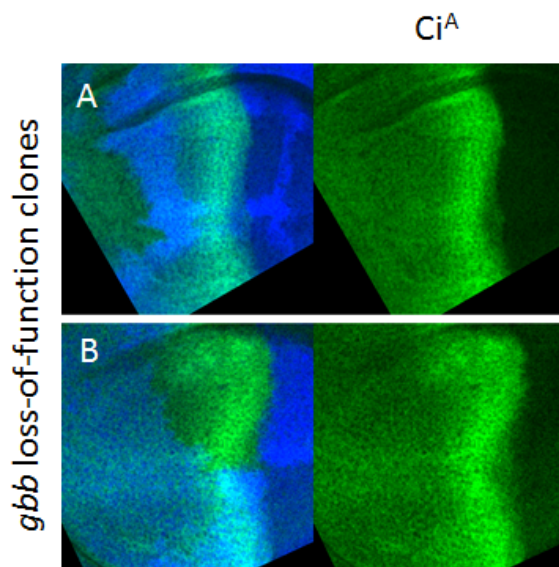
*ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* males were crossed to *w ; UAS-ci-RNAi* females to generate *ap-Gal4, UAS-GFP/ UAS-ci-RNAi ; dpp-lacZ/ +* larvae

*ap-Gal4, UAS-GFP/+ ; dpp-lacZ/+* males were crossed to *w ; UAS-ci-RNAi/+ ; UAS-tnv<sup>QD</sup>/+* females to generate *ap-Gal4, UAS-GFP/ UAS-ci-RNAi ; dpp-lacZ/ UAS-tnv<sup>QD</sup>* larvae

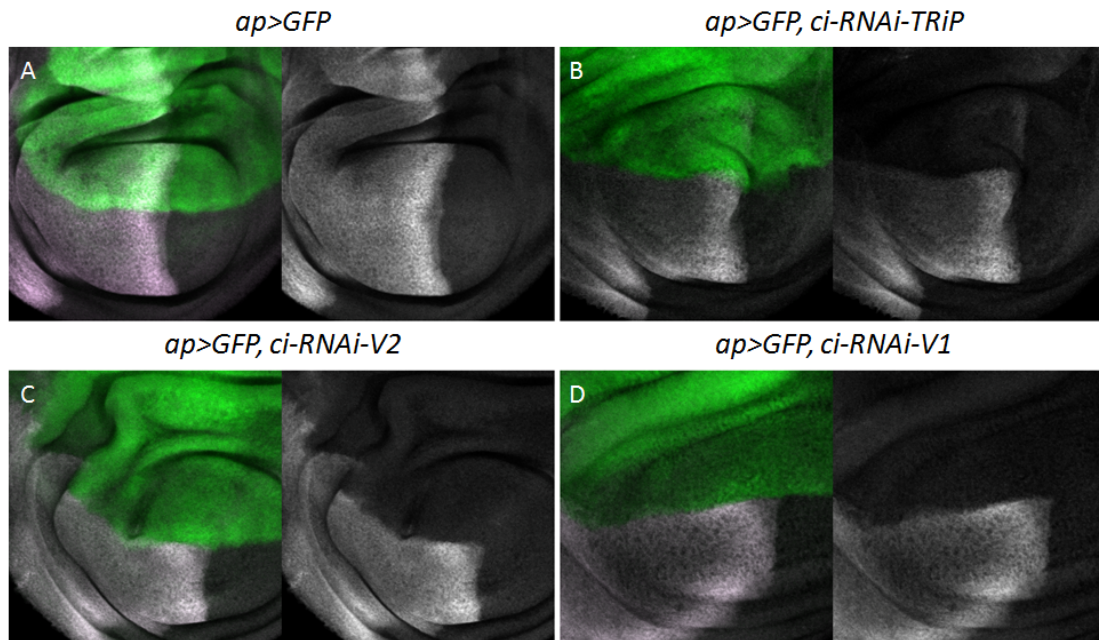
### Results and Conclusions:

In our experiments examining the distribution of Ci<sup>A</sup> protein with reduced BMP signaling, we were not able to observe a clear change in Ci<sup>A</sup> (Fig 7.30). Based on these experiments, it was unclear if Ci<sup>A</sup> was altered as staining did not appear completely wild-type in *gbb* loss-of-function clones but there was variability in the deviation from wild-type (Fig 7.30B). We cannot conclude that there is any change in Ci<sup>A</sup> with reduced BMP signaling. This is in contrast to cells with increased BMP signaling where a reproducible, albeit subtle, change in Ci<sup>A</sup> was observed.

To remove Ci protein from cells, we made use of available *UAS-ci-RNAi* lines. We obtained three



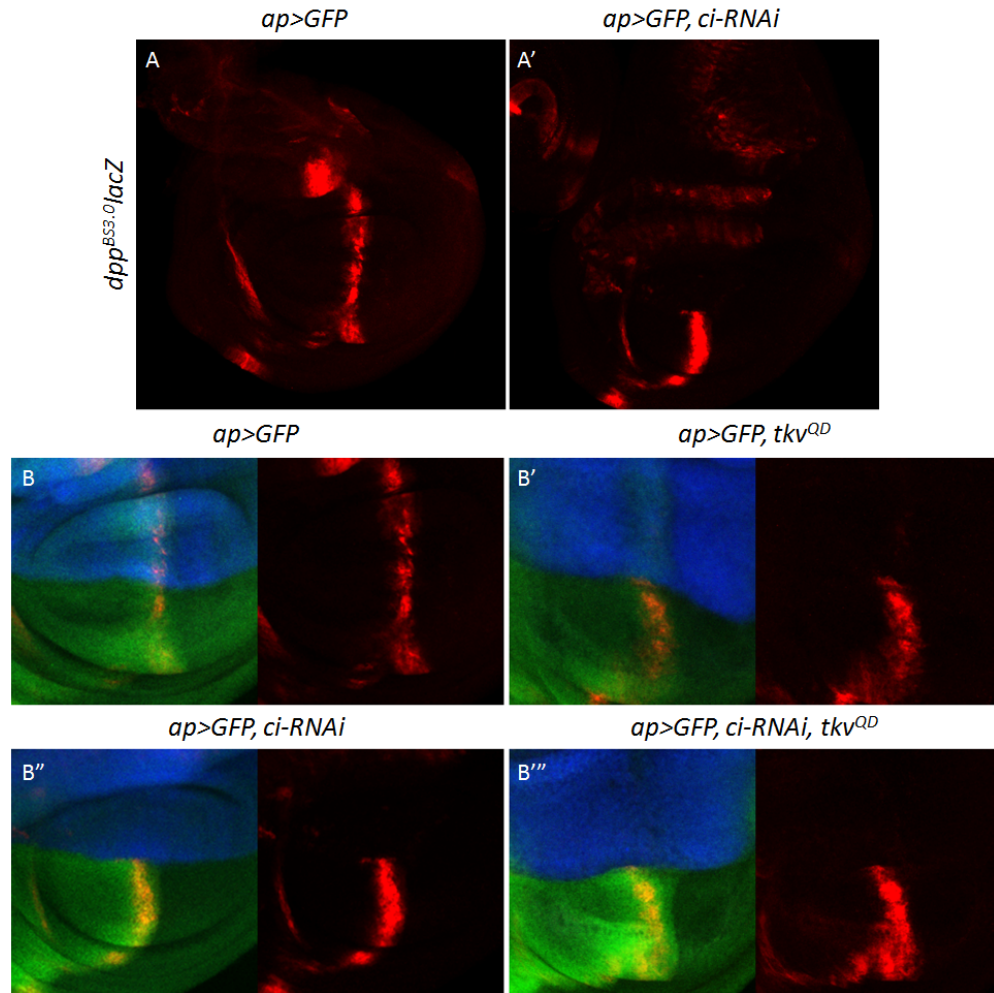
**Figure 7.30 – Ci<sup>A</sup> levels are mostly unchanged in *gbb* loss-of-function clones.** *gbb*<sup>2</sup> loss-of-function clones were generated during first instar and observed in wandering third instar larvae. Clones are marked by the absence of GFP (in blue). Shown in green is staining of Ci<sup>A</sup> protein. **A.** Control pouch with no clones crossing the domain of high Ci<sup>A</sup> levels. Ci<sup>A</sup> is high along the anterior-posterior boundary and low throughout the remainder of the anterior compartment. **B.** A *gbb*<sup>2</sup> loss-of-function clone covering the dorsal portion of the anterior-posterior boundary in the anterior compartment has a mostly wildtype Ci<sup>A</sup> pattern, with a possibly subtle change in its distribution or levels.



**Figure 7.31. Testing several *UAS-ci-RNAi* lines for their ability to knock-down Ci levels.** We received one RNAi line from the TRiP collection (B) and two lines from the VDRC, labeled V1 (C) and V2 (D). To test the efficiency of these lines, we expressed each line in the dorsal compartment of the wing pouch using *ap-Gal4* and examined  $Ci^A$  protein levels (in white). *UAS-GFP* was co-expressed (shown in green) to mark the *ap-Gal4* expression domain. **A.** Wildtype pattern of  $Ci^A$  in an *ap>GFP* wing pouch. **B.**  $Ci^A$  levels are reduced but not eliminated in *ap>GFP, ci-RNAi-TRiP* wing pouches. **C.**  $Ci^A$  levels are almost completely reduced in *ap>GFP, ci-RNAi-V2* wing pouches. **D.**  $Ci^A$  levels are almost completely reduced in *ap>GFP, ci-RNAi-V1* wing pouches.

such lines, one from the TRiP collection called *UAS-ci-RNAi-TRiP* and two from the VDRC called *UAS-ci-RNAi-V1* and *UAS-ci-RNAi-V2*. To determine the strength of these lines, we observed  $Ci^A$  in wing pouches expressing the line under the control of *ap-Gal4* (Fig 7.31) and adult phenotypes of animals from these crosses. *ap>ci-RNAi-TRiP* animals had reduced but not eliminated  $Ci^A$  protein in the dorsal compartment (Fig 7.31B), indicating that this line resulted in incomplete knock-down. *ap>ci-RNAi-V1* and *ap>ci-RNAi-V2*, on the other hand, led to an almost complete loss of  $Ci^A$  stain in the dorsal compartment (Fig 7.31C-D), with *ap>ci-RNAi-V1* displaying a more consistent loss of  $Ci^A$ . Adult phenotypes were consistent with  $Ci^A$  observations (date not shown). Progeny from all crosses displayed some pupal lethality, but the degree correlated with the strength of the RNAi. Wings in *ap>ci-RNAi-V1* flies were the most severe, displaying fluid-filled bubbles and blisters without any clear pattern, while *ap>ci-RNAi-TRiP* wings were less severe, displaying the held-out phenotype, smaller blisters, and the presence of clear vein pattern. In addition, all progeny had notum defects such as loss of bristles along the midline, ectopic lateral bristles, and reduced

or absent scutellum but the severity of these defects correlated with the severity of Ci reduction as observed with antibody staining. We conclude that *UAS-ci-RNAi-TRiP* is the least strong line, *UAS-ci-RNAi-V2* is a moderate line, and *UAS-ci-RNAi-V1* is the strongest line.



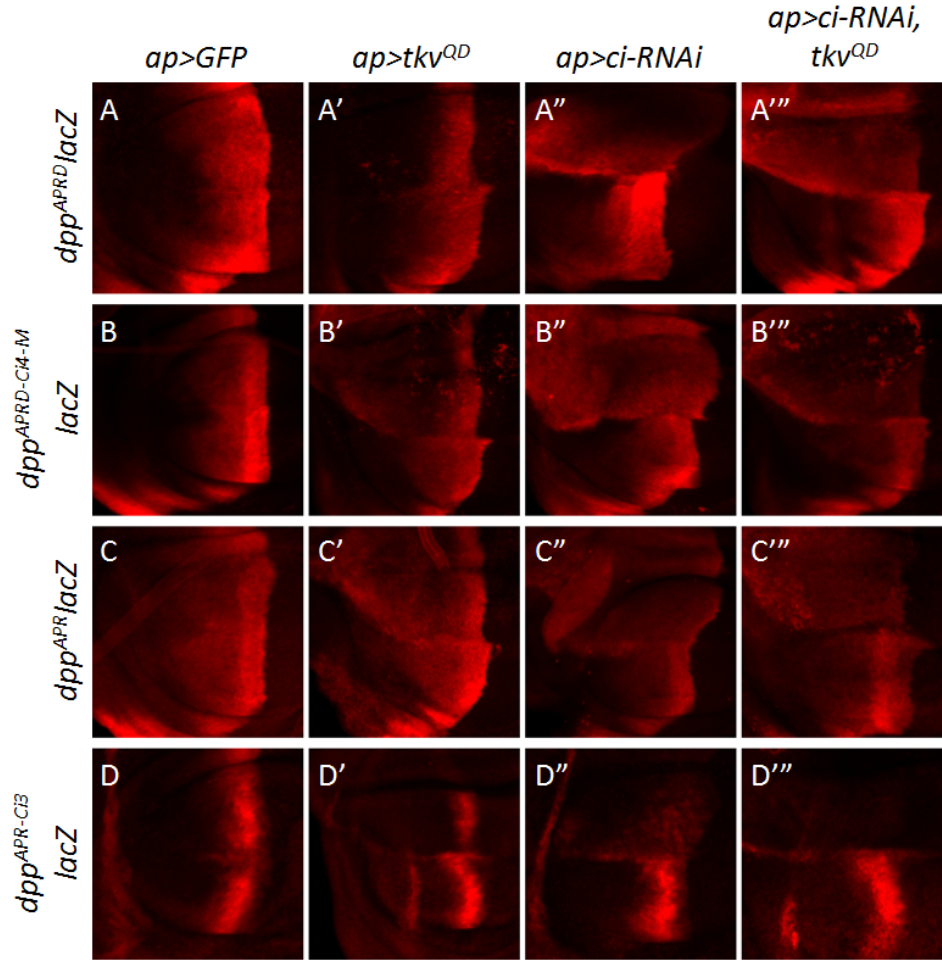
**Figure 7.32. Expression of  $dpp^{BS3.0} lacZ$  is lost in the cells of the wing pouch without Ci protein.** To test a requirement for Ci in regulating negative transcriptional feedback of *dpp*, we directed expression of *UAS-ci-RNAi-V1* with or without co-expression of *UAS-tkv<sup>QD</sup>* in the dorsal compartment of the wing pouch through *ap-Gal4* and monitored the expression of  $dpp^{BS3.0} lacZ$  (displayed in red).  $Ci^A$  stain is shown in green and *UAS-GFP* expression in blue marks the *ap-Gal4* expression domain. Expression of  $dpp^{BS3.0} lacZ$  is completely lost in the dorsal compartment of pouches without Ci. **A.** whole-pouch expression of  $dpp^{BS3.0} lacZ$  in control (**A**) and *ap>ci-RNAi* (**A'**) wing pouches. In the absence of Ci protein,  $dpp^{BS3.0} lacZ$  expression is expanded into the anterior notum but is lost from the wing pouch. **B.** A comparison of  $dpp^{BS3.0} lacZ$  expression in the wing pouch of *ap>GFP* control (**B**), *ap>GFP, tkv<sup>QD</sup>* (**B'**), *ap>GFP, ci-RNAi* (**B''**), and *ap>GFP, tkv<sup>QD</sup>, ci-RNAi* (**B'''**) wing pouches. In all but control pouches, expression of  $dpp^{BS3.0} lacZ$  is completely absent in the dorsal compartment.

Our initial attempts to test the requirement for Ci in regulating  $dpp^{BS3.0} lacZ$  were unsuccessful as, to our surprise, expression of this reporter was completely lost in the absence of Ci protein (Fig 7.32).

Whole wing disc images of *dpp<sup>BS3.0</sup>lacZ* in control (Fig 7.32A) and *ap>ci-RNAi-V1* (Fig 7.32A') revealed that, although expression of *dpp<sup>BS3.0</sup>lacZ* was expanded in the notum, it was not expressed in the wing pouch in the absence of Ci. Wing pouch images confirm that *dpp<sup>BS3.0</sup>lacZ* expression is completely lost in the dorsal compartment of *ap>tkv<sup>QD</sup>* (Fig 7.32B'), *ap>ci-RNAi* (Fig 7.32B''), and *ap>tkv<sup>QD</sup>, ci-RNAi* (Fig 7.32B''') wing pouches. Without a baseline of *dpp<sup>BS3.0</sup>lacZ* expression in the absence of Ci, this system cannot be used to address our concerns. Similar experiments could be performed with *dpp<sup>p10638</sup>lacZ* though (shown in figure 3.5) and they revealed that Ci protein is not required for negative feedback regulation of *dpp<sup>p10638</sup>lacZ*.

As a final test for a role for Ci in regulating *dpp*, we examined the expression of our *dpp<sup>APRD</sup>lacZ* and *dpp<sup>APR</sup>lacZ* reporter line series in the absence of Ci protein with and without increased BMP signaling. In addition to the *dpp<sup>APRD</sup>lacZ*, *dpp<sup>APRD-Ci5-M</sup>lacZ*, *dpp<sup>APRD-Ci5</sup>lacZ* lines examined in chapter three (Fig 3.9), we also analyzed expression of *dpp<sup>APRD-Ci4-M</sup>lacZ* (Fig 7.33B), *dpp<sup>APR</sup>lacZ* (Fig 7.33C), and *dpp<sup>APR-Ci3</sup>lacZ* (Fig 7.33D). The expression pattern for *dpp<sup>APRD</sup>lacZ* is included for reference (Fig 7.33A). As previously reported, expression of each of these reporter lines is reduced along the anterior-posterior boundary in response to expression of *UAS-tkv<sup>QD</sup>* under the control of *ap-Gal4* (Fig 7.33B',C',D'). When *ci-RNAi* was expressed in the dorsal compartment expression levels of all three reporters was reduced and expression was expanded well into the anterior compartment (Fig 7.33B'',C'',D''). In the case of *dpp<sup>APRD-Ci4-M</sup>lacZ* and *dpp<sup>APR</sup>lacZ* we observed uniform expression throughout the anterior compartment, while with *dpp<sup>APR-Ci3</sup>lacZ* expression in the anterior compartment was unevenly distributed and did not extend to the lateral regions of the wing pouch. This result indicates that the Ci protein must play a role in regulating expression of all three reporter lines, including *dpp<sup>APR-Ci3</sup>lacZ* that presumably does not bind Ci. Ci must either have an indirect role in regulating *dpp* or bind the *dpp<sup>APR</sup>lacZ* sequences at an unappreciated site.

We next tested the effect of both increasing BMP signaling and decreasing Ci protein on expression of *dpp<sup>APRD-Ci4-M</sup>lacZ*, *dpp<sup>APR</sup>lacZ*, and *dpp<sup>APR-Ci3</sup>lacZ*. In *ap>tkv<sup>QD</sup>, ci-RNAi* wing pouches, expression of *dpp<sup>APRD-Ci4-M</sup>lacZ* and *dpp<sup>APR</sup>lacZ* was still observed throughout the anterior compartment but at slightly reduced levels compared to *ap>ci-RNAi* wing pouches (Fig 7.33B''',C'''). Expression of *dpp<sup>APR-Ci3</sup>lacZ*, however, is almost completely lost in *ap>tkv<sup>QD</sup>, ci-RNAi* wing pouches (Fig 7.33D'''). We do not believe that there is necessarily a stronger change in expression of *dpp<sup>APR-Ci3</sup>lacZ* compared to *dpp<sup>APRD-Ci4-</sup>*



**Figure 7.33 Ci indirectly regulates *dpp* but is not required for negative feedback regulation of *dpp*.** We observed expression of *dpp*<sup>APRD</sup>*lacZ* (A), *dpp*<sup>APRD-Ci4-M</sup>*lacZ* (B), *dpp*<sup>APR</sup>*lacZ* (C), *dpp*<sup>APR-Ci3</sup>*lacZ* (D) in *ap>GFP* control pouches (A-D), *ap>tkv*<sup>QD</sup> pouches with increased BMP signaling (A'-D'), *ap>ci-RNAi* pouches without Ci (A''-D''), and *ap>GFP*, *tkv*<sup>QD</sup>, *ci-RNAi* pouches with increased BMP signaling and without Ci protein (A'''-D'''). For each reporter, images were taken at the same confocal settings to allow comparison of *lacZ* levels between genotypes. As previously observed, expression of all reporters is reduced in response to increased BMP signaling. Expression of all reporters was reduced along the anterior-posterior boundary and expanded into the anterior compartment in the absence of Ci protein, indicating that Ci plays a role in regulating the expression of these reporters. In pouches with both increased BMP signaling and reduced Ci proteins, expression of *dpp-lacZ* is further reduced from *ap>ci-RNAi* pouches indicating that neither Ci binding or Ci protein is required for negative feedback regulation of *dpp*.

*M**lacZ* and *dpp*<sup>APR</sup>*lacZ* through. For each reporter, we must consider the strength of repression in the face of *tkv*<sup>QD</sup> expression and the amount of expression in *ap>ci-RNAi* pouches. Given that expression of *dpp*<sup>APR-Ci3</sup>*lacZ* is already expressed at lower levels in *ap>ci-RNAi* wing pouches than either *dpp*<sup>APRD-Ci4-M</sup>*lacZ* or *dpp*<sup>APR</sup>*lacZ*, it is not surprising that it appears almost absent in *ap>tkv*<sup>QD</sup>, *ci-RNAi* wing pouches. Together, these experiments indicate to us that neither Ci binding or Ci protein are required for negative feedback

regulation of *dpp*. Our result with *dpp*<sup>APR-Ci3</sup>*lacZ* has raised the intriguing possibility that Ci plays an indirect role in regulating *dpp* or that it may be binding to the *cis*-regulatory sequences at unknown sites.



#### Appendix 7.14– Reduced *gbb* influences wing phenotypes but not *dpp<sup>BS3.0</sup>lacZ* expression in *ci<sup>W</sup>* animals.

##### Rationale:

We reasoned that if *Ci* was required for negative feedback regulation of *dpp*, a reduction in *gbb* copy number in a *ci* mutant background would not result in negative regulation of *dpp*. To test this, we used a dominant gain-of-function allele of *ci* known as *ci<sup>W</sup>* (Dominguez, 1999; Locke and Tartof, 1994; Slusarski, 1995). The lesion associated with this allele is an insertion in the regulatory sequences of *ci* that affects expression of *ci* in the wing pouch. *ci<sup>W</sup>* has been reported to give ectopic *ci* activity in the posterior compartment, but the wing phenotypes associated with this allele resemble reduced *dpp* activity. We reduced *gbb* levels in a *ci<sup>W</sup>* mutant background and asked if we could observe a rescue of wing defects, possible indicative of an increase in *dpp* expression. We also observed expression of the *dpp<sup>BS3.0</sup>lacZ* reporter in the wing pouch to directly test the effect of *gbb* and *ci<sup>W</sup>* on expression of *dpp*.

##### Approach:

We observed wing phenotypes and expression of *dpp<sup>BS3.0</sup>lacZ* in animals with one copy of *gbb<sup>2</sup>*, one copy of *ci<sup>W</sup>*, or both.

We performed the following crosses:

*OR* flies were crossed to *w ; AyGal4 UAS-GFP ; dpp<sup>BS3.0</sup>lacZ / T(2;3) SM5-TM6b* flies to generate

*AyGal4 UAS-GFP /+ ; dpp<sup>BS3.0</sup>lacZ/+* larvae and adults

*OR* flies were crossed to *w ; FRT<sup>G13</sup> gbb<sup>2</sup> ; dpp<sup>BS3.0</sup>lacZ / T(2;3) SM5-TM6b* flies to generate

*FRT<sup>G13</sup> gbb<sup>2</sup> /+ ; dpp<sup>BS3.0</sup>lacZ/+* larvae and adults

*Ci<sup>W</sup>* flies were crossed to *w ; AyGal4 UAS-GFP ; dpp<sup>BS3.0</sup>lacZ / T(2;3) SM5-TM6b* flies to generate

*AyGal4 UAS-GFP /+ ; dpp<sup>BS3.0</sup>lacZ/+ ; Ci<sup>W</sup> /+* larvae and adults

*Ci<sup>W</sup>* flies were crossed to *w ; FRT<sup>G13</sup> gbb<sup>2</sup> ; dpp<sup>BS3.0</sup>lacZ / T(2;3) SM5-TM6b* flies to generate

*FRT<sup>G13</sup> gbb<sup>2</sup> /+ ; dpp<sup>BS3.0</sup>lacZ/+ ; Ci<sup>W</sup> /+* larvae and adults

Adult wings were mounted and scored blindly for patterning defects. Wings were scored as one of the following classes: Class I - wildtype; Class II – small breaks in L4, straight PCV; Class III – small breaks in L4, curved PCV; Class IV – large gaps in L4, straight PCV; Class V – large gaps in L4, curved PCV; Class VI – no



distal L4, straight PCV; Class VII – no distal L4, curved PCV; Class VIII – fusion of L4 and L5, no PCV, little to no L4; Class IX – blistered. Numbers were then pooled to reflect L4 and PCV defects.

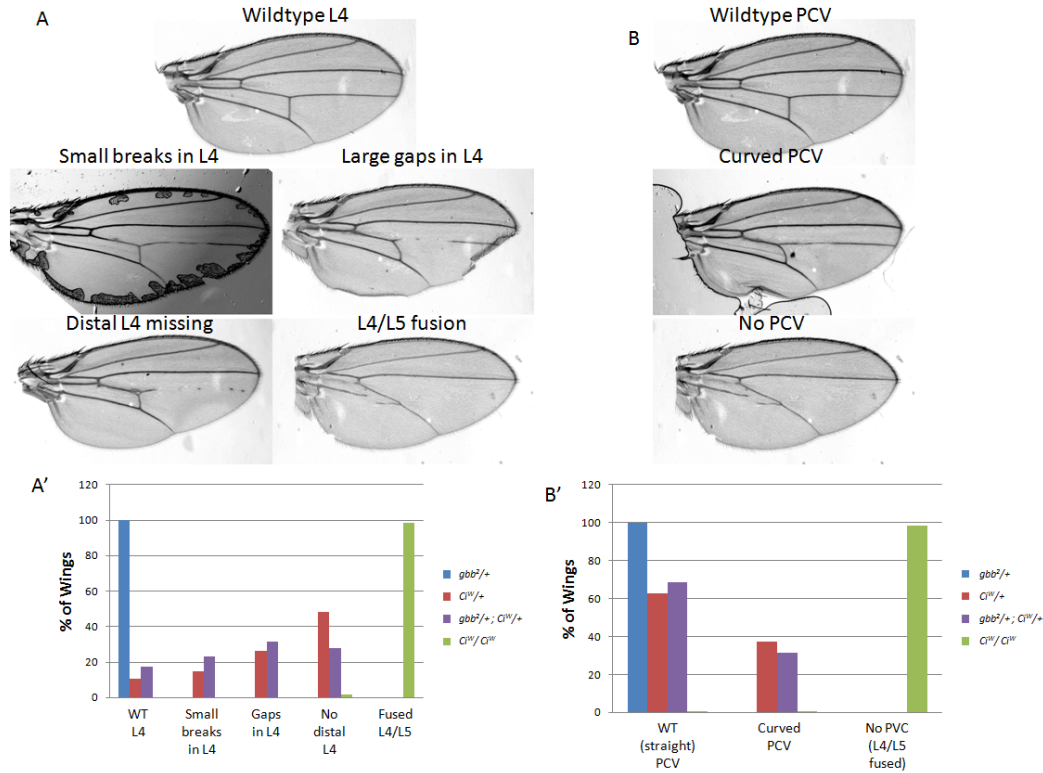
Larval tissue was dissected as third instar crawlers. Anti- $\beta$ Gal and anti-Ci<sup>A</sup> antibodies were used to visualize *lacZ* and Ci, respectively. Images were not taken on the same day or at the same settings so only the pattern of expression and not absolute levels can be compared.

### Results and Conclusions:

Adult wings could be classified based on abnormal L4 or PCV phenotypes. We observed a variety of L4 defects that include small gaps in the vein, large gaps in the vein, or loss of the entire distal vein (Fig 7.34A). We also observed fusion of the L4 and L5 veins at the approximate location of the PCV, resulting in a complete loss of L4. Figure 7.34A' displays the distributions of adult wing phenotypes that we observed. Animals with only one copy of *gbb* removed displayed 100% wildtype-like wings while those with two copies of *ci<sup>W</sup>* displayed close to 100% of the most severe phenotype of L4/L5 fusion. With only one copy of *ci<sup>W</sup>*, a distribution of moderate phenotypes was observed. A reduction in *gbb* dosage in the *ci<sup>W</sup>* background results in a slight shift to less severe wing phenotypes in comparison to *ci<sup>W</sup>/+* alone.

We also observed defects in the PCV in animals with reduced *ci* function, with many displaying a curved PCV. In animals with L4/L5 fusions, the PCV was missing altogether (Fig 7.34B). As with our L4 defects, animals with only one copy of *gbb* had completely wildtype PCV phenotypes while those with two copies of *ci<sup>W</sup>* almost always lacked a PCV. In *ci<sup>W</sup>/+* animals, approximately 37% displayed the curved PCV while the other 63% had the wildtype straight PCV. Reduction of *gbb* in these animals gave a slight rescue in PCV defects to only 31% with the curved PCV and 69% wildtype. The distribution of PCV phenotypes is displayed in figure 7.34B'. The results of both the L4 and PCV data suggest that a reduction in *gbb* can lead to a very slight rescue in *ci<sup>W</sup>* phenotypes. If, indeed, *ci<sup>W</sup>* animals have reduced *dpp* function, the observed rescue could indicate an up-regulation of *dpp*.

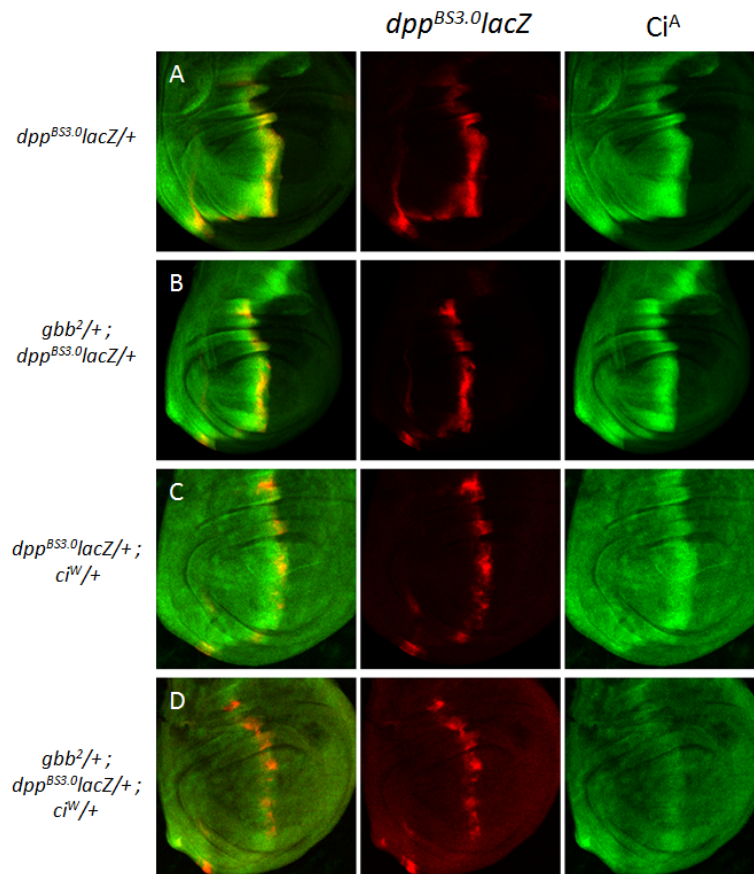
We next examined expression of *dpp<sup>BS3.0</sup>lacZ* in these genotypes (Fig 7.35). Expression of *dpp<sup>BS3.0</sup>lacZ* is indistinguishable from control in *gbb<sup>2</sup>/+* animals, indicating that a reduction of *gbb* by one copy does not drastically alter expression of *dpp<sup>BS3.0</sup>lacZ* (Fig 7.35A,B). In *ci<sup>W</sup>/+* animals, expression of *dpp<sup>BS3.0</sup>lacZ* is greatly reduced in comparison to control animals. Expression of this reporter is patchy along



**Figure 7.34. Reduced *gbb* copy number in a *ci*<sup>W</sup> background slightly rescues wing phenotypes.** We observed wing phenotypes in animals with reduced *gbb* copy number, reduced *ci* function, or both. *ci*<sup>W</sup> animals display a variety of defects in L4 and the PCV. **A.** L4 defects range from small gaps, large gaps, loss of most of the distal region, or complete loss of the distal region after L4/L5 fusion. **A'.** *gbb*<sup>2/+</sup> wings display 100% wildtype phenotypes while *ci*<sup>W/ci<sup>W</sup> animals display 100% fused L4/L5 phenotypes. *ci*<sup>W/+</sup> animals display a moderate phenotype with 11% wildtype, 15% showing small L4 breaks, 26% showing large gaps in L4, and 28% with most of the distal L4 lost. Introduction of one copy of *gbb*<sup>2</sup> in the genotype *gbb*<sup>2/+</sup>; *ci*<sup>W/+</sup> leads to a slight rescue in these phenotypes and display 17% wildtype, 23% with small L4 gaps, 32% with large L4 gaps, and 28% with no distal L4. **B.** Observed PCV defects range from mostly wildtype (straight), to curved, to lost in the case of L4/L5 fusion. **B'.** Animals with only one copy of *gbb* display wildtype PCVs. Animals with two copies of *ci*<sup>W</sup> display complete loss of the PCV. One copy of *ci*<sup>W</sup> results in a mild phenotype, with 37% of PCVs curved. *gbb*<sup>2/+</sup>; *ci*<sup>W/+</sup> animals display 31% curved PCV.</sup>

the anterior-posterior axis, suggesting that Ci is not properly activating it in this tissue. This tissue also displays an altered Ci<sup>A</sup> distribution with reduced levels along the anterior-posterior axis and increased levels in the posterior compartment (Fig 7.35C). When we examined expression of *dpp*<sup>BS3.0</sup>*lacZ* in *ci*<sup>W</sup> animals with a reduction in *gbb*, achieved by examining *gbb*<sup>2/+</sup>; *ci*<sup>W/+</sup> wing pouches, there was no obvious change in *dpp*<sup>BS3.0</sup>*lacZ* expression in comparison to *ci*<sup>W/+</sup> wing pouches (Fig 7.35D). This suggests that expression of *dpp*<sup>BS3.0</sup>*lacZ* is not being up-regulated in *ci*<sup>W/+</sup> in response to reduced *gbb* levels.

Our results examining the effect of *gbb* on *ci<sup>W</sup>* wing phenotypes and *ci<sup>W</sup> dpp<sup>BS3.0</sup>lacZ* expression were conflicting. A slight rescue in wing phenotypes was observed with a reduction in *gbb* but we did not observe an increase in *dpp<sup>BS3.0</sup>lacZ*. It is possible that the change in *dpp* that occurs in response to *gbb* reduction is too subtle to realize by the methods used in this experiment. We had also hoped to use this experiment to address a requirement for Ci in negative feedback regulation of *dpp*. However, through the course of our analysis it became clear that the function of the *ci<sup>W</sup>* allele was unknown. This mutation clearly affects the expression of *ci*, but it is unclear if it also affects the activity of the *ci* that is present. Without knowing whether there was actually any active *ci* present, we could not draw any conclusions regarding the requirement for Ci based on these results.



**Figure 7.35 A reduction in *gbb* copy number does not rescue reduced *dpp<sup>BS3.0</sup>lacZ* associated with the *ci<sup>W</sup>* allele.** We observed expression of *dpp<sup>BS3.0</sup>lacZ* in animals with endogenous and reduced *gbb* and *ci* copy number. Expression of *dpp<sup>BS3.0</sup>lacZ* is shown in red, levels of *Ci<sup>A</sup>* are shown in green. Images were not taken at the same settings, precluding comparisons of absolute expression levels. **A.** Wildtype patterns of *dpp<sup>BS3.0</sup>lacZ* expression and *Ci<sup>A</sup>*. **B.** Reduced *gbb* levels give *dpp<sup>BS3.0</sup>lacZ* and *Ci<sup>A</sup>* patterns that are indistinguishable from wildtype. **C.** In a *ci<sup>W</sup>/+* animal, expression of *dpp<sup>BS3.0</sup>lacZ* is reduced along the anterior-posterior axis and is seen in a patchy distribution. *Ci<sup>A</sup>* displays a less pronounced anterior-posterior stripe and staining is observed in the posterior compartment. **D.** In a *gbb<sup>2</sup>/+ ; ci<sup>W</sup>/+* animal, expression of *dpp<sup>BS3.0</sup>lacZ* is indistinguishable from *ci<sup>W</sup>/+* animals.

### Appendix 7.15 –A *UAS-shn-RNAi* construct results in efficient knock-down of Shn protein levels

#### Rationale:

As Shn is a well-established co-repressor for Mad (Dai et al., 2000; Pyrowolakis et al., 2004; Udagawa et al., 2000), we wished to test whether Shn protein is required for negative feedback of *dpp* expression. To do this, we reduced Shn protein levels by expressing a *UAS-shn-RNAi* line and asked if *dpp* will be reduced in these cells when BMP signaling is increased. First, we tested a *UAS-shn-RNAi* line received from the VDRC for its effectiveness in reducing Shn levels. As we do not have a Shn antibody, we took an indirect approach by observing expression of a gene regulated by Shn and phenotypes that occur in response to *UAS-shn-RNAi* expression.

#### Approach:

We observed expression of *brk-lacZ*, a gene that is repressed by Shn, in clones of cells expressing *shn-RNAi* by crossing:

*brk-lacZ/Y ; UAS-shn-RNAi/CyO-GFP* males to *y w FLP122 ; AyGal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *brk-lacZ/ y w FLP122 ; UAS-shn-RNAi/ AyGal4 UAS-GFP* larvae

Crosses were pre-mated in glass vials and then moved to apple juice plates with yeast paste and brooded every 24 hours. To induce the expression of FLP recombinase, apple juice plates containing larvae were sealed and heat shocked at 37°C by submersion in a water bath for 10-15 minutes at either 48-72 or 54-76 hours post lay to generate early or mid second instar clones. Clones were identified by the expression of GFP.

Phenotypes of wing pouches with *shn-RNAi* expressed in the dorsal compartment were observed by crossing:

*dpp<sup>p10638</sup>lacZ/Sm6a* males to *w ; ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ* larvae

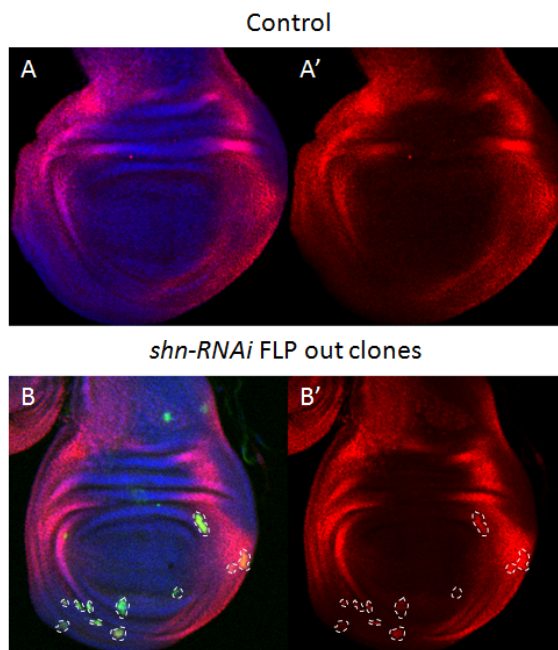
*dpp<sup>p10638</sup>lacZ/+ ; UAS-*tkv*<sup>QD</sup>/+* males to *w ; ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ ; UAS-*tkv*<sup>QD</sup>/+* larvae

*dpp<sup>p10638</sup>lacZ UAS-shn-RNAi/Sm6a* males to *w ; ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ UAS-shn-RNAi* larvae

*dpp<sup>p10638</sup>lacZ UAS-shn-RNAi/CyO-GFP ; UAS-*tkv<sup>QD</sup>*/TM6b* males to *w ; ap-Gal4 UAS-GFP ; +/T(2;3)SM5-TM6b* females to generate *ap-Gal4 UAS-GFP/ dpp<sup>p10638</sup>lacZ UAS-shn-RNAi ; UAS-*tkv<sup>QD</sup>*/+* larvae

### Results and Conclusions:

As a test of the ability of our *UAS-shn-RNAi* line to knock-down Shn levels we examined expression of a gene regulated by *dpp*, *brk-lacZ*. In the medial region of the wing pouch, Shn forms a repressive complex with pMad and Med and represses expression of *brk* (Gao et al., 2005; Marty et al., 2000; Pyrowolakis et al., 2004). In the lateral regions of the wing pouch where pMad levels are very low or absent, *brk* is expressed (Fig 7.36A). If our *shn-RNAi* line is reducing Shn protein levels, then we expect to see an increase in *brk-lacZ* within *shn-RNAi* expressing clones. Indeed, we did observe a cell-autonomous

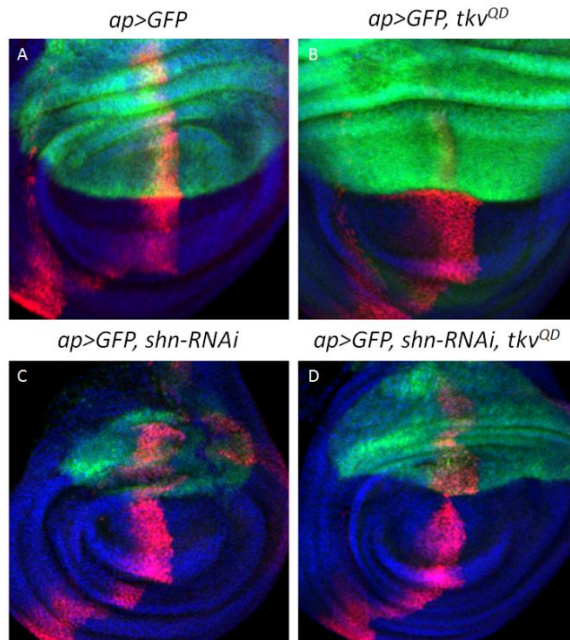


**Figure 7.36. Expression of *brk-lacZ* is cell-autonomously up-regulated in *shn-RNAi* expressing FLP-out clones.** Expression of *brk-lacZ* (in red) was observed in wildtype (A) pouches and pouches with *shn-RNAi* FLP-out clones (B). Clones are marked by GFP expression, in green. Hoechst staining nuclei is shown in blue. *brk-lacZ* displays a cell-autonomous up-regulation of expression in *shn-RNAi* clones (outlined in B). This increase is seen in both the medial and lateral regions of the wing pouch.

increase in *brk-lacZ* expression within *shn-RNAi* FLP-out clones (Fig 7.36B) in the medial region of the wing pouch as well as in the lateral portion. The slight up-regulation of *brk-lacZ* that we observed in the lateral pouch suggests that Shn is playing a role in repressing *brk* in these cells, a region where it is generally accepted that pMad and the Shn/pMad/Med complex is not present at discernible levels.

We attempted to express *UAS-shn-RNAi* throughout the dorsal compartment under the control of *ap-Gal4*. This could give us more control over the location and breadth of *shn*-null tissue. Dorsal compartments of *ap>GFP* and *ap>tkv<sup>QD</sup>* are normal and we observed the expected expression behavior of *dpp<sup>p10638</sup>lacZ* (Fig 7.37A,B). However,

when *shn-RNAi* was expressed alone in *ap>shn-RNAi* tissue or in combination with activated receptor in



**Figure 7.37. Expression of *shn-RNAi* by *ap-Gal4* results in loss of dorsal tissue.** Attempts to observe expression of *dpp<sup>p10638</sup>lacZ* in dorsal cells without Shn were unsuccessful as the dorsal compartment of *ap>shn-RNAi* pouches is lost. Expression of *dpp<sup>p10638</sup>lacZ* (in red) was observed in *ap>GFP* (A), *ap>GFP, tkv<sup>QD</sup>* (B), *ap>GFP, shn-RNAi* (C), and *ap>GFP, tkv<sup>QD</sup>, shn-RNAi* (D) wing pouches. The dorsal compartments of all wing pouches expressing *UAS-shn-RNAi* are reduced or eliminated (C-D), indicating that we cannot address a requirement for Shn using this method. However, these results suggest that *UAS-shn-RNAi* expression does lead to a reduction in Shn levels.

*ap>shn-RNAi, tkv<sup>QD</sup>* wing pouches, we were not able to observe dorsal wing pouch tissue (Fig 7.37C,D). Instead, we observe a mostly-normal ventral compartment juxtaposed to what we believe is notum tissue. These experiments indicate that future studies will require the use of *shn-RNAi* FLP-out clones. They also offered further support that our *UAS-shn-RNAi* line led to efficient knock-down as a loss of dorsal tissue in *ap>shn-RNAi* pouches is consistent with loss of expression of a factor important in wing disc development such as Shn.



### Appendix 7.16 – Expression of *brk-lacZ* is completely repressed in *ap>tkv<sup>QD</sup>* wing discs.

#### Rationale:

We examined the expression of *brk-lacZ* in *ap>tkv<sup>QD</sup>* wing discs to gain insight into the expression patterns of BMP targets with high levels of BMP signaling throughout the dorsal compartment.

#### Approach:

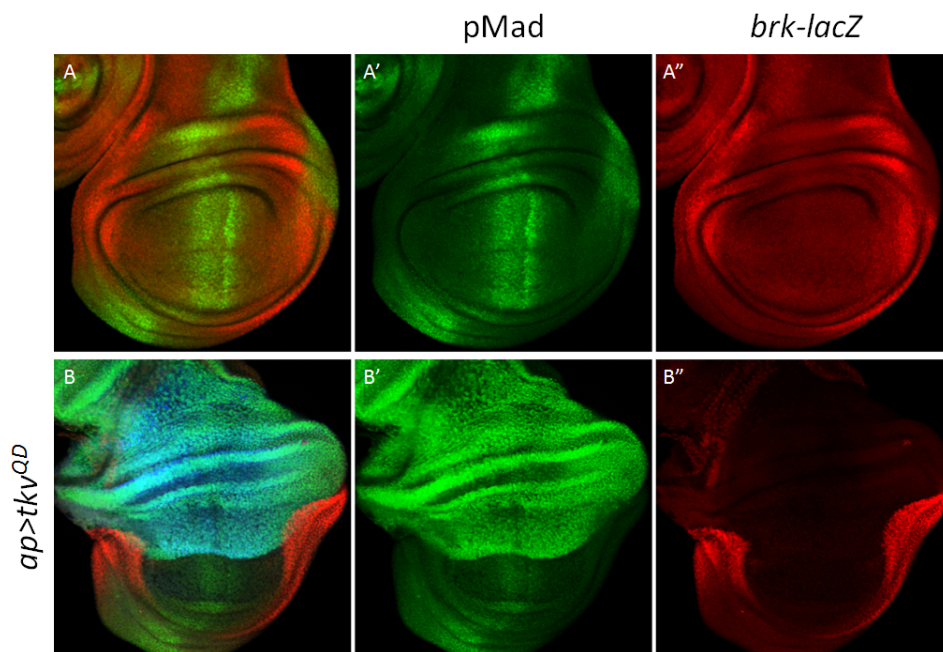
To observe *brk-lacZ* in *ap>tkv<sup>QD</sup>* wing discs, we crossed:

*brk-lacZ/Y* ; *ap-Gal4 UAS-GFP/CyO-GFP* males to *w* ; *UAS-tkv<sup>QD</sup>* females to generate *brk-lacZ/+* ;

*ap-Gal4 UAS-GFP/+* ; *UAS-tkv<sup>QD</sup>/+* larvae

#### Results and Conclusions:

Expression of *brk-lacZ* is strongly reduced in the dorsal compartment of *ap>tkv<sup>QD</sup>* wing discs (Fig 7.38B) compared to wildtype expression of *brk-lacZ* (Fig 7.38A). This result suggests that expression of any BMP target gene that is sensitive to Brk levels and repressed by it should be up-regulated in response to *tkv<sup>QD</sup>*.



**Figure 7.38 Expression of *brk-lacZ* is reduced in response to increased BMP signaling.** *brk-lacZ* expression was observed in wildtype (A) and *ap>GFP, tkv<sup>QD</sup>* (B) wing discs. Expression of *brk-lacZ* is displayed in red, pMad is displayed in green, and GFP is shown in blue. **A.** Wildtype expression of *brk-lacZ* is seen in the lateral regions of the wing disc in a reverse gradient from pMad. **B.** Expression of *tkv<sup>QD</sup>* in the dorsal compartment results in an increase in pMad and a complete loss of *brk-lacZ* expression.

### Appendix 7.17. An increase in *dpp* copy number does not influence expression of *dpp<sup>p10638</sup>lacZ*.

#### Rationale:

We have always noted a striking difference in the expression patterns of *dpp<sup>p10638</sup>lacZ* and *dpp<sup>BS3.0</sup>lacZ*. The expression pattern of *dpp<sup>p10638</sup>lacZ* is significantly wider and more graded in the anterior compartment than *dpp<sup>BS3.0</sup>lacZ*, which is seen in a uniform stripe of cells along the anterior-posterior boundary. As *dpp<sup>p10638</sup>lacZ* is a weak allele of *dpp* (Karpen and Spradling, 1992; Spradling et al., 1999; Twombly et al., 1996), we considered the possibility that expression of *lacZ* from the enhancer trap *dpp<sup>p10638</sup>lacZ* is increased above the level of endogenous *dpp* expression in response to reduced *dpp* function. If so, this may explain some of the differences in expression observed for *dpp<sup>BS3.0</sup>lacZ* and *dpp<sup>p10638</sup>lacZ*. To test this, we increased the copy number of *dpp* using the *Dp(2;2)DTD48* duplication, which contains one extra copy of *dpp* (Irish and Gelbart, 1987; Spencer et al., 1982), and examined expression of *dpp<sup>p10638</sup>lacZ*.

#### Approach:

We performed the following crosses:

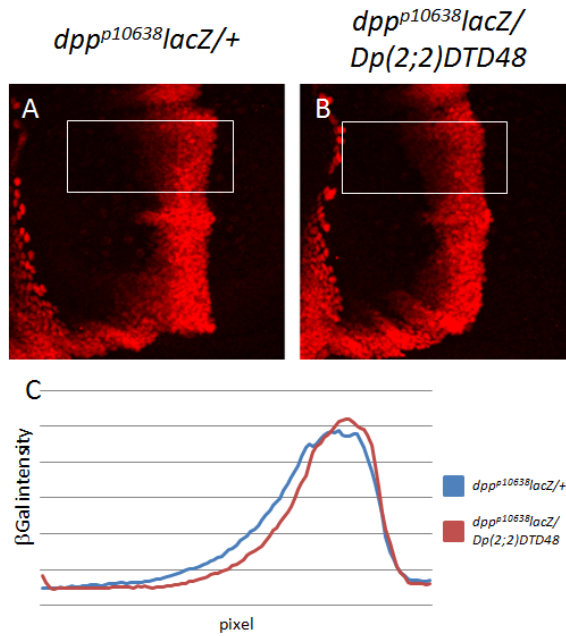
*W<sup>1118</sup>* males to *yw ; dpp<sup>p10638</sup>lacZ/SM6a* females to generate *dpp<sup>p10638</sup>lacZ/+* larvae with one functional copy of *dpp* and one hypomorphic allele of *dpp*.

*Dp(2;2)DTD48* males to *yw ; dpp<sup>p10638</sup>lacZ/SM6a* females to generate *dpp<sup>p10638</sup>lacZ/ Dp(2;2)DTD48* larvae with two functional copies of *dpp* and one hypomorphic allele of *dpp*.

#### Results and Conclusions:

Expression of *dpp<sup>p10638</sup>lacZ* was unchanged between animals with one wildtype copy of *dpp* (*dpp<sup>p10638</sup>lacZ/+*, Fig 7.39A) and animals with two wildtype copies of *dpp* (*dpp<sup>p10638</sup>lacZ/Dp(2;2)DTD48*, Fig 7.39B). Intensity profiles representing an average of several discs from each genotype reveal almost identical expression patterns of *dpp<sup>p10638</sup>lacZ* (Fig 7.39C). Based on this result, I conclude that expression of *dpp<sup>p10638</sup>lacZ* most likely does recapitulate the endogenous expression of *dpp*.





**Fig 7.39. Expression of *dpp<sup>p10638</sup>lacZ* is unchanged with one or two copies of *dpp*.** We examined expression of *dpp<sup>p10638</sup>lacZ*, in red, with one wildtype copy of *dpp* (**A**, *dpp<sup>p10638</sup>lacZ/+*, n=5) or two wildtype copies of *dpp* (**B**, *dpp<sup>p10638</sup>lacZ/Dp(2;2)DTD48*, n=3). **C**. Average intensity profiles of *dpp<sup>p10638</sup>lacZ* expression in these two genotypes, generated from the boxed regions in **A** and **B**, reveals no change in *dpp<sup>p10638</sup>lacZ* expression.

### Appendix 7.18. Activating BMP signaling by ACVR1<sup>R206H</sup> leads to a repression of *dpp*<sup>BS3.0</sup> *lacZ* expression.

#### Rationale:

In addition to expression of *UAS-tkv<sup>QD</sup>* to generate strong cell-autonomous increased BMP signaling, we made use of a constitutively active form of the mammalian Sax homolog, ACVR1/ALK12, known as ACVR1<sup>R206H</sup>. This mutation was identified based on its role in the bone disorder, Fibrodysplasia Ossificans Progressiva (FOP), and leads to increased BMP signaling in vertebrate systems (Billings et al., 2008; Fukuda et al., 2009; Kaplan et al., 2009a; Kaplan et al., 2009b; Shen et al., 2009; Shore et al., 2006; van Dinther et al., 2010). Work in our lab has recently determined that this protein leads to increased BMP signaling when expressed in *Drosophila* tissue as well (Le and Wharton, 2012). To test ACVR1<sup>R206H</sup>-mediated increases in BMP signaling for the ability to repress expression of *dpp*, we examined expression of *dpp*<sup>BS3.0</sup> *lacZ* in tissue with over-expression of *UAS-ACVR1<sup>R206H</sup>*.

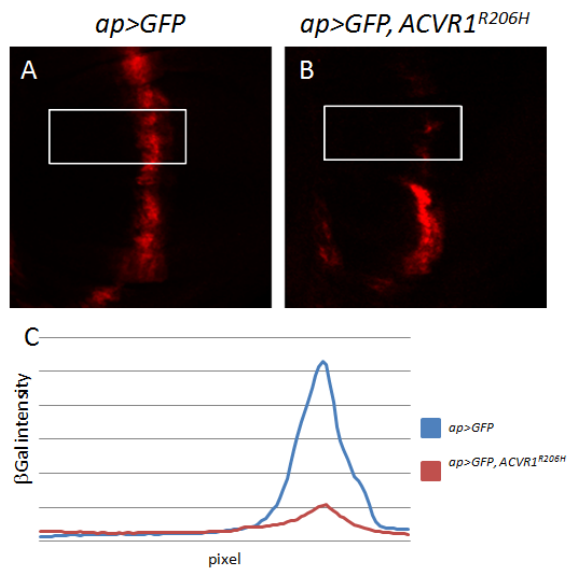
#### Approach:

We performed the following crosses:

*UAS-ACVR1<sup>R206H</sup>/(CyO) ; (MKRS)/(TM6b)* males were crossed to *w ; Ap-Gal4 UAS-GFP ; dpp<sup>BS3.0</sup> lacZ/T(2;3)* females to generate *Ap-Gal4 UAS-GFP/ UAS-ACVR1<sup>R206H</sup> ; dpp<sup>BS3.0</sup> lacZ/+* larvae  
*w<sup>1118</sup>* males were crossed to *w ; Ap-Gal4 UAS-GFP ; dpp<sup>BS3.0</sup> lacZ/T(2;3)* females to generate *Ap-Gal4 UAS-GFP/ + ; dpp<sup>BS3.0</sup> lacZ/+* larvae

#### Results and Conclusions:

As with expression of the activated receptor Tkv, the active form of the mammalian ACVR1 protein also leads to a strong reduction in expression of *dpp*<sup>BS3.0</sup> *lacZ* (Fig 7.40A-C). Weak expression of *dpp*<sup>BS3.0</sup> *lacZ* remains when *UAS-ACVR1<sup>R206H</sup>* is expressed under the control of *ap-Gal4* (Fig 7.40B), but this level of expression is far lower than *dpp*<sup>BS3.0</sup> *lacZ* in control discs (Fig 7.40A,C).



**Fig 7.40. Expression of *dpp<sup>BS3.0</sup>lacZ* is reduced in response to expression of *UAS-ACVR1<sup>R206H</sup>*.** We examined expression of *dpp<sup>BS3.0</sup>lacZ*, in red, control *ap>GFP* wing pouches (**A**, n=2) and in *ap>GFP, ACVR1<sup>R206H</sup>* wing pouches (**B**, n=5). **C**. Average intensity profiles of *dpp<sup>BS3.0</sup>lacZ* expression in these two genotypes, generated from the boxed regions in **A** and **B**, reveals a reduction in reporter expression in the presence of *ACVR1<sup>R206H</sup>*.

### Appendix 7.19. Reducing activin signaling in border cell clusters influences migration

#### Rationale:

Based on our observations in the lab that BMP signaling influences border cell migration and based on the reciprocal roles that the BMP and activin/TGF- $\beta$  signaling pathways often play in cell migration, we hypothesized that Activin signaling may play a role in regulating migration of the border cell cluster as well. To test this, we reduced activin signaling in border cells or polar cells and examined stage ten egg chambers for migration defects.

#### Approach:

Activin signaling was reduced by expressing *RNAi* directed against *babo*, *dSmad2*, or *wit* specifically in the border cells, by *slbo-Gal4*, or specifically in the polar cells, by *upd-Gal4*. Stage 10 egg chambers were evaluated for defects, including delayed migration and dissociation of the border cell cluster. We tested several *RNAi* lines to determine the ones that give the strongest phenotypes, the results of which are presented here. In the main test of chapter six, “dissociation” specifically referred to clusters that had lost their tight associations and appear elongated or clusters from which at least two border cells had been left behind. The dissociation phenotypes presented here mostly represent dissociation of just one border cell. This particular phenotype was not further analyzed due to its high prevalence in our control genotypes and we, instead, focused on more severe dissociation phenotypes. The following *RNAi* lines were tested:

|                          |                                                                                 |
|--------------------------|---------------------------------------------------------------------------------|
| <i>wit-RNAi-R1:</i>      | <i>UAS-wit-RNAi</i> (NIG #10776R-1)                                             |
| <i>wit-RNAi-R2:</i>      | <i>UAS-wit-RNAi</i> (NIG #10776R-2)                                             |
| <i>wit-RNAi-V1:</i>      | <i>w ; ; UAS-wit-RNAi</i> (VDRC line # 42244)                                   |
| <i>wit-RNAi-V2:</i>      | <i>w ; UAS-wit-RNAi</i> (VDRC line # 865)                                       |
| <i>dSmad2-RNAi-R1:</i>   | <i>w ; (CyO) ; UAS-dSmad2-RNAi</i> (NIG # 2262R-1)                              |
| <i>dSmad2-RNAi-R2:</i>   | <i>UAS-dSmad2-RNAi</i> (NIG # 2262R-2, exact genotype unknown)                  |
| <i>dSmad2-RNAi-V1:</i>   | <i>w ; UAS-dSmad2-RNAi</i> (VDRC line # 105687)                                 |
| <i>dSmad2-RNAi-TRiP:</i> | <i>y<sup>1</sup>v<sup>1</sup> ; ; p(TRiP.JF02320) AttP2/TM3, Sb<sup>1</sup></i> |
| <i>babo-RNAi-R3:</i>     | <i>w UAS-babo-RNAi</i> (NIG #8224R-3)                                           |

|                        |                                                                                     |
|------------------------|-------------------------------------------------------------------------------------|
| <i>babo-RNAi-V1:</i>   | <i>w ; ; UAS-babo-RNAi</i> (VDRC line # 853)                                        |
| <i>babo-RNAi-V2:</i>   | <i>w ; ; UAS-babo-RNAi</i> (VDRC line # 106092)                                     |
| <i>babo-RNAi-V3:</i>   | <i>w ; ; UAS-babo-RNAi</i> (VDRC line # 3825)                                       |
| <i>babo-RNAi-TRiP:</i> | <i>y<sup>1</sup>v<sup>1</sup> ; ; p(TRiP.JF01953) AttP2/TM3, Sb<sup>1</sup></i>     |
| <i>babo-RNAi-M1:</i>   | <i>UAS-babo RNAi 10E2,6E2/CyO-GFP</i> (a generous gift from Dr Mike O'Connor)       |
| <i>babo-RNAi-M2:</i>   | <i>UAS-babo RNAi 1A3,58B3/TM6c - Sb, Tb</i> (a generous gift from Dr Mike O'Connor) |

The following crosses were performed:

*RNAi* or males were crossed to *w ; slbo-Gal4 UAS-GFP/CyO* females to reduce activin signaling in the border cells

*yw* males were crossed to *w ; slbo-Gal4 UAS-GFP/CyO* females as controls

*RNAi* males were crossed to *w Upd-Gal4* females to reduce activin signaling in the polar cells

*yw* males were crossed to *w Upd-Gal4* females as controls

Crosses involving *slbo-Gal4* were raised at 29°C until eclosion. To avoid complications from *upd-gal4* expressed in other tissues and times in development, crosses involving *upd-Gal4* were raised at 18°C until eclosion. After eclosion, all flies were cleared from vials daily and moved to 29°C to age for two days before dissection.

Methods for dissection, immunostaining, and analysis are described in chapter six.

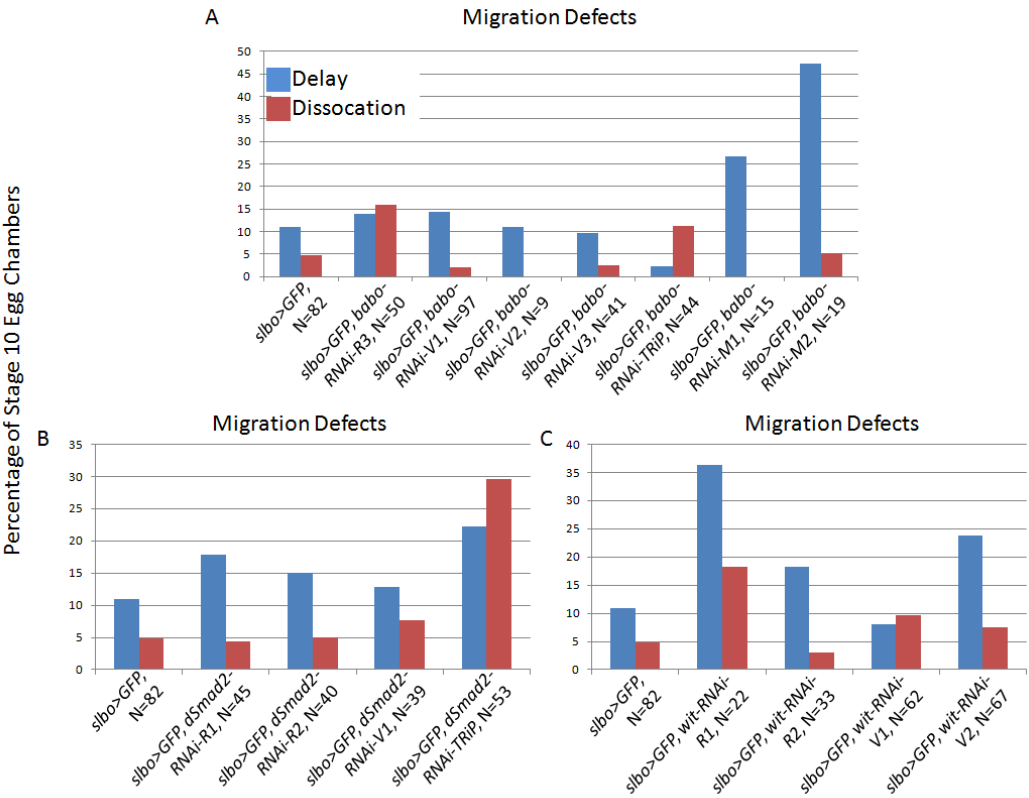
### Results and Conclusions:

When we reduced activin signaling in the border cells using all of the available *RNAi* lines, we observed a variable response on border cell migration (Fig 7.41). Some *RNAi* lines did not affect migration of the border cells while several did. As some lines led to a clear migration defect phenotype, we attribute this variability to efficiency of knock-down for each line. Reduction of either Babo (Fig 7.41A) or dSmad2 (Fig 7.41B) in the border cells led to delayed migration and increased dissociation of the border cell clusters. *slbo>wit-RNAi* egg chambers also displayed these phenotypes (Fig 7.41C), but Wit was not chosen for further study due to potential complications with BMP signaling. As the BMP and activin

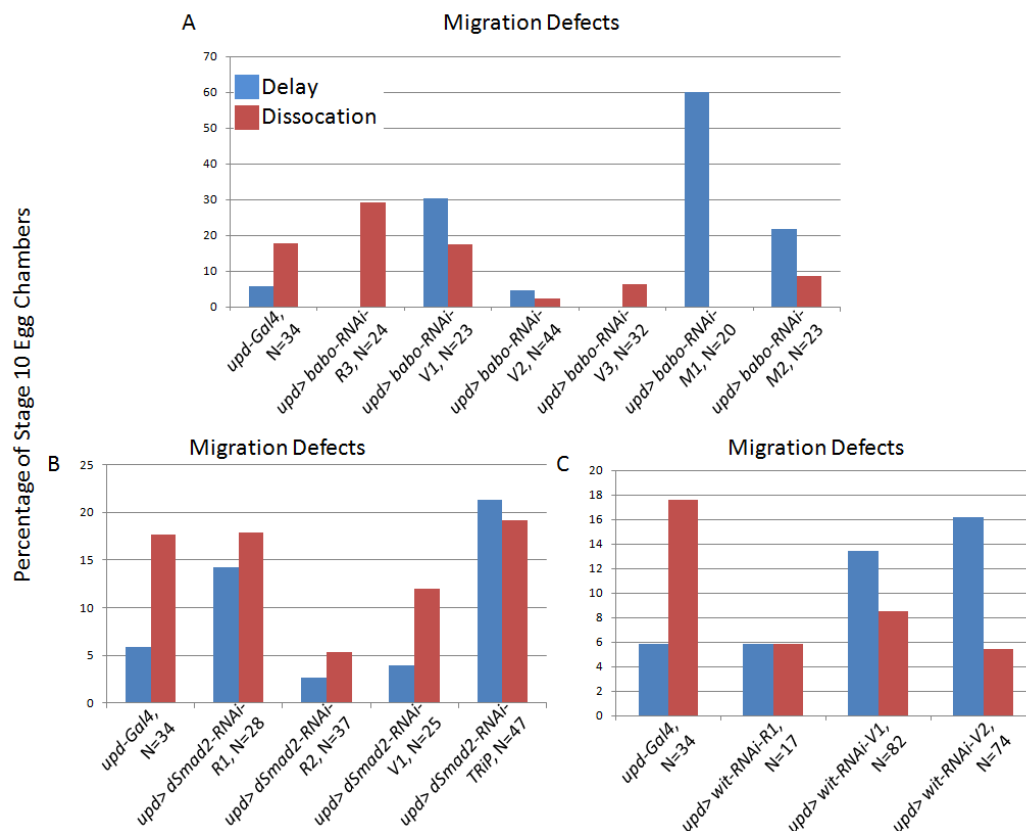
pathways can signal through either Wit or Punt, depending on cellular context, we could not be sure what a reduction in the type II receptor Wit would do.

Reduction of activin pathway components specifically in the polar cells also led to migration defects, primarily an increase in stage ten egg chambers with delayed migration (Fig 7.42). *Upd-Gal4* alone displays a high prevalence of single border cell dissociation, so this phenotype was not further studied. In both *upd>babo-RNAi* (Fig 7.42A) and *upd>dSmad2-RNAi* (Fig 7.42B) genotypes, delays in migration were observed. Reduction of *wit* in polar cells also influences border cell migration (Fig 7.42C).

Based on these observations, we chose four *RNAi* lines for further study: *UAS-babo-RNAi-V1*, *UAS-babo-RNAi-M1*, *UAS-babo-RNAi-M2*, and *UAS-dSmad2-RNAi-TRiP*.



**Fig 7.41. Reduced activin signaling in border cells leads to migration defects.** Activin signaling was reduced specifically in the border cells through use of the *slbo-Gal4* driver and *RNAi* lines directed against *babo*, *dSmad2*, or *wit* and stage ten egg chambers were examined. We observed two phenotypes: delayed migration of the border cell cluster and dissociation of one or several cells from the border cell cluster. Expression of *RNAi* directed against *babo* (A), *dSmad2* (B), or *wit* (C) led to variable defects in border cell migration and cluster dissociation. For each, the percentage of each defect observed out of the total stage ten egg chambers scored is displayed.



**Fig 7.42. Reduced activin signaling in polar cells leads to migration defects.** Activin signaling was reduced specifically in the polar cells through use of the *upd-Gal4* driver and *RNAi* lines directed against *babo*, *dSmad2*, or *wit* and stage ten egg chambers were examined. We observed two phenotypes, delayed migration of the border cell cluster, and dissociation of one or several cells from the border cell cluster. Expression of *RNAi* directed against *babo* (A), *dSmad2* (B), or *wit* (C) led to variable defects in border cell migration and cluster dissociation. For each, the percentage of each defect observed out of the total stage ten egg chambers scored is displayed.

**Appendix 7.20. List of fly stocks used in experiments**

| <u>Stock ID</u> | <u>Genotype</u>                                            |
|-----------------|------------------------------------------------------------|
| 35              | <i>b pr cn bw</i>                                          |
| 81              | <i>Ci<sup>W</sup></i>                                      |
| 150             | <i>Dp(2;2)DTD48</i>                                        |
| 188             | <i>dpp<sup>d12</sup> bw gbb<sup>1</sup>/CyO wg-lacZ</i>    |
| 194             | <i>dpp<sup>d5</sup> bw gbb<sup>1</sup>/SM6a</i>            |
| 196             | <i>dpp<sup>d5</sup>/CyO</i>                                |
| 197             | <i>dpp<sup>d6</sup> bw gbb<sup>1</sup>/Sm6a</i>            |
| 199             | <i>dpp<sup>d6</sup>/CyO</i>                                |
| 221             | <i>dpp<sup>hr27</sup> cn bw/Sm6a</i>                       |
| 222             | <i>dpp<sup>hr4</sup> cn bw/Sm6a</i>                        |
| 224             | <i>dpp<sup>hr56</sup> cn bw/Sm6a</i>                       |
| 226             | <i>dpp<sup>hr92</sup> cn bw/Sm6a</i>                       |
| 256             | <i>hhMRT/TM3 Sb</i>                                        |
| 322             | <i>Ore-R</i>                                               |
| 356             | <i>P[ry+?;ptc-lacZ] / CyO ; Sb / TM6b Hu Tb?</i>           |
| 495             | <i>w[1118] ; dpp[d12] / CyO-GFP</i>                        |
| 522             | <i>w* ; bw gbb<sup>1</sup>/CyO-GFP</i>                     |
| 576             | <i>w* P[w+;lacW]biPol-1 (omb-lacZ)</i>                     |
| 593             | <i>w* ; P[w+m*;ptc=Gal4]</i>                               |
| 597             | <i>w? ; P[w+mC;UAS-dpp]weak</i>                            |
| 599             | <i>w? ; P[w+mC ; UAS=saxA]4B2</i>                          |
| 604             | <i>w? ; P[w+mC;UAS=tkvA]B23</i>                            |
| 607             | <i>w* ; p{w+mC=UAS-GFP.S65T}T2</i>                         |
| 615             | <i>w* ; P[w+mW;FRT]G13 shaIN gbb1/CyO P[w+; Act5C=GFP]</i> |
| 649             | <i>w* ; P[w[+mW]; sal=Gal4]</i>                            |



|      |                                                                                     |
|------|-------------------------------------------------------------------------------------|
| 623  | <i>w* P[w{+mW}=GawB]A9</i>                                                          |
| 629  | <i>w* P[w{+mW}=GawB]MS-1096</i>                                                     |
| 718  | <i>w<sup>1118</sup></i>                                                             |
| 780  | <i>y w P[ry+;hsp70:FLP]1 ; P[w+mW;FRT]G13 P[ry+;y+]44B</i>                          |
| 784  | <i>y w* P[ry+;hsp70:FLP]122 ; P[w+mW;FRT]G13 2X-P[w+?;Ubi=nls-GFP]</i>              |
| 796  | <i>y w* ; p P[w+mC=AyGal4]25 P[w+m*=UAS-GFP.S65T]T2 / CyO y+</i>                    |
| 808  | <i>y*w* p[w+mW.hs=GawB]ombmd653/(FM7a)</i>                                          |
| 881  | <i>y w1118 ; P[w+mW;FRT]G13 sha[IN] bw gbb[2] / SM6a</i>                            |
| 917  | <i>y w1118 P[w+;UAS=gbb]9.7 ; P{ry[+t7.2]=PZ}dpp[P10638] cn / CyO wg-lacZ</i>       |
| 918  | <i>y w1119 P[w+;UAS=gbb]9.7</i>                                                     |
| 928  | <i>y w1118 ; P[w+;UAS-gbb]9.9</i>                                                   |
| 942  | <i>yw1118 ; wg[sp]/SM6a (no longer in stock list)</i>                               |
| 943  | <i>y w1118</i>                                                                      |
| 967  | <i>yw ; dpp[blk]-Gal4</i>                                                           |
| 1075 | <i>y w ; dpp[P10638] dp Sp cn / SM6a</i>                                            |
| 1250 | <i>w ; FRTG13 sax[4] sha[IN] ; BS3.0 / T(2;3)</i>                                   |
| 1251 | <i>w ; 2xGFP FRT40A ; BS3.0 / T(2;3)</i>                                            |
| 1252 | <i>w; AyGal4 UAS-GFP; dpp<sup>BS3.0</sup>lacZ /T(2;3) SM5-TM6b</i>                  |
| 1254 | <i>w ; FRT40 tkv[a12] Df(2L)flp147E /CyO-TM6b</i>                                   |
| 1257 | <i>+/(CyO wg-lacZ) ; hh[MRT] BS3.0/TM3Sb (original stock used by Lorena Soares)</i> |
| 1289 | <i>P{ry[+];BS3.1.1Bgal} ry[506] /TM2</i>                                            |
| 1290 | <i>y w ; P{ry[+]; BS3.21 Bgal} ry[506] /TM3[Ser Bgal w[+]]</i>                      |
| 1291 | <i>P{ry[+]; BS3.23 Bgal} ry[506] /TM3 Sb</i>                                        |
| 1292 | <i>P{ry[+]; BS3.2 Bgal} ry[506]</i>                                                 |
| 1293 | <i>w ; P{ry[+]; BS3.22 Bgal} ry[506]</i>                                            |
| 1303 | <i>w brk-lacZ ; FRT[G13] 2xUbi-GFP M /CyO-GFP</i>                                   |
| 1335 | <i>y w ; dpp[d5]/CyO ; P{w{+mW}, gbb6.8}3.3</i>                                     |

|      |                                                                                           |
|------|-------------------------------------------------------------------------------------------|
| 1432 | <i>y w FLP122; Sp/CyO-GFP ; MKRS/TM6b, Hu, Tb</i>                                         |
| 1435 | <i>w ; FRT<sup>G13</sup> gbb<sup>2</sup> ; dpp<sup>BS3.0</sup> lacZ / T(2;3) SM5-TM6b</i> |
| 1458 | <i>y w f FLP122 ; Gl4/CyO-GFP</i>                                                         |
| 1461 | <i>y w f FLP122 ; FRT[G13] 2xGFPM /CyO-GFP</i>                                            |
| 1462 | <i>w ; dpp<sup>p10638</sup> lacZ FRT<sup>G13</sup> 2X ubi-nlsGFP/CyO-GFP</i>              |
| 1574 | <i>(y) w ; ap-Gal4/CyO-GFP</i>                                                            |
| 1650 | <i>y[1] w[1118] ; P{w[+mC]=dpp-lacZ.Exel.1}2</i>                                          |
| 1651 | <i>y[1] w[1118]; P{w[+mC]=dpp-lacZ.Exel.2}2</i>                                           |
| 1652 | <i>y[1] w[1118]; P{w[+mC]=dpp-lacZ.Exel.2}3</i>                                           |
| 1679 | <i>yw? ; dpp<sup>p10638</sup> lacZ ; UAS-dpp<sup>w</sup> /T(2;3) SM5-TM6b</i>             |
| 1719 | <i>w ; babo-RNAi (R3)</i>                                                                 |
| 1720 | <i>UAS-wit-RNAi/TM3 (R2)</i>                                                              |
| 1760 | <i>w; P{w[inc]=GAL4-slbo26}, 1206{w[1nc]=UAS-GFP S65T}/CyO</i>                            |
| 1762 | <i>upd-Gal4</i>                                                                           |
| 1767 | <i>UAS-dSmad2-RNAi (R1)</i>                                                               |
| 1784 | <i>UAS-dSmad2-RNAi (R2)</i>                                                               |
| 1852 | <i>UAS-GFP ; dpp[blk]-GAL4 / T(2;3)</i>                                                   |
| 1857 | <i>w ; ptc&gt;GFP / T(2;3)</i>                                                            |
| 1864 | <i>w; ap-Gal4 UAS-GFP ; + / T(2;3)SM5-TM6b</i>                                            |
| 2310 | <i>w ; Sb/TM3 Ser-GFP</i>                                                                 |
| 2311 | <i>+/(AyGal4 UAS-GFP) ; hhMRT BS3.0 / TM3 Sb (new recombinant)</i>                        |
| 2312 | <i>w? ; sal-Gal4 UAS-GFP / T(2;3)</i>                                                     |
| 2315 | <i>w ; sp ; dpp<sup>BS3.0</sup> lacZ /T(2;3)</i>                                          |
| 2319 | <i>w ; p[w+mW, dpp-CiKO-GFPnls]59</i>                                                     |
| 2320 | <i>w ; p[w+mW, dpp-CiKO-GFPnls]19</i>                                                     |
| 2322 | <i>w ; p[w+mW, dpp-CiWT-GFPnls]30</i>                                                     |
| 2323 | <i>w ; p[w+mW, dpp-CiWT-GFPnls]10</i>                                                     |

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|------|---------------------------------------------------------------------------------------------|
| 2324 | <i>y[1] v[1] ; P{y[+t7.7] v[+t1.8]=TRiP.JF01953}attP2 (UAS-babo-TRiP RNAi)</i>              |
| 2325 | <i>w ; UAS-baboA-ΔI 5A2,8A2 ; UAS-baboA-ΔI 16A3,20A3</i>                                    |
| 2326 | <i>w ; p[w+mW, dpp-CiHiAf-GFPnls]3</i>                                                      |
| 2329 | <i>w ; p[w+mW, dpp-CiHiAf-GFPnls]50</i>                                                     |
| 2333 | <i>y w FLP122 ; UAS-tkv<sup>QD</sup></i>                                                    |
| 2334 | <i>dpp<sup>AP<sup>Rd</sup></sup>lacZ</i>                                                    |
| 2335 | <i>dpp<sup>AP<sup>rd</sup></sup>lacZ</i>                                                    |
| 2336 | <i>p[TG5-286, ry+ lacZ] ry506 / TM2 ry (BS3.1)</i>                                          |
| 2338 | <i>dpp<sup>AP<sup>RD</sup></sup>lacZ</i>                                                    |
| 2340 | <i>UAS-dSmad2-RNAi (V1)</i>                                                                 |
| 2341 | <i>dpp<sup>AP<sup>R</sup></sup>lacZ (old)</i>                                               |
| 2342 | <i>w ; dpp<sup>Exel-1</sup>lacZ ; UAS-tkvA/T(2;3)</i>                                       |
| 2343 | <i>w ; babo-RNAi (V2)</i>                                                                   |
| 2344 | <i>w[1118] ; babo-RNAi (V3)</i>                                                             |
| 2345 | <i>w[1118] ; UAS-wit-RNAi (V1)</i>                                                          |
| 2346 | <i>w[1118] ; UAS-wit-RNAi (V2)</i>                                                          |
| 2347 | <i>w[1118] ; UAS-babo-RNAi (V1)</i>                                                         |
| 2348 | <i>y[1] v[1] ; P{y[+t7.7] v[+t1.8]=TRiP.JF02320}attP2 / TM3, Sb[1] (UAS-Smox-TRiP RNAi)</i> |
| 2352 | <i>y[1] v[1] ; P{y[+t7.7] v[+t1.8]=TRiP.JF01715}attP2 (UAS-ci-TRiP RNAi)</i>                |
| 2358 | <i>UAS-babo RNAi 10E2,6E2/CyO-GFP (M1)</i>                                                  |
| 2359 | <i>UAS-babo RNAi 1A3,58B3/TM6c - Sb, Tb (M2)</i>                                            |
| 2360 | <i>dpp<sup>AP<sup>-D</sup></sup>lacZ</i>                                                    |
| 2362 | <i>(y) w ; dpp<sup>p10638</sup>lacZ, UAS-ci-RNAi/Sm6a</i>                                   |
| 2363 | <i>w ; ap-Gal4 UAS-GFP ; dpp<sup>BS3.0</sup>lacZ / T(2;3)SM5-TM6b</i>                       |
| 2365 | <i>w ; UAS-baboB-ΔI 7A2,6A2; UAS-baboB-ΔI 11E3,11H3</i>                                     |
| 2370 | <i>w[1118] ; UAS-ci-RNAi (v2)</i>                                                           |

|      |                                                                                        |
|------|----------------------------------------------------------------------------------------|
| 2373 | <i>Gla/CyO ; UAS-Flag-dSmad2</i>                                                       |
| 2375 | <i>w[1118] ; UAS-ci-RNAi (V1)</i>                                                      |
| 2397 | <i>w ; UAS-shn-RNAi</i>                                                                |
| 2398 | <i>y w ; dpp<sup>p10638</sup>lacZ, UAS-shn-RNAi/Sm6a</i>                               |
| 2402 | <i>yw ; APRD / (Sm6a)</i>                                                              |
| 2405 | <i>dpp<sup>p10638</sup>lacZ, UAS-ci-RNAi/CyO-GFP ; UAS-tkv<sup>QD</sup>/TM6b</i>       |
| 2408 | <i>w ; dpp<sup>p10638</sup>lacZ, UAS-shn-RNAi/CyO-GFP ; UAS-tkv<sup>QD</sup>/ TM6b</i> |
| 2410 | <i>y w ; dpp<sup>APRD</sup>lacZ/(TM3 Sb Ser)</i>                                       |
| 2411 | <i>y w ; dpp<sup>APRD-Ci3</sup>lacZ / (TM3 Sb Ser)</i>                                 |
| 2412 | <i>y w ; dpp<sup>APRD-Ci4-M</sup>lacZ/(TM3 Sb Ser)</i>                                 |
| 2413 | <i>y w ; dpp<sup>APRD-Ci5</sup>lacZ/(TM3 Sb Ser)</i>                                   |
| 2414 | <i>y w ; dpp<sup>APR</sup>lacZ/(TM3 Sb Ser)</i>                                        |
| 2415 | <i>y w ; dpp<sup>APR-Ci3</sup>lacZ/(TM3 Sb Ser)</i>                                    |
| 2416 | <i>y w ; dpp<sup>APRD-Ci5-M</sup>lacZ/(TM3 Sb Ser)</i>                                 |
| 2417 | <i>y w ; dpp<sup>BS3.0-MS2</sup>lacZ/(Tm3 Sb Ser)</i>                                  |
| 2418 | <i>y w ; dpp<sup>BS3.0</sup>lacZ/(TM3 Sb Ser), line F3 (new one made by me)</i>        |
| 2419 | <i>w ; dpp<sup>p10638</sup>lacZ ; UAS-tkv<sup>QD</sup>/ T(2;3)SM5-TM6b</i>             |
| 2629 | <i>dpp<sup>APRd</sup>lacZ</i>                                                          |

Stocks not found in the current fly list:

*UAS-wit-RNAi (R1)*

*UAS-dpp<sup>w</sup>, dpp<sup>BS3.0</sup>lacZ/TM6b*

*dpp<sup>hr56</sup>/Sm6a*

*dpp<sup>hr4</sup>/Sm6a*

*w ; TM3 Sb Ser/TM6b Hu Tb*

Chapter 8:  
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