Molecular dissection of the dual functioning *Drosophila* ALK1/2 BMP type I receptor ortholog Saxophone

by

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B.S. Tufts University, 2003

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Division of Biology and Medicine at Brown University

Providence, RI

May 2014

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This dissertation by Viet Q. Le 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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Twombly V., Bangi E., Le V.Q, Malnic B., Singer M.A., & Wharton K.A. (2009) Functional analysis of *saxophone*, the *Drosophila* gene encoding the BMP type I receptor ortholog of human ALK1/ACVRL1 and ACVR1/ALK2. Genetics 183, 563-79. doi: 10.1534/genetics.109.105585.

Wilson J.M., Le V.Q., Zimmerman C., Marmorstein R., & Pillus L. (2006) Nuclear export modulates the cytoplasmic Sir2 homologue Hst2. EMBO reports 7, 1247-51. doi: 10.1038/sj.embor.7400829.

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ACKNOWLEDGEMENTS

This thesis dissertation was an endeavor that would not have been possible without the guidance and support of my family, friends, and mentors. I would like to acknowledge and express my gratitude to the following people:

My advisor, Kristi Wharton, for being a mentor that has allowed me to make mistakes and learn from them. She has instilled in me a love and appreciation for the beauty of data. I try to share this with those around me everyday.

My committee members Alison DeLong, Michael McKeown, and Rebecca Page whose insight and advice have been instrumental in my development as a scientist. Alison, for continually pushing me to do better and more rigorous science. She has been incredibly generous with her time and graciously hosted me in her lab when I needed to perform experiments there. Whenever other graduate students ask for thesis committee recommendations, Alison is always at the top of my list. Rebecca, for teaching me to always strive for excellence ("This is an A, let's push it to an A+." "You're at a 95, let's get to 100."). Her words of encouragement were always kind and timely. Michael, whose knack for anticipating where a project is going has taught me to always keep the next step in sight. Whenever he asked a question during my presentations and the answer would be just a few slides ahead, I knew I was headed in the right direction.

Vicki Rosen, for being my outside reader. It was our conversation at a FOP workshop that sparked many of the questions and experiments that proved vital for my research.

My many labmates, for making the Wharton lab a place filled with the fondest of memories. I have enjoyed learning from my colleagues and passing on that knowledge. In particular, I would like to thank Takuya Akiyama for setting the standard of scientific research that I aim to accomplish.

My father, Tam, whose overwhelming interest in my thesis research was a wellspring of inspiration. Our conversations pushed me to find new and better ways to communicate my research. My mother, Han, who made sure I never got too sick or too thin and was always eager to help me with my “homework.” My brothers Huan, Lam, and Quang, for your support and for reminding me to slow down and enjoy a day of fishing once in awhile. My niece, Jessica, whose laughter was always the antidote for the doldrums of the bench.

Richard, Juan, Youssef, Jeffrey, Catherine, Lindsay, and Stephen, one could not ask for better friends. Thank you for making every day better than the last.

Last but certainly not least, Maryanna, without whom nothing would make sense.

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Chapter 4 – Hyperactive BMP signaling induced by ALK2^{R206H} requires type II receptor function in a *Drosophila* model for classic Fibrodysplasia Ossificans Progressiva

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Chapter 6 – Appendices

Abstract of “Molecular dissection of the antagonistic function of the *Drosophila* ALK1/ALK2 BMP Type I receptor ortholog Saxophone” by Viet Q. Le, Ph.D., Brown University, May 2014.

The bone morphogenetic protein (BMP) signaling pathway is an essential regulator of developmental and cellular processes. Secreted ligands initiate BMP signaling by binding to a quadripartite receptor complex consisting of two type I and two type II receptor kinases. The type I receptor kinase, which is activated by the type II receptor in the signaling complex, phosphorylates R-Smad transcription factors to elicit cellular responses via target gene regulation. The existence of multiple BMP receptors raises the possibility that different combinations of receptors can affect signaling output of receptor complexes. This concept is supported by studies in cell culture, zebrafish, and *Drosophila* indicating that type I receptor permutations within a complex can dramatically impact signaling activity. Although it has been suggested that type I receptors influence each other’s activity, the precise mechanism is unknown.

In *Drosophila*, two type I receptors, Tkv and Sax, and two type II receptors, Punt and Wit, transduce signals initiated by the BMP ligands Dpp, Gbb, and Scw. The interplay between these components is essential in a variety of developmental contexts. During wing development, a dual function has been ascribed to Sax where it can facilitate or antagonize signaling. Genetic characterization of this behavior led to a model in which Sax mediates signaling in a complex with Tkv, but inhibits BMP signals when paired with another Sax receptor.

From experiments described here, we have identified protein subdomains that determine type I receptor signaling activity. These regions are important in type II receptor-mediated activation of type I receptor kinases and dimerization of type I receptors. In a Drosophila model for the bone disease fibrodysplasia ossificans

progressiva (FOP), we have demonstrated that the constitutive activity of the FOP mutant receptor ALK2[R206H] is type II receptor-dependent, further underscoring the importance of the type II receptor. We propose that Sax:Sax dimers inhibit signaling by adopting a configuration that is incompatible for activation by type II receptors. Our work provides a backdrop for understanding how type I receptors can influence signaling activity of a receptor complex by affecting interactions with the type II receptor.

Chapter 1

Introduction

INTRODUCTION

Cell-to-cell communication during development

One of the greatest challenges a multicellular organism faces during development is producing an organism that consists of numerous cell types, tissues, and complex organs derived from just one cell—the zygote. As the embryo undergoes development, certain cells in the organism will undergo a variety of processes like proliferation, migration, apoptosis, alterations in cell morphology, and changes in cell fate, which together generate complex structures in the organism. Added to this complexity is the task of regulating these processes both spatially and temporally. How are these functions coordinated and delegated to different cells? How do cells know when and which task they must perform? Cell-to cell communication is critical in coordinating all of these events.

During development certain cells will communicate with other cells in the surrounding tissues and direct their behavior. Cells communicate with each other via networks of proteins and molecules called signal transduction pathways. Generally speaking, these pathways consist of signaling molecules that transmit information extracellularly from cell to cell, and cell-surface proteins that act as receptors/antennas to receive these "messages." The receptors in turn relay this information internally by interacting and modifying proteins known as intracellular transducers. In response to signaling cues, these effectors elicit diverse cellular responses—often times, changes in gene expression—that can initiate, modulate, or terminate cellular processes during development.

Cells use a diverse array of signaling pathways composed of specific sets of proteins for coordinating and regulating developmental processes. Most of these pathways are identified by the family of their specific signaling molecules, some of which

include the fibroblast growth factor (FGF) family, the Hedgehog family, the Wnt family, and the TGF-β family. Although many of these transduction pathways were originally identified in directing a particular process, it is clear that each pathway has multiple regulatory roles during development (Clevers, 2006; Dorey and Amaya, 2010; Ingham and McMahon, 2001; Wu and Hill, 2009). Furthermore, signal transduction pathways are not wholly confined or discrete systems and can interact with each other (Itasaki and Hoppler, 2010; Javelaud et al., 2012; Miraoui and Marie, 2010). Therefore, by using a combination of these networks, cells can generate highly-specific messages that elicit diverse responses in other cells (Perrimon et al., 2012).

The cell-surface receptors of signaling pathways represent a critical nexus between the inner-workings of the cell and the extracellular environment. They function as antennas that receive the messages sent between cells and relay that information intracellularly to induce a response. Precise control of their function and activity is critical for the proper development of an organism. Consequently, mutations that alter or disrupt their function can cause cellular miscommunication that may contribute to disease and developmental disorders (Abdalla and Letarte, 2006; Gu et al., 2006; Horton and Lunstrum, 2002; Johnson et al., 1996; Kaplan et al., 2009; Lehmann et al., 2003; Robitaille et al., 2002; Webster and Donoghue, 1996; Xie et al., 1998; Zhou et al., 2001). More and more, however, we are also seeing cases in which receptors can have roles outside of their respective, canonical pathways (Lee-Hoeflich et al., 2004; Lee et al., 2007; Moustakas and Heldin, 2005; Nohe et al., 2002). Therefore, further characterizing the functional roles of these receptors and how their activities are controlled can provide us insight into potentially new mechanisms that regulate developmental processes, as well as increasing our understanding of the diseases associated with dysregulated receptor activity.

BMP Signaling Pathway

One of the best characterized cell-to cell communication system is the bone morphogenetic protein (BMP) signaling pathway, the signaling molecules of which were originally identified for their ability to induce bone formation in vertebrates (Sampath et al., 1987; Urist, 1965; Wozney et al., 1988). The role of BMP signaling, however, has greatly expanded to include the regulation of diverse biological processes ranging from dorsal-ventral axis specification, limb patterning, cell growth, cell proliferation, and apoptosis (Wu and Hill, 2009). Furthermore, the function of BMP signaling extends far beyond vertebrates, as conserved components of the pathway also control aspects of development in invertebrates such as *Drosophila* and *C. elegans* (Newfeld et al., 1999; Parker et al., 2004; Patterson and Padgett, 2000).

BMPs, along with TGF-βs and Activins, are growth factors that compose the TGF-β superfamily of signaling molecules. These secreted cytokines activate their respective signaling pathways by coordinating the formation of a heterotetrameric receptor complex consisting of two type I and two type II receptors. Type I and type II receptors are distantly related single-pass, transmembrane, serine/threonine kinases that are distinguishable by the presence of a glycine-serine rich (GS) domain immediately upstream of the type I receptor kinase domain (Bragdon et al., 2011; Miyazono et al., 2010; Mueller and Nickel, 2012).

Receptor complex formation relays the signal intracellularly through a cascade of phosphorylation events. First, the type II receptor kinase phosphorylates the type I receptor in the GS domain, relieving the autoinhibitory conformation adopted by the type I receptor and thereby activating its kinase (Franzén et al., 1995; Huse et al., 2001; Wrana et al., 1994a). In turn, the activated type I receptor phosphorylates a conserved SSXS motif in the C-terminus of receptor-mediated Smads (R-Smads), a group of intracellular transducers of the pathway (Abdollah et al., 1997b; Kretzschmar et al., 1997;

Souchelnytskyi et al., 1997). Phosphorylated R-Smads interact with the related co-Smad, Smad4, forming a heteromeric complex that accumulates in the nucleus and regulates target gene expression in conjunction with other transcriptional regulators(Feng and Derynck, 2005; Massagué et al., 2005; Wu and Hill, 2009). Homomeric complexes composed of only R-Smads have also been observed to form, however, their function remains unclear (Schmierer and Hill, 2007)

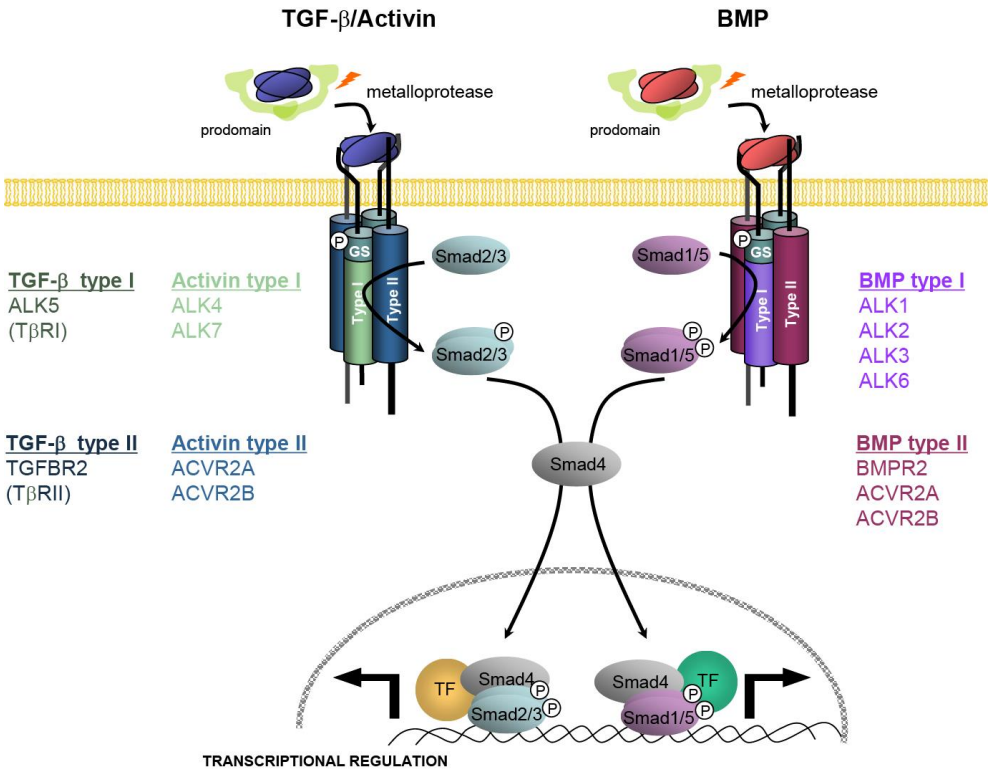


Figure 1.1. The TGF-β superfamily signaling pathway. The core components of the Smad-dependent signaling pathways for TGF-βs, Activin, and BMPs are shown. TGF-βs, Activins, and BMPs are secreted dimers that bind to a heterotetrameric complex consisting of two type I and two type II receptor kinases. Upon complex formation the constitutively active type II receptor transphosphorylates the GS domain of type I receptor to activate its kinase. The activated type I receptor phosphorylates intracellular effectors of the pathway called Smads. Phospho-Smads forms a complex with the co-Smad, Smad4, that accumulates in the nucleus and regulates target gene expression. Each branch of the TGF-β superfamily of ligands signals through a specific suite of type I and type II receptors as indicated (the exception being ACVR2A/B, which are used by both Activin and BMPs).TGF-β and Activin type I receptors specifically phosphorylate Smad2/3, whereas BMP type I receptors phosphorylate Smad1/5/8. Adapted from (Wharton and Derynck, 2009).

Although the overall signaling pathway architecture is conserved between each TGF-β superfamily branch, BMPs, TGF-βs, and Activins primarily signal through their own distinct suite of receptors and effectors. BMP signals culminate in phosphorylation of the Smad1/5/8 group of R-Smads, whereas TGF-β and Activin signaling converge on the Smad2/3 group of R-Smads. This divergence in pathway components allows each branch of the TGF-β superfamily to take up different—and sometimes overlapping—roles in regulating cellular and developmental processes.

Regulation of BMP signaling

The importance of precisely regulating BMP signaling output during development is underscored by a number of diseases associated with mutations that dysregulate the pathway such as juvenile polyposis syndrome (JPS) (Howe et al., 2001; Howe et al., 2004; Kim et al., 2003; Zhou et al., 2001), hereditary hemorrhagic telangiectasia type 2 (HHT2) (Abdalla and Letarte, 2006; Bayrak-Toydemir et al., 2006; Gu et al., 2006; Olivieri et al., 2007; Wehner et al., 2006), and fibrodysplasia ossificans progressiva (FOP) (Fukuda et al., 2008; Gregson et al., 2011; Kaplan et al., 2009; Petrie et al., 2009; Shore et al., 2006). As such, BMP signaling activity is extensively regulated, and control mechanisms can be found at nearly every level of the pathway (**Fig. 1.2**).

Secreted factors such as the vertebrate proteins Chordin and Noggin, and the *Drosophila* proteins Short gastrulation (Sog), Twisted gastrulation (Tsg), Crossveinless (CV), and Crossveinless-2 (CV-2) bind and prevent BMP ligands from interacting with their receptors (Ashe and Levine, 1999; Conley et al., 2000; Ross et al., 2001; Serpe et al., 2008; Shimmi et al., 2005a; Shimmi and O'Connor, 2003; Walsh et al., 2010; Zakin and De Robertis, 2010). Some of these extracellular inhibitors are cleaved by secreted metalloproteases (e.g. Xolloid/Tolloid), resulting in the release of the BMP ligand (Marqués et al., 1997; Piccolo et al., 1997; Shimmi et al., 2005b; Shimmi and O'Connor,

2003). Therefore, the interplay between these components can establish the signaling range of BMP ligands by facilitating their diffusion across a field of cells.

At the cell surface, BMPs can interact with other proteins in addition to the type I and type II receptors. Some of these cell surface proteins act as co-receptors that help mediate BMP signaling. For instance, heparin sulfate proteoglycans, such as Dally and Dally-like in *Drosophila*, interact with BMPs and facilitate binding of the ligands to BMP receptors (Akiyama et al., 2008; Fujise et al., 2003; Jackson et al., 1997; Raftery and Umulis, 2012). Interestingly, the recently identified extracellular regulators Pentagone and Larval Translucida impact the signaling range of BMP ligands through interactions with Dally and Dally-like, respectively (Raftery and Umulis, 2012; Szuperák et al., 2011; Vuilleumier et al., 2010). On the other hand, some cell surface proteins can downregulate the pathway. For example, the pseudo-receptor BAMBI, which resembles a type I receptor that lacks an intracellular kinase domain, can bind and sequester BMPs away from BMP receptor complexes (Onichtchouk et al., 1999).

Intracellular factors that regulate BMP signaling include a class of Smads separate from the R-Smads and Smad4, known as the inhibitory Smads (I-Smads). I-Smads, such as Smad6/7 and the *Drosophila* protein Dad (Daughters against dpp), inhibit R-Smad phosphorylation by competing for type I receptor binding thereby preventing signal transduction (Goto et al., 2007; Hata et al., 1998; Hayashi et al., 1997; Inoue et al., 1998; Kamiya et al., 2008; Nakao et al., 1997; Tsuneizumi et al., 1997). Additionally, Smad6 has been reported to compete for Co-Smad binding, which prevents the formation of R-Smad/Co-Smad oligomers that regulate target gene expression (Hata et al., 1998).

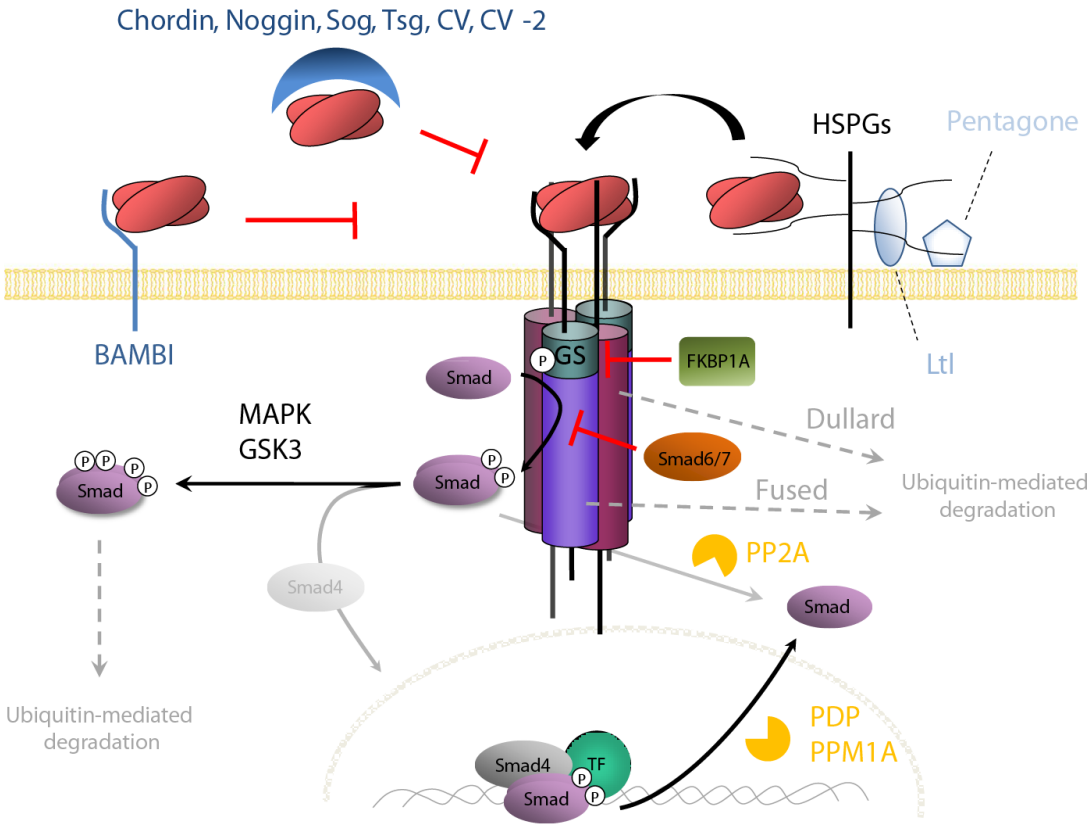


Figure 1.2. The BMP signaling pathway is extensively regulated. The availability of BMP ligands can be modulated by secreted factors such as Chordin, Noggin, Sog, Tsg, CV, and CV-2 as well as cell surface proteins such as the pseudo-receptor BAMBI. Other cell surface proteins such as HSGPs (e.g. Dally and Dally-like in *Drosophila*) act as co-receptors to facilitate binding of BMP ligands to the receptor complex. Intracellular regulators such as FKBP1A and the inhibitory Smad6/7 proteins negatively regulate phosphorylation of the type I receptor GS domain and receptor-mediated Smads, respectively. Furthermore, ubiquitin-mediated turnover and dephosphorylation of the core BMP signaling components can attenuate BMP signaling. For instance, phospho-Smads can be targeted for degradation by MAPK- and GSK3-mediated phosphorylation. Additionally, phospho-Smads are dephosphorylated by a host of phosphatases that include PP2A, PDP, and PPM1A. Various factors, such as Fused and Dullard, have also been identified that target type I and type II receptors for ubiquitin-mediated degradation.

Post-translational modifications further regulate BMP signaling. For example, ubiquitin-mediated degradation of BMP receptors and Smads attenuates signaling activity. In the germline stem cell niche of the *Drosophila* ovary, Fused (Fu), a serine/threonine kinase that regulates Hedgehog signaling, and the E3 ligase Smurf target the type I receptor Tkv for degradation (Xia et al., 2010). In *Xenopus*, the phosphatase Dullard promotes ubiquitination and degradation of BMPRII (Satow et al., 2006). On the other hand, ubiquitin-mediated turnover of Smads requires sequential phosphorylation by both MAP Kinases and GSK3, a kinase in the Wnt signaling pathway (Fuentealba et al., 2007). Degradation facilitated by MAPK and GSK3 is thought to control the duration of phospho-Smad signaling since it is dependent on BMP-induced phosphorylation of the Smad protein. Furthermore, dephosphorylating the GS domain of the type I receptor may function to deactivate type I receptor kinase activity, whereas removing phosphate groups from Smads can affect the nuclear localization of these effector proteins. As such, PP2A, PP1, PPM1A, pyruvate dehydrogenase phosphatase (PDP), and Dullard have all been implicated in dephosphorylating either BMP receptors or Smads to attenuate BMP signaling (Bengtsson et al., 2009; Chen et al., 2006; Duan et al., 2006; Satow et al., 2006; Shi et al., 2007).

Impact of type I receptor combinations on BMP signaling

Although mechanisms exist to precisely control BMP signaling activity, it is becoming increasingly clear that the composition of the signaling complex itself dictates to a large degree the extent of BMP signaling activity. In humans/mammals, BMP signaling is transduced by four BMP type I receptors (ALK1, ALK2, ALK3, & ALK6) and three BMP type II receptors (BMPR2, ACVR2A, and ACVR2B) (**Table 1 & Fig. 1.1**). Since the receptor complex is composed of two type I and two II receptors, it is possible that the receptors interact in various combinations to form distinct complexes with wide-

ranging signaling activities. Several studies have indicated that the identity of the type I receptors within a signaling complex can have very different and profound impacts on the output of BMP signaling activity.

TGF-β Superfamily	Type I Receptor	Alternative Names	*Drosophila* Ortholog	*Drosophila* Ligand
BMP, TGF-β	**ALK1** (activin receptor-like kinase 1)	**ACVRL1** (activin A receptor type II-like 1) **TSR1** (TGF-β superfamily receptor type I)	Saxophone	Gbb, Scw
BMP	**ALK2** (activin receptor-like kinase 2)	**ACVR1** **ACTRI** (activin A receptor, type 1)	Thick Veins	Dpp
	ALK3 (activin receptor-like kinase 3)	**BMPR1A** (bone morphogenetic protein receptor, type IA)		
	ALK6 (activin receptor-like kinase 6)	**BMPR1B** (bone morphogenetic protein receptor, type IB)		
Activin, GDF, Nodal	**ALK4** (activin receptor-like kinase 4)	**ACTRIB** (activin A receptor, type 1B)	Baboon	Dawdle, dActivin, Maverick, Myoglianin
TGF-β, GDF9	**ALK5** (activin receptor-like kinase 5)	**TβRI** or **TGFBR1** (transforming growth factor, beta receptor 1)		
Activin, Nodal	**ALK7** (activin receptor-like kinase 7)	**ACVR1C** **ACTRIC** (activin A receptor, type 1C)		

Table 1. Type I receptors of the TGF-β superfamily. The seven human/vertebrate type I receptors and the corresponding branch of TGF-β superfamily signaling that they transduce. Also indicated are the alternative names for the receptors, their *Drosophila* orthologs and the corresponding *Drosophila* ligands. In the text of this study, the seven TGF-β superfamily type I receptors are referred to as ALK1-7. Their alternative names are noted parenthetically if the cited reference uses that particular nomenclature.

Some of the earliest evidence that different type I receptor permutations affect signaling activity was observed in *Drosophila* studies. In these experiments, coexpression of the *Drosophila* BMP type I receptors Tkv (ALK3/6 orthlog) and Sax (ALK1/2 ortholog) resulted in embryonic and wing phenotypes consistent with

synergistic signaling activity (Haerry et al., 1998; Neul and Ferguson, 1998; Nguyen et al., 1998). Similarly, cotransfection of ALK2 and ALK3/6 in mouse C2C12 cells synergistically induced alkaline phosphatase activity—a common reporter of BMP signaling activity (Aoki et al., 2001). ALK2 and ALK3 were also observed to synergistically induce transcription of a BMP-responsive luciferase reporter in these same cells. These *Drosophila* and mouse cell culture studies, however, relied on the use of constitutively active mutant forms of the type I receptors without addressing the composition of the receptor complex. Thus it could not be concluded whether the synergistic activity was a function of heteromeric type I receptor interactions (i.e. ALK2:ALK3/6 or Tkv: Sax) within a receptor signaling complex.

Recent work in zebrafish more directly implicates the importance of a heteromeric BMP receptor complex for signaling (Little and Mullins, 2009). Dorsal-ventral (DV) patterning in the zebrafish embryo is regulated by BMP signaling and requires both ALK3/6 and ALK8 (zebrafish ALK2 ortholog) type I receptors non-redundantly (the zebrafish ortholog of ALK2), as well as the BMP2b and BMP7 ligands. Using a combination of crosslinking followed by immunoprecipitation, Little and Mullins demonstrate that ALK3:ALK8 heteromeric complexes require the presence of both BMP2b and BMP7. Morpholino-induced knockdown of either BMP2b or BMP7 abolished the interaction between ALK3 and ALK8. Furthermore, only purified BMP2-BMP7 heterodimers were sufficient to induce BMP signaling in the BMP2b morpholino knockdown embryos. Neither BMP2b nor BMP7 purified homodimers were capable of activating the pathway. Taken together, these results highlight the importance of forming receptor complexes with type I receptors in heteromeric combinations. Homomeric ALK3 or ALK8 receptor complexes, if formed, must not be contributing to BMP signaling since neither ALK3 nor ALK8 alone is genetically sufficient for normal DV patterning. Instead, BMP2b-BMP7 heterodimers appear to mediate the formation of obligateALK3:

ALK8 –containing heteromeric complexes to transduce signaling. Although it was proposed that binding of BMP2b-7 heterodimer would be best accommodated by a heteromeric ALK3:ALK8-containg receptor complex, the mechanism by which ALK3 and ALK8 homomeric complexes are prevented from transducing BMP2b or BMP7 homodimer signals is not known.

Insight as to why some homomeric receptor complexes might not be able to signal comes from studies conducted in mouse embryonic endothelial cells and human chondrocytes. In these cell types, it was observed that TGF-β ligands were recruiting the BMP type I receptor ALK1 into mixed complexes with the TGF-β type I receptor, ALK5 (**Fig. 1.1 & Table 1**) (Finnson et al., 2008; Goumans et al., 2003). Within these mixed complexes, however, both type I receptors maintained their respective Smad-specificities indicating that the interaction with a TGF-β ligand did not switch ALK1 into a transducer of the TGF-β signaling pathway. Importantly, the full signaling activity of the BMP type I receptor ALK1 was found to be dependent on ALK5 kinase activity, indicating that type I receptors can influence each other's signaling activity (Goumans et al., 2003). Furthermore, ALK1 signaling through Smad1/5 acts in direct opposition to ALK5-mediated signaling through Smad2/3. These results represent a scenario by which signaling components from different TGF-β superfamily branches can interact with each other to regulate signaling output. The observation that ALK1-mediated signaling was dependent on ALK5 kinase activity suggested that ALK1 is a substrate for ALK5, however ALk5-mediated phosphorylation of ALK1 was not observed. The mechanism by which ALK5 promotes ALK1 signaling activity, therefore, remains unidentified.

The *Drosophila* ALK1/ALK2 ortholog Saxophone is a dual functioning type I receptor that exhibits antagonistic behavior

Drosophila also provides another example where receptor combinations can potentially affect signaling outcomes. The type I receptor Saxophone has been characterized as a receptor with dual behaviors (Bangi and Wharton, 2006b). Under certain circumstances Sax can facilitate BMP signaling, whereas in other contexts it can antagonize the pathway. A model has been proposed in which the type I receptor combination within a signaling complex dictates whether Sax antagonizes or promotes BMP signaling (Bangi and Wharton, 2006b). Signaling complexes consisting of Tkv:Tkv or Sax:Tkv are considered to be competent for signal transduction, whereas Sax:Sax complexes are incompetent (**Fig. 1.3**). How Sax's kinase activity remains silent or inactive in homomeric complexes, and whether or not it becomes activated within a Sax:Tkv complex remains unknown.

The primary focus of this thesis work is to elucidate the mechanism underlying Sax's dual function, as well as its impact in a developmental context. What are the factors that dictate whether Sax antagonizes or facilitates BMP signaling? Under what contexts does Sax behave as an inhibitor or a promoter of BMP signaling? How are some homomeric combinations of type I receptors (e.g.ALK3:ALK3, ALK8:ALK8, Sax:Sax) incompatible for signaling? Is it a function of how homomeric type I receptor combinations interact with type II receptors in the signaling complex? And if so, are type II receptors prevented from activating homomeric combinations like Sax:Sax? Moreover, can Tkv promote Sax signaling as ALK5 does for ALK1? Answering these questions will further our understanding of Sax's role in *Drosophila* BMP signaling and development, and also may provide insight into a general mechanism by which different combinations of type I receptors can dictate the signaling output of a receptor complex. Lastly, understanding what determines the specific behavior of Sax and whether this dual

function is conserved in its orthologs, ALK1 /2, could have a profound impact on our understanding of the mechanisms underlying diseases associated with dysregulated ALK1/2 activity.

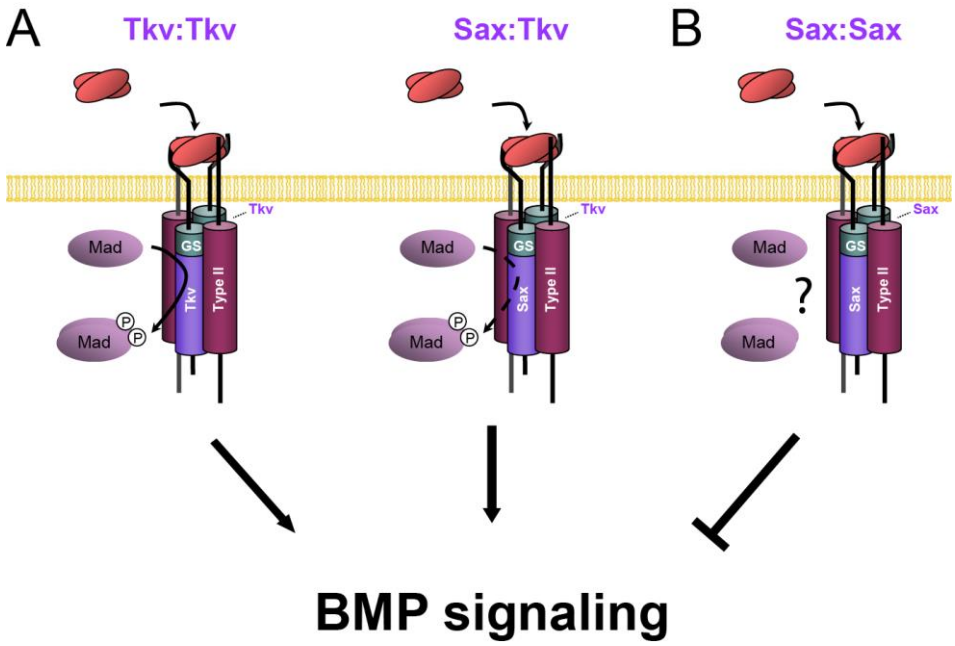


Figure 1.3. Model of signaling competent and signaling incompetent *Drosophila* BMP receptor complexes. A. Receptor complexes in which the type I receptor combination is either Tkv with Tkv or Tkv with Sax are competent for transducing BMP signals. Although Tkv can phosphorylate Mad, it is unknown whether Sax can phosphorylate Mad (dashed line) within a Sax:Tkv containing complex (middle). **B**. Receptor complexes in which the type I receptor combination is Sax:Sax are incompetent for transducing BMP signals. As a result, Sax:Sax complexes inhibit BMP signaling by titrating away BMP ligand. The mechanism by which Sax signaling activity remains silent in this context has not yet been identified.

BMP ligands

Bone morphogenetic proteins were first identified, as their name implies, on the basis of their ability to induce bone formation. The first evidence of proteins with osteogenic potential was observed in 1965 when Marshall Urst demonstrated that demineralized bone matrix could induce bone formation when implanted into the soft

tissue and bones of various animals (Urist, 1965). It would be another 20 years, however, before the first BMP, osteogenin (BMP3) was isolated from demineralized bovine bone matrix, followed shortly by the identification and cloning of the first human BMPs (Sampath et al., 1987; Wozney et al., 1988).

Thus far the total number of BMPs identified in humans/mammals has expanded to roughly ~20 proteins, easily making BMPs the largest branch of the TGF-β superfamily (**Fig. 1.4**). In contrast, only 3 TGF-βs and 4 members of the Activin family have been identified. However, several TGF-β superfamily ligands that fall outside of these branches such as Nodal Lefty 1, and Lefty 2 (not shown) are considered to be part of the Activin family (Mueller and Nickel, 2012; Schmierer and Hill, 2007).

BMPs can be divided into multiple phylogenetic subgroups each containing several BMP family members with the best characterized being the BMP2/4 and BMP5/6/7/8 subgroups (**Fig. 1.4**). BMPs are also well conserved outside of vertebrates. For example, the *Drosophila* BMPs consist of the BMP2/4-ortholog Decapentaplegic (Dpp), the BMP5/6/7/8-ortholog Glass bottom boat (Gbb), and the Gbb-paralog Screw (Scw) (Doctor et al., 1992; Fritsch et al., 2010; Kingsley, 1994; Newfeld et al., 1999; Padgett et al., 1993; Sampath et al., 1993; Wharton et al., 1991). In C. *elegans,* at least one BMP-like molecule is encoded by the *dbl-1* (*dpp, BMP-like*) gene, and potentially two more TGF-β-like ligands are represented by the *daf-7* and *unc-129* genes (Morita et al., 2002; Newfeld et al., 1999; Patterson and Padgett, 2000; Suzuki et al., 1999). Although many members of the BMP branch have osteogenic potential, BMP ligands also regulate other processes including organogenesis, limb patterning, and early embryonic development (Bandyopadhyay et al., 2006; Chang et al., 2002; Wu and Hill, 2009).

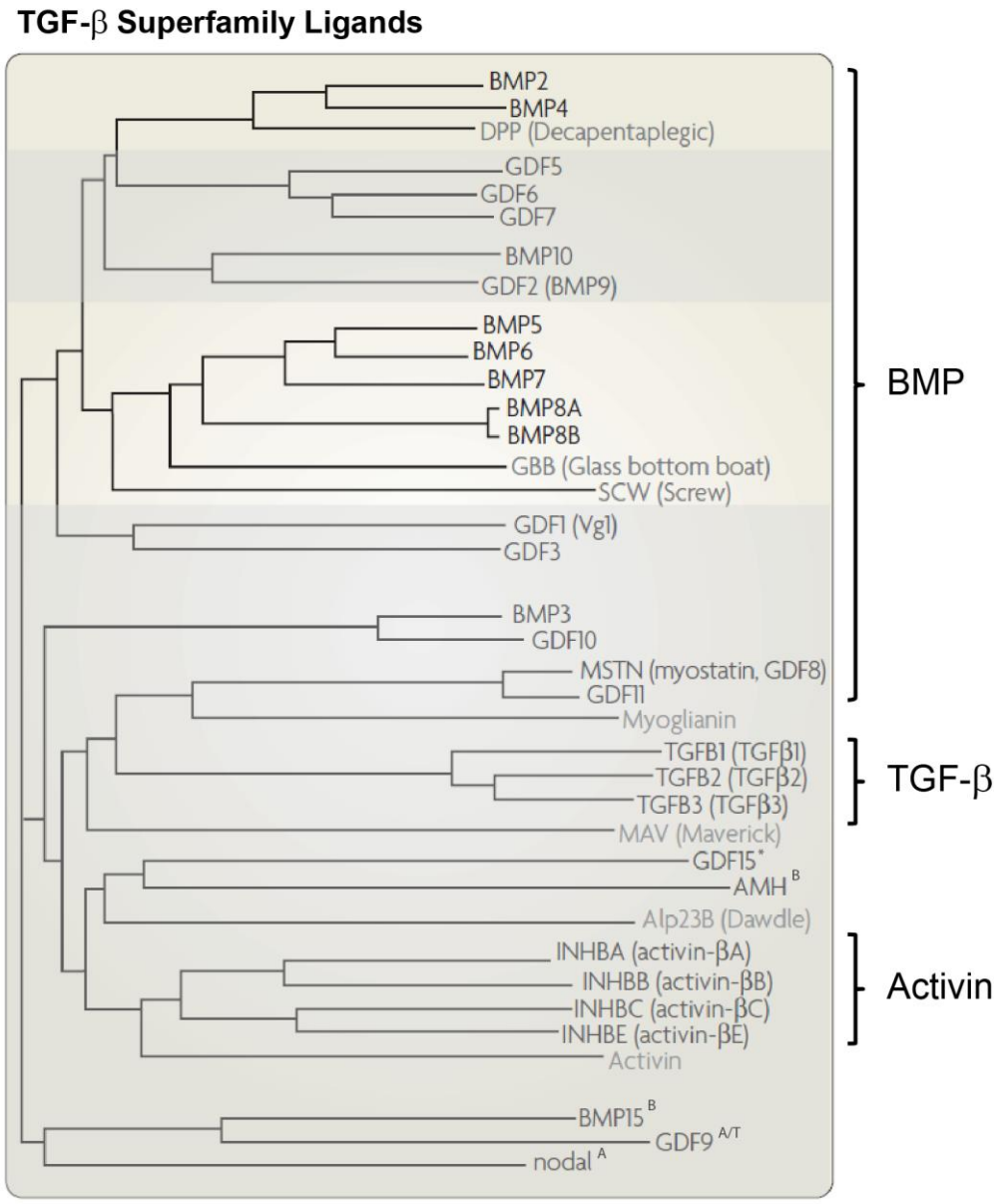


Figure 1.4. **Phylogeny of TGF-β superfamily ligands.** Phylogenetic tree based on protein alignments of human TGF-β superfamily ligands The BMP branch contains the most family members and includes the GDFs (growth differentiation factors), thus named because they were originally identified in regulating growth and differentiation. The BMP2/4 and BMP5/6/7/8 subgroups are highlighted in light gray. For ligands that fall outside of their branch, a superscript designates the subfamily to which they belong (A = Activin, B = BMP, T= TGF-β, * = unclassified). Included in the phylogeny are the *Drosophila* BMP ligands Dpp, Gbb, and Scw, as well as the *Drosophila* TGF-β/Activin-like ligands Myoglianin, Mav, Dawdle, and Activin. Adapted from (Schmierer and Hill, 2007).

BMPs are secreted signaling molecules that function as either homodimers or heterodimers. Each monomer in the mature BMP dimer has been described as a downturned left hand characterized by 2 "fingers," each of which is composed of a pair of antiparallel β-strands, extending from an alpha-helical "wrist" (**Fig. 1.5**). The monomers interact via their "palms" to form a dimer whose shape resembles a winged butterfly.

Like all TGF-βs, BMPs are initially produced as inactive propeptide chains (Bragdon et al., 2011; Ramel and Hill, 2012). The proprotein is characterized by an N-terminal signal peptide, a poorly-conserved prodomain region, a linker domain, and a C-terminal mature ligand domain that constitutes the monomer in the BMP dimer. The mature BMP ligand domain contains 7 conserved cysteines, six of which participate in intramolecular disulfide bonds that form the basis of the cystine knot structure characteristic of BMPs (Griffith et al., 1996; Scheufler et al., 1999). The remaining cysteine forms a disulfide bond between BMP monomers that is critical to stabilize the dimer.

Release of the biologically active mature ligand domain requires processing of the BMP propeptide through a series of proteolytic cleavage events in the linker region (Ramel and Hill, 2012) . These cleavages are mediated by subtilisin-like proprotein convertase, such as furin, which recognize and cleave dibasic residue motifs found in the linker region (Constam and Robertson, 1999; Cui et al., 1998; Cui et al., 2001; Ramel and Hill, 2012). Recently, a cleavage site (called NS) was identified in the N-terminal prodomain of Gbb, which yields a novel, large form of the Gbb ligand with distinct signaling activity (Akiyama et al., 2012; Fritsch et al., 2012). This NS site is conserved in many of the TGF-β ligands indicating that cleavage at alternative sites may be a general strategy for generating multiple ligand forms with different signaling outputs from one BMP propeptide.

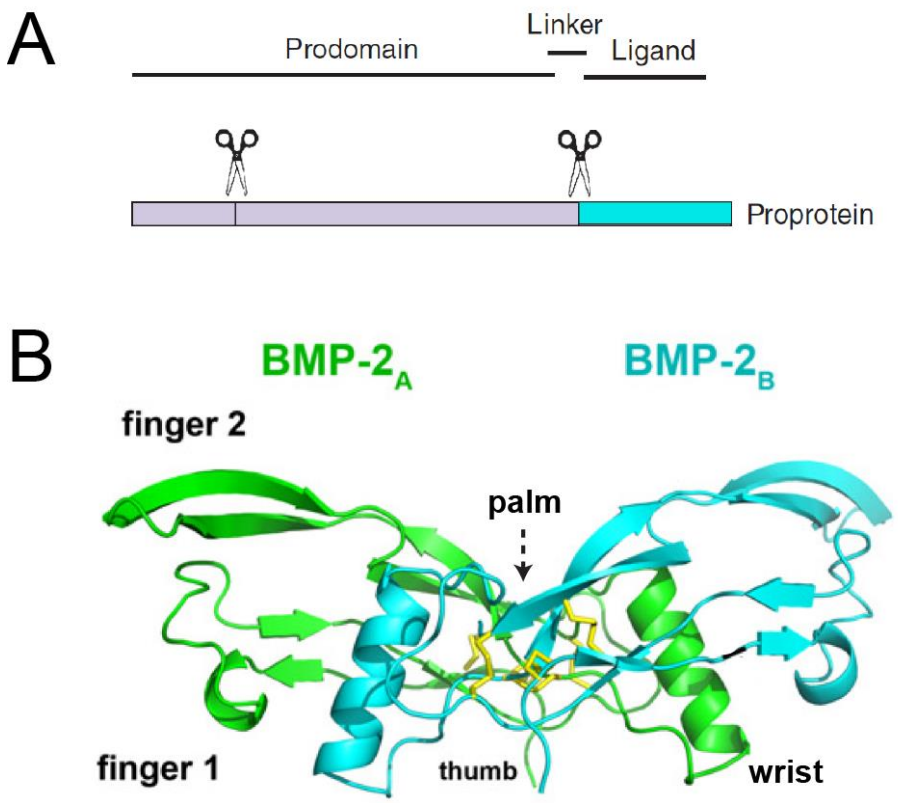


Figure 1.5. Structure of the BMP dimer. A. BMPs are produced as long proprotein chains consisting of a prodomain, linker region, and ligand domain (cyan). Cleavage events (scissors) in the linker domain release the mature ligand. Recently, cleavage sites have been identified in the N-terminal prodomain of the BMP proprotein. **B.** "Butterfly" view of the BMP2 homodimer. Each monomer ($BMP\text{-}2_{A,}$ green. $BMP\text{-}2_{B,}$ cyan). resembles a downturned left hand and interacts with each other via their "palms." Fingers 1 and 2 are pairs of anti-parallel β-strands extending perpendicularly from the "wrist" alpha helix. The N-terminus extends down resembling the "thumb." Yellow residues indicate cysteine residues that form the cystine knot.

Although ultimately dissociated from the mature ligand, the prodomain regulates BMP activity. In some cases, the prodomain can remain non-covalently associated with the mature ligand domain even after cleavage and regulate the stability, activity, or trafficking of the ligand. For instance, BMP-4 in association with its prodomain is targeted for degradation (Degnin et al., 2004). On the other hand, BMP-7 remains associated with its prodomain when secreted and is directed to the extracellular matrix potential via interactions between the BMP-7-prodomain complex and ECM proteins

such as fibrillin-1 (Gregory et al., 2005). Furthermore, the prodomain may act as a molecular chaperone as it is required for the proper folding and secretion of the closely – related Activin and TGF-β ligands. Expressing only the C-terminal ligand domains of either Activin or TGF-β1 forms protein aggregates that fail to be secreted (Gray and Mason, 1990). Given the relatedness of the TGF-β superfamily ligands, this requirement may also be true for BMP ligands.

Ligand-mediated receptor complex formation

TGF-β superfamily ligands coordinate the interaction of type I and type II receptors to form signaling complexes that phosphorylate the R-Smad intracellular transducers. The number of TGF-β superfamily ligands (>30) far outweighs the number of type I receptors (6) suggesting that the extracellular domains of type I receptors bind ligands promiscuously (Mueller and Nickel, 2012). Even so, type I receptors can be generally divided into groups largely based on which specific TGF-β superfamily branch of ligands they bind and transduce **(Figure 1.1, 1.6, & Table 1)**. In humans, BMP signals are mediated by four type I receptors: ALK1, ALK2, ALK3, and ALK6; and three type II receptors: BMPR2, ACVR2A, and ACVR2B. On the other hand, TGF-β ligands signal through ALK5 (TβRI) and TGFBR2 (TβRII), whereas Activins bind to the ALK4/7 type I receptors and the ACVR2A/2B type II receptors.

Exceptions to these ligand-receptor specificities, however, do exist. For instance, ALK1 binds BMP9 with high affinity, but can also bind TGF-β1 and modulate TGF-β-specific signaling (Brown et al., 2005; Finnson et al., 2008; Goumans et al., 2003; Mahlawat et al., 2012; Oh et al., 2000). Despite sharing sequence similarity with BMPs, several GDFs bind and signal through the Activin branch of receptors rather than BMP-specific receptors (Mueller and Nickel, 2012). Furthermore, the Activin type II receptors display greater promiscuity and participate in both BMP and Activin signaling.

TGF-βs and BMPs also exhibit different affinities for each receptor type. For example, TGF-βs bind to the TGF-β type II receptor (TβRII) with higher affinity than to the type I receptor (TβRI/ALK5) (Ehrlich et al., 2011). Furthermore, TGF-β receptor complex formation requires that the TGF-β ligand bind TβRII type II receptor first before being able to interact with TβRI/ALK5. In contrast, BMPs do not follow such a rigid paradigm and have been observed to bind both receptor types. However, receptor preferences are evident within the BMP family receptor. For example, BMP-2 exhibits high affinity for the type I receptors ALK3 and ALK6, but low affinity for the type II receptors BMPRII, ActRII, and ActRIIB (Ehrlich et al., 2011; Heinecke et al., 2009; Kirsch et al., 2000a; Nickel et al., 2001; Saremba et al., 2008; Sebald et al., 2004).

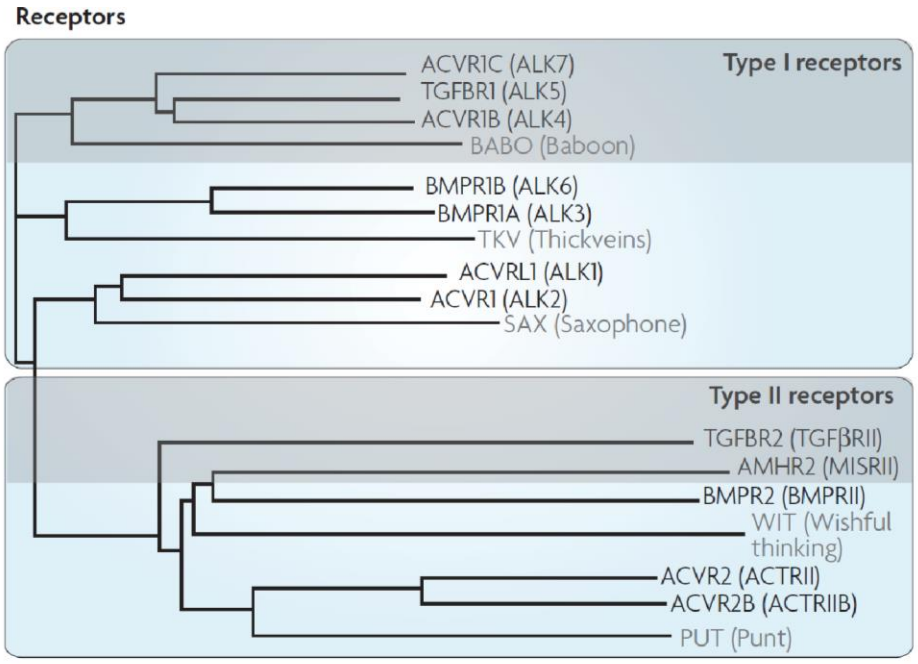
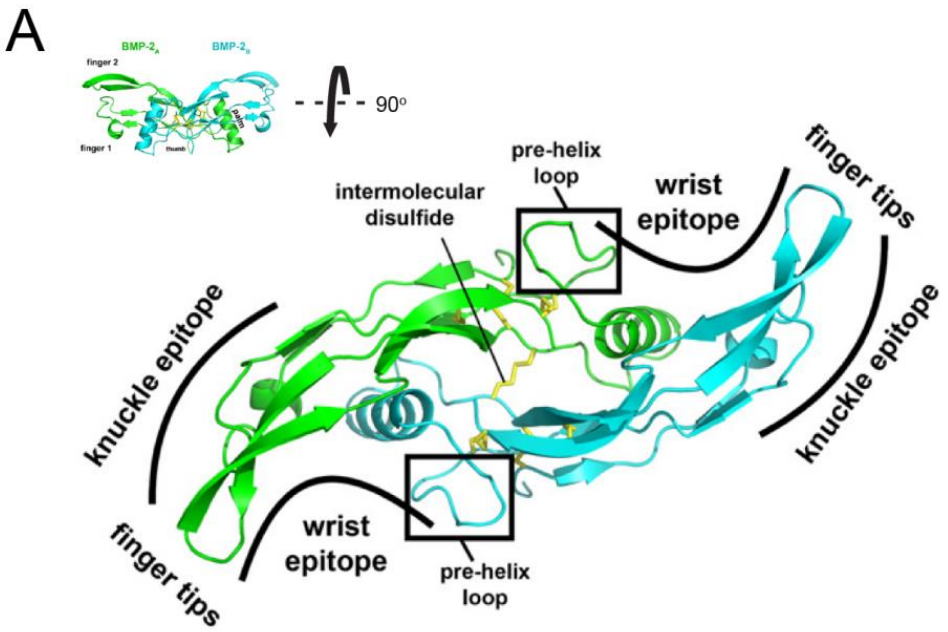


Figure 1.6. Phylogeny of the TGF-β superfamily receptors. Phylogenetic tree based on protein alignments of human TGF-β superfamily receptors. Type I and type II are distantly related receptor serine/threonine kinases. The type I receptors are grouped above of the type II receptors. TGF-β and Activin receptors are in the box shaded gray. BMP receptors are in the box shaded light blue. *Drosophila* orthologs of the TGF-β superfamily receptors are indicated in a lighter shade of gray. Adapted from (Schmierer and Hill, 2007).

In contrast, BMP-7 binds the type II receptors ActRII, ActRIIB, and BMPRIIB with intermediate affinities, but exhibits low affinity for BMPRII and the type I receptors BMPRIA and BMPRIB (Ehrlich et al., 2011; Greenwald et al., 2003; Heinecke et al., 2009; Sebald et al., 2004). Both BMP-6 and BMP-7 can also bind to ALK2, albeit with low affinities (Greenwald et al., 2003; Heinecke et al., 2009; Saremba et al., 2008). The affinity of BMP-7 for ALK2, however, is improved by 5-fold in the presence of the type II receptor ActRII (Greenwald et al., 2003).

Structural studies suggest that a signaling complex consists of a ligand dimer bound to two type I and two type II receptors (a 1:2:2 ratio) (Allendorph et al., 2006; Groppe et al., 2008; Lin et al., 2006), and the binding epitopes for these receptors have been mapped onto the BMP dimer (**Fig. 1.7A**). Each BMP type II receptor in the signaling complex binds to one BMP monomer via the "knuckle" epitope (Greenwald et al., 2003). The binding epitope for type I receptors, however, consists of inputs from both monomers—the interface between the "wrist" epitope from one monomer and the inside of the "fingers" of the other monomer (Kirsch et al., 2000b; Nickel et al., 2001). In contrast, the type II receptor for TGF-βs interacts with the "fingertips" of the TGF-β monomer. Moreover, structural studies indicate that the extracellular domains of the BMP receptors do not physically contact each other in the signaling complex, whereas the extracellular domains of the TGF-β type I and II receptors do (**Fig. 1.7B**) (Allendorph et al., 2006; Greenwald et al., 2003; Groppe et al., 2008; Hart et al., 2002; Weber et al., 2007; Zúñiga et al., 2005).

In addition to ligand-mediated complex formation, type I and type II receptors have also been observed to interact with each other in pre-formed complexes (PFC) independently from ligand (Ehrlich et al., 2011; Gilboa et al., 2000; Marom et al., 2011). Evidence exists for both homotypic (e.g., typeI:typeI) and heterotypic (typeI:type II) PFCs. Studies involving PFCs propose that the mode of receptor complex formation has



B

	Predicted signaling complex	Type I receptor binding site	Type II receptor binding site
TGF-β		Wrist	Fingertips
Activin		Wrist	Knuckle
BMP		Wrist	Knuckle

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Figure 1.7. Location of the type I and type II receptors binding epitope. Adapted from (Lin et al., 2006; Mueller and Nickel, 2012) **A.** The BMP2 homodimer rotated 90° with respect to the view in (**Fig. 1.5A**). Type I receptors bind to the wrist epitope which is composed of residues from both monomers. Type II receptors bind to the knuckle epitope of one monomer. The pre-helix loop is important for BMP-type I receptor specificity (Keller et al., 2004; Kotzsch et al., 2009; Mueller and Nickel, 2012). Intermolecular disulfide bond that stabilizes the dimer shown in yellow. **B.** Comparison of the ternary structure of ligand-receptor complexes for TGF-β, Activin, and BMP. Ligand dimers are in the same view as (**A**). Type I receptors (yellow) and type II receptors (red).

profound effects on signaling. For instance, BMP ligands that bind to PFCs appear to activate the Smad1/5/8-dependent, canonical BMP signaling pathway (Nohe et al., 2002; Hassel et al., 2003). In contrast, it has been suggested that BMP-induced signaling complexes (BiSCs), in which BMP ligands coordinate "free/unassociated" receptors into a signaling complex, activate a non-canonical p38/MAP kinase pathway (Nohe et al., 2002; Hassel et al., 2003).

Distinct roles for BMP Type I receptors

The existence of multiple BMP type I receptors (four in humans/mammals, two in *Drosophila*, etc.) lends itself to the genetic and functional redundancy of type I receptors in certain cell and tissue types (Murali et al., 2005; Rajagopal et al., 2009; Wine-Lee et al., 2004; Yoon et al., 2005). Distinct roles and functions, however, have been ascribed to each type I receptor in various developmental contexts. In some cases, redundancy is avoided by discrete, spatiotemporal expression patterns of the receptors (Dewulf et al., 1995; Li et al., 2011; Nikaido et al., 1999a; Nikaido et al., 1999b; Sanyal et al., 2002). For example, *in situ* hybridization studies reveal that nearly all tissues of the adult mouse express ALK-3 mRNA, whereas ALK-6 mRNA is restricted to the brain and lung (Dewulf et al., 1995).

Other mechanisms, however, must account for the non-redundant functions of different BMP type I receptors that are expressed in the same cells or tissues. Although ALK2 and ALK3 are expressed in the developing mouse heart, a conditional knockout of ALK3 leads to defects in cardiac morphogenesis indicating that ALK2 does not compensate for the absence of ALK3 in this process (Gaussin et al., 2002; Gu et al., 1999; Ramsdell and Yost, 1999). In the mouse-derived 2T3 cells, ALK3 (BMPRIA) induces adipocyte differentiation, whereas ALK6 (BMPRIB) induces osteoblast differentiation (Chen et al., 1998a).

These disparities in function may reflect differences in ligand-receptor affinities. For instance, BMP-2 and BMP-4 preferentially bind ALK3/6, whereas BMP-6/7 exhibit a preference for ALK2 (ten Dijke et al., 1994; Ebisawa et al., 1999; Heinecke et al., 2009; Kirsch et al., 2000a; Lavery et al., 2008; Saremba et al., 2008). Binding studies also indicate that BMP-2 can further discriminate between its two preferred receptors as it binds with even higher affinity to ALK3 than ALK6 (Saremba et al., 2008). Consistent with these reported binding affinities, BMP-2 has been observed to signal preferentially through ALK3 over ALk6 in cell culture (Ho and Bernard, 2009). Alternatively, type I receptors that have overlapping expression patterns might elicit diverse responses by differentially phosphorylating Smad1/5/8, each of which may have different roles in a cellular context.

Role of the BMP type I receptors during *Drosophila* development

The highly conserved BMP signaling pathway plays important roles in the development of the fruit fly *Drosophila melanogaster*. BMP signaling has been shown to regulate diverse processes such as apoptosis, embryonic dorsal-ventral axis specification, neuromuscular junction development, stem cell niche maintenance and wing patterning. The number of BMP signaling components, however, is markedly fewer in *Drosophila* than humans—the most dramatic difference being in the number of BMP ligands (**Fig. 1.8**). Whereas humans have ~20 BMPs, the *Drosophila* genome contains genes for just three: *decapentaplegic (dpp), glass bottom boat (gbb), and screw (scw)*. Similarly, there are fewer type I and type II receptors that mediate BMP signaling. In *Drosophila*, the genes *thick veins (tkv)* and *saxophone (sax)* encode the two BMP type I receptors, whereas *punt (put)* and *wishful thinking* (*wit*) encode the two type II receptors. Lastly, the BMP-specific group of R-Smads, Smad1/5/8, is represented by the sole *Drosophila* ortholog Mothers against Dpp (Mad), which when phosphorylated interacts

with the Smad4 (co-Smad) ortholog Medea and other transcription factors , such as Schnurri, to regulate target gene expression (Blitz and Cho, 2009; Marty et al., 2000; Massagué et al., 2005; Wu and Hill, 2009).

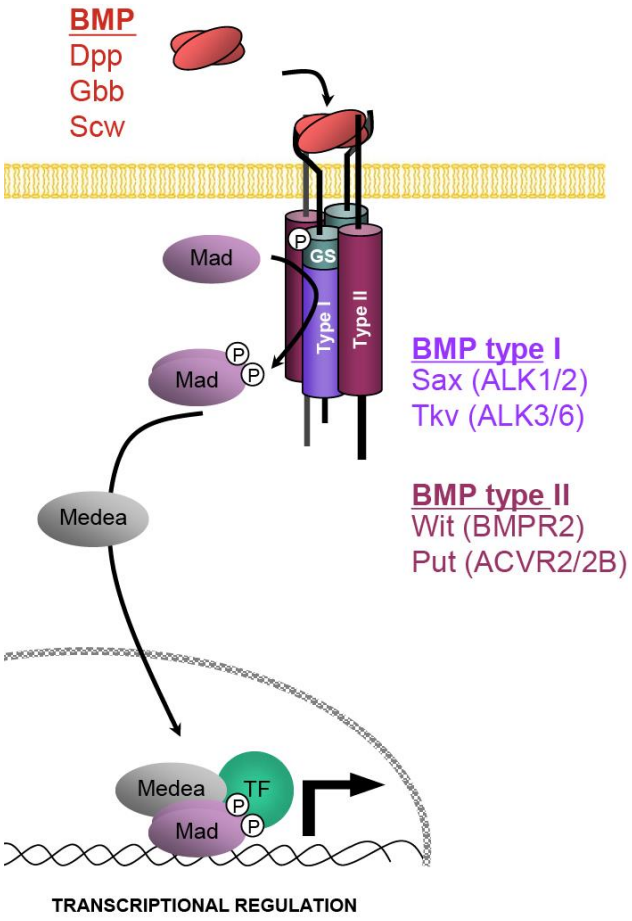


Figure 1.8. The *Drosophila* BMP signaling pathway. In contrast to humans, the BMP signaling pathway in *Drosophila* consists of far fewer components. Three *Drosophila* ligands Decapentaplegic (Dpp), Glass bottom boat (Gbb), and Screw (Scw) signal through the type I receptors Saxophone (Sax) and Thickveins (Tkv), and the type II receptors Wishful thinking (Wit) and Punt (Put). Receptor complex formation activates the type I receptor which phosphorylates the Mad, the sole orthlog of Smad1/5/8. pMad complexes with the Smad4 ortholog Medea and regulates target gene expression in the nucleus. The human orthologs of the receptors are indicated in parentheses.

Phylogenetic analyses indicate that Tkv is the *Drosophila* ortholog of ALK3/6, whereas Sax is the ALK1/2 ortholog. One of the main differences between Tkv and Sax are respective affinities for Dpp, Gbb, and Scw. In the wing, phenotypes associated with Dpp overexpression are preferentially suppressed by overexpression of dominant negative Tkv (Haerry et al., 1998; Nguyen et al., 1998). In contrast, wing phenotypes associated with either Gbb or Scw overexpression are preferentially suppressed by overexpression of dominant negative Sax (Haerry et al., 1998; Nguyen et al., 1998). These genetic experiments suggest that Tkv is the high affinity receptor for Dpp, whereas Sax exhibits higher affinity for Gbb and Scw. Similar results were observed in the embryo. Embryonic injection of dominant negative *tkv* or *sax* RNA preferentially suppressed the dorsalizing effects of injecting *dpp* or *s*cw RNA, respectively (Nguyen et al., 1998). Additionally, coimmunoprecipitation experiments support a model in which Tkv functions as the high affinity receptor for Dpp, and Sax functions as the high affinity receptor for Gbb (Haerry, 2010; Le and Wharton, 2012).

The *Drosophila* BMP ligands Dpp and Gbb have human orthologs as well. Based on phylogenetic analysis Dpp clusters with the BMP2/4 subgroup of BMP ligands, whereas Gbb is most similar to the BMP5/6/7/8 subgroup (Newfeld et al., 1999). Several cross-species studies demonstrating functional conservation within the BMP subgroups support this phylogenetic classification. Like their human orthologs, purified recombinant Dpp and Gbb induced osteogenesis in a rat subcutaneous bone induction model (Sampath et al., 1993). Conversely, human BMPs can functionally replace their *Drosophila* orthologs. For example, a chimeric transgene in which human BMP4 ligand sequence replaced that of *dpp* in the *dpp* locus rescued dorsal-ventral patterning defects exhibited by *dpp* mutant embryos (Padgett et al., 1993). Additionally, Tkv binds to BMP2 with high affinity in binding assays performed in cell culture, and neither excess BMP7 nor excess Gbb could compete with BMP2 for Tkv-binding (Penton et al., 1994). This

result suggests that a binding preference for Tkv is conserved in the BMP2/4/Dpp subfamily of BMP ligands.

Similarly, members of the BMP5/6/7/8 subfamily of ligands can function in place of Gbb. For instance, chimeric constructs of the BMP5, -6, & -7 ligand domains fused to the Gbb prodomain rescued of lethality associated with *gbb* mutants (gbb^{D20}/Df) (Fritsch et al., 2010). Importantly, full-length BMP7 exhibited only partial rescue of *gbb* lethality suggesting that divergence of the prodomain sequence accounts for deviations in the activity of Gbb and BMP7. (Fritsch et al., 2012).

Screw, on the hand, clusters with neither the BMP2/4 nor the BMP5/6/7/8 subgroups and was originally proposed to be the ortholog of GDF-3 (Newfeld et al., 1999). Recent data, however, suggest that *screw* is a rapidly evolving paralog of *gbb* resulting from a gene duplication of *gbb* in Diptera (Fritsch et al., 2010; Van der Zee et al., 2008). The inability of the BMP5,-6,-7, &-8 ligand domains to rescue *scw* mutant lethality further supports the functional divergence of *scw* from the BMP5/6/7/8/(Gbb) subgroup (Fritsch et al., 2010).

Tkv and Sax are essential mediators of BMP signaling that regulate a variety of tissue and organ development in *Drosophila*. These include dorsal-ventral axis specification during embryogenesis (Affolter et al., 1994; Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Schupbach and Wieschaus, 1989; Terracol and Lengyel, 1994; Xie et al., 1994), regulating synaptic growth and development at the neuromuscular junction(McCabe et al., 2004; Rawson et al., 2003), and patterning the adult wing (Bangi and Wharton, 2006b; Brummel et al., 1994; Haerry et al., 1998; Nellen et al., 1994; Penton et al., 1994; Ray and Wharton, 2001; Singer et al., 1997; Tanimoto et al., 2000; Terracol and Lengyel, 1994). Accordingly, loss of either receptor during these processes is associated with a concomitant decrease in phosphorylation of Mad, the intracellular transducer of the BMP signaling pathway and type I receptor substrate

(Bangi and Wharton, 2006b; Dorfman and Shilo, 2001; McCabe et al., 2004; Rawson et al., 2003; Tanimoto et al., 2000). However, despite the requirement for Sax and Tkv in overlapping domains, these receptors are not functionally equivalent in a number of tissues, underscoring their differing contributions during development. The specific findings regarding the distinct contributions of Sax and Tkv in several developmental processes are outlined in the following sections.

Embryonic dorsal-ventral axis specification

During embryogenesis BMP signaling is required for dorsal-ventral axis specification. The BMP ligands Dpp and Scw signal through Tkv and Sax to specify dorsal fates such as the dorsal ectoderm and amnioserosa (O'Connor et al., 2006; Shimmi et al., 2005b). This BMP signaling activity can be visualized as a gradient of pMad distribution that is initially refined to a sharp peak running along the dorsal midline during the blastoderm stage (Dorfman and Shilo, 2001). This domain of high BMP signaling coincides with the cells that give rise to the dorsal-most fate, the amnioserosa. During germ band extension pMad then becomes more broadly distributed, expanding dorsolaterally.

Both Sax and Tkv are required for dorsal-ventral axis specification because embryos that lack function of either receptor display "ventralized" phenotypes. Ventralized embryos have defects in dorsally derived structures such as the head, filzkorper, and dorsal hairs, as well as expansion of ventral features such as the denticle belts (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Schupbach and Wieschaus, 1989; Terracol and Lengyel, 1994). However, ventralization phenotypes of embryos mutant for *sax* are weaker than *tkv* mutant embryos (Brummel et al., 1994; Nellen et al., 1994).

These "ventralized" phenotypes are reminiscent of embryos mutant for the BMP ligands *dpp* and *scw* (Arora et al., 1994; Irish and Gelbart, 1987; Wharton et al., 1993). *dpp* is required for all dorsal fates in the embryo, whereas *scw* is only required to specify amnioserosa (Arora et al., 1994; Irish and Gelbart, 1987; Wharton et al., 1993). Subsequent studies suggested that Tkv and Sax mediate Dpp and Scw signals in the embryo, respectively (Neul and Ferguson, 1998; Nguyen et al., 1998). These studies, however, also highlight intrinsic differences in the signaling activities between Sax and Tkv. RNA injection of a constitutively active form of *sax* alone could not induce dorsal structures in embryos "ventralized" by loss of *dpp*. Injection of constitutively active *tkv* RNA, however, could rescue dorsal structures in *dpp* mutant embryos in a dose-dependent manner. These results suggest that the signaling activity of constitutively-active Tkv is higher than that of constitutively-active Sax, since only constitutively-active Tkv was sufficient to rescue *dpp* mutant embryo phenotypes.

Interestingly, coinjection of RNA coding for constitutively-active forms of *sax* and *tkv* synergistically promoted the formation of dorsal structures and rescued nearly 100% of ventralized *dpp* mutant embryos (Neul and Ferguson, 1998; Nguyen et al., 1998). These results were some of the first data to suggest that Sax and Tkv may form heteromeric signaling complexes that function to synergistically activate BMP signaling. Subsequently, purified Scw:Dpp heterodimers were shown to have 10-fold higher signaling activity in cell culture than their homodimer counterparts (Shimmi et al., 2005b). Furthermore, signaling induced by Scw:Dpp heterodimers was dependent on both Sax and Tkv, as RNAi knockdown of either receptor or both receptors dramatically reduced the level of Mad phosphorylation induced by Scw:Dpp heterodimers. Taken together, these data suggest that Scw:Dpp heterodimers coordinates the formation of Sax:Tkv-containing receptor complexes that exhibit synergistic signaling activity.

Neuromuscular junction development

Several studies have demonstrated the importance of BMP signaling in regulating growth and formation of the synapses between muscles and the motoneurons that innervate them (Aberle et al., 2002; Marqués et al., 2002; Marqués, 2005; McCabe et al., 2003; McCabe et al., 2004; Rawson et al., 2003). Synapses at the NMJ are initially established during embryogenesis but undergo significant changes to accommodate the dramatic growth during larval stages. Communication between the motoneurons and muscle fibers is critical for proper NMJ development. Gbb acts as a retrograde signal originating postsynpatically from the muscle to activate BMP signaling in the presynaptic motoneuron (Marqués et al., 2003; McCabe et al., 2003). Activation of BMP signaling by Gbb in the motoneuron is dependent on the presence of both type I receptors Tkv and Sax as well as the type II receptor Wit. Consistent with the importance of BMP signaling in NMJ development, loss of *gbb, tkv, sax,* or *wit* reduces pMad in embryonic motoneurons, impairs synaptic transmission, decreases the size of the NMJ, and reduces the number of synaptic boutons (structures at the NMJ that contain the active zones of synaptic transmission) (Aberle et al., 2002; Marqués et al., 2002; McCabe et al., 2003; McCabe et al., 2004; Rawson et al., 2003).

Genetic experiments indicate that Sax and Tkv participate with Wit to activate BMP signaling. For instance, removing one copy of either *sax* or *tkv* in a *wit* heterozygous background significantly reduces the size of the synapse at the NMJ (McCabe et al., 2004). This genetic interaction in either transheterozygous combination suggests that both type I receptors participate with *wit* in the same pathway. Furthermore, loss of either *sax* or *tkv* leads to a loss of pMad accumulation in the nuclei of embryonic motoneurons, which phenocopies *wit* mutant embryos. These results suggest that Sax, Tkv, and Wit signal together.

Differences in Sax and Tkv function, however, are also apparent at the NMJ despite both being essential for the proper growth and development of the NMJ. As *sax* and *tkv* are type I receptors that function downstream of *wit*, experiments were performed to test whether *sax* or *tkv* could rescue NMJ defects associated with *wit* mutants (McCabe et al., 2004). Chimeric receptors consisting of the extracellular domain of Tkv fused to the intracellular domain of Wit (Tkv::Wit) and the extracellular domain of Wit fused to the intracellular domain of Tkv (Wit::Tkv) were constructed. Swapping the intracellular and extracellular domains of the type I and type II receptors renders the chimeric receptors incompatible with the endogenous receptors. Therefore, changes in BMP signaling that result from expressing these chimeras should originate from complexes composed solely of the chimeric receptors. Coexpression of Tkv::Wit and Wit::Tkv rescued both the size of the NMJ and lethality associated with *wit* mutants. Coexpression of the corresponding Sax::Wit and Wit::Sax chimeras, however, did not rescue *wit* mutants. These results suggest that the intracellular domain of Tkv is sufficient to propagate signals from Wit, whereas Sax cannot despite being required for both BMP signaling and proper growth at the NMJ.

Wing vein patterning

BMP signaling also plays an important role during wing development. The adult wing is derived from the larval precursor known as the wing imaginal disc. Both Tkv and Sax play critical roles in mediating BMP signals that regulate cell proliferation and patterning along the anterior-posterior axis of the wing imaginal disc. It is during wing development however, that Sax and Tkv display perhaps the greatest disparity in their BMP signaling behaviors.

The wing imaginal disc is a tear-shaped, sac-like structure composed of a single-cell epithelium with distinct dorsal-ventral and anterior-posterior axes (**Fig. 1.9**). The

wing disc gives rise to three different adult structures: the notum, the hinge, and the wing. The notum is the dorsal segment of the adult thorax, and the hinge is where the wing attaches to the notum. The wing disc can therefore be divided into regions corresponding to these structures. The cells that will be specified to become the notum are located in the dorsal half of the wing disc, whereas the cells that will become the adult wing are located in the oval-shaped wing pouch at the center the ventral portion of the disc. Surrounding the wing pouch are the cells that will give rise to the hinge.

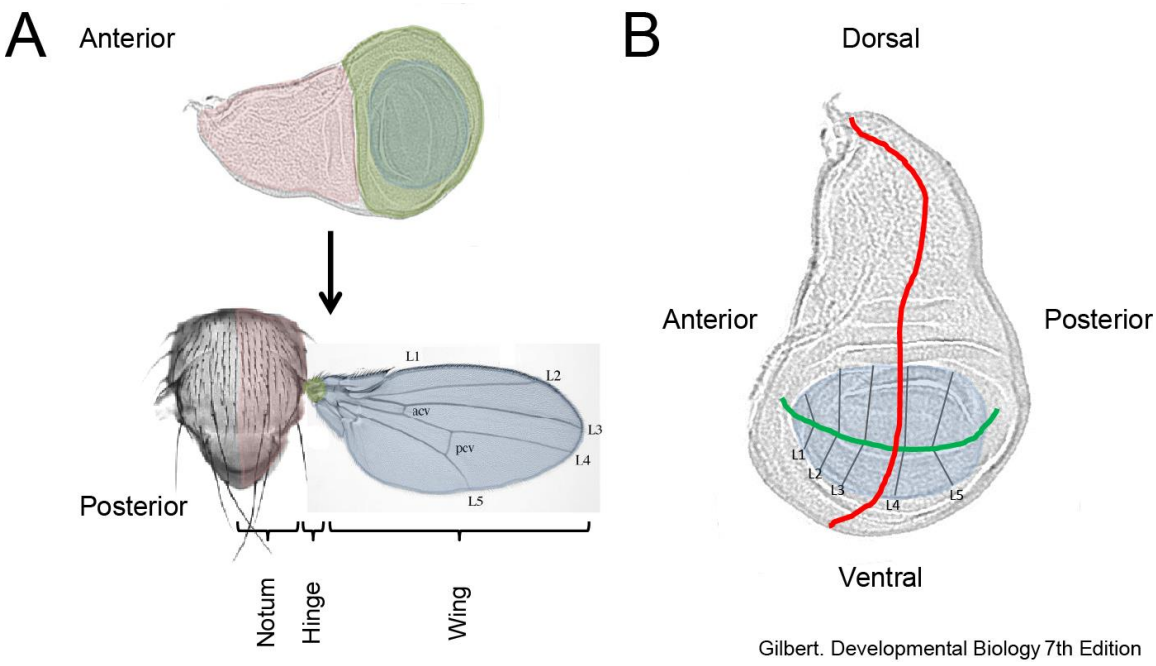


Figure 1.9 The *Drosophila* wing imaginal disc. A. The adult notum (pink), hinge (green), and wing blade (blue) are derived from cells in specific regions of the larval wing imaginal disc. The adult wing is characterized by an invariant pattern of five longitudinalveins (L1-L5), an anterior crossvein (acv) between L3 and L4, and a posterior crossvein (pcv) between L4 and L5. **B**. The wing imaginal disc is a tear-shaped, single-celled epithelium with distinct anterior/posterior and dorsal/ventral axes the boundaries of which are shown in red and green, respectively. The position of the vein primordia (L1-L5) that give rise to the L1-L5 veins in the adult wing are indicated in the wing pouch (shaded blue).

The adult wing is characterized by an invariant pattern of five longitudinal veins (L1-L5) that run proximodistally, an anterior crossvein, and a posterior crossvein (**Fig. 1.9A**). The tissues between the longitudinal veins are known as intervein regions. BMP signaling plays a critical role in larval wing disc patterning, which will determine the positioning of the longitudinal veins in the adult wing (Blair, 2007; Raftery and Umulis, 2012). In the larval wing disc, *sax* is reportedly expressed uniformly, whereas expression of *tkv* is low in the medial portion of the disc and increases laterally in both the anterior and posterior compartments (Brummel et al., 1994; Ogiso et al., 2011; Tanimoto et al., 2000). Sax and Tkv mediate signals from Gbb, Dpp, and potentially Gbb:Dpp heterodimers to establish a gradient of BMP signaling activity represented by the graded distribution of pMad (Affolter and Basler, 2007; Bangi and Wharton, 2006a; Bangi and Wharton, 2006b; Raftery and Umulis, 2012; Tanimoto et al., 2000).

This pMad gradient consists of a sharp peak running along the A/P boundary in the posterior compartment and a lower, broad peak in the anterior compartment (**Fig. 1.10A**). The anterior and posterior peaks are separated by a "trough" of low BMP signaling activity that coincides with the A/P boundary—a region where *tkv* expression is lowest. As pMad is a transcriptional regulator, the pMad gradient differentially affects the expression of prepatterning genes, which include *spalt (sal), optomoter blind (omb), and brinker (brk),* that are critical for the position of L2 and L5 (Blair, 2007; Raftery and Umulis, 2012). BMP signaling therefore imparts positional information that cells "read" to be specified properly as either vein or intervein tissue.

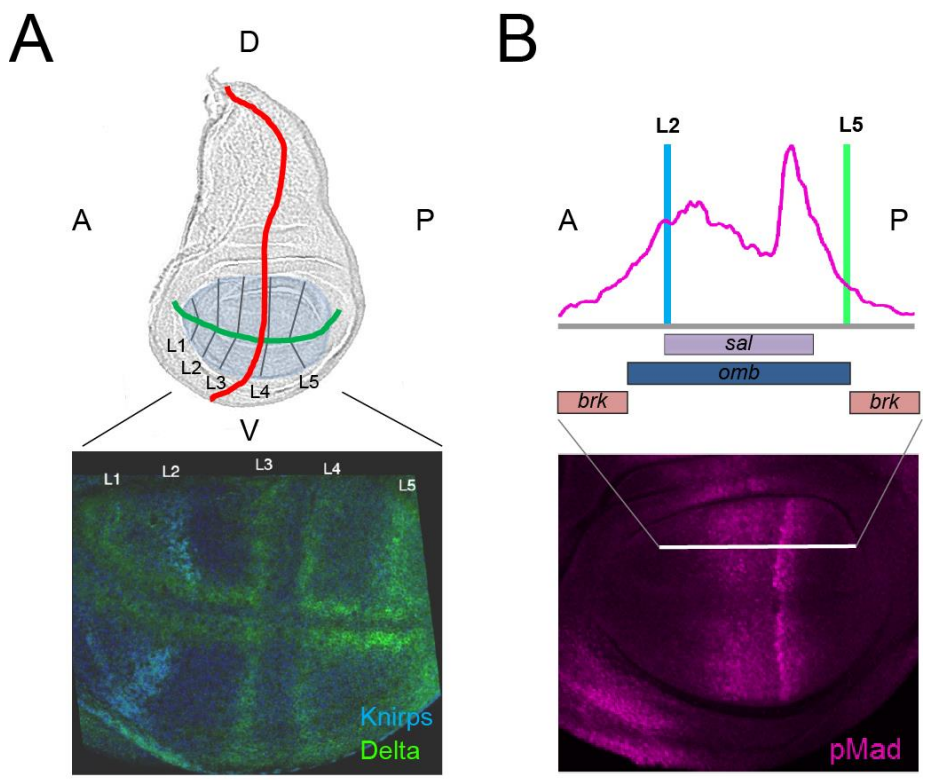


Figure 1.10 BMP signaling patterns the wing imaginal disc. A. (*top*) Positions of the L1-L5 vein primordial in the wing pouch of the wing imaginal disc (top). A/P boundary (red). D/v boundary (green). **A**. (*bottom*) L2 primordium is marked by α-Knirps staining (blue). L1, L3-L5 primorida is marked by α-Delta staining (green). **B.** (top) The positions of the L2 and L5 primordia is established at the anterior edge of the *sal* (purple rectangle) expression domain and the posterior boundary of *omb* (blue rectangle) and *brk* (pink rectangles) expression, respectively. A BMP signaling activity gradient is critical in establishing the expression domains of *sal*, *omb*, and *brk*. The BMP signaling activity gradient is depicted here as a pMad intensity profile corresponding to the white line in the wing disc stained with α-pMad, **B.** (bottom). The intensity profile reveals the characteristic shape of the pMad gradient marked by a sharp, peak of intensity in the posterior compartment, and a low but broad peak of intensity in the anterior compartment. The peaks are separated by a "trough" of low pMad over the A/P boundary.

Regulation by the pMad gradient creates the nested expression of *sal, omb,* and *brk* (**Fig. 1.10B**). BMP signaling directly and indirectly regulates *sal* expression, which is confined to the medial domain of the wing pouch (de Celis and Barrio, 2000; de Celis and Barrio, 2009). In contrast, the expression domain of *brk* is restricted to the lateral

regions of the wing disc as a result of being directly repressed by BMP signaling in the medial wing disc (Jazwinska et al., 1999; Moser and Campbell, 2005; Müller et al., 2003). *omb* is expressed in a wide domain that encompasses the *sal* domain, and repression by Brk sets the boundaries of the *omb* expression domain (Grimm and Pflugfelder, 1996; Sivasankaran et al., 2000). Thus, BMP signaling indirectly regulates *omb*.

The positions for the L2 and L5 vein primordia correspond to the expression domain boundaries of these prepatterning genes (**Fig. 1.10**). For instance, patterning genes such as *knirps (kni)* are activated at the anterior edge of the *sal* domain where they will specify L2 (de Celis and Barrio, 2000). Conversely, L5 is specified in the posterior compartment by factors such as *abrupt (abr)* which is activated at the boundary between *omb* and *brk* (Cook et al., 2004).

Altering the function or gene dosage of either *sax* or *tkv* can impact the shape of the pMad gradient, expression of BMP target genes, as well as the vein pattern and morphology of the adult wing, suggesting that both Sax and Tkv are essential for proper BMP signaling during wing development (Bangi and Wharton, 2006b; Brummel et al., 1994; de Celis, 1997; Khalsa et al., 1998; Ogiso et al., 2011; Penton et al., 1994; Ray and Wharton, 2001; Singer et al., 1997; Tanimoto et al., 2000; Terracol and Lengyel, 1994). Mitotic clones in wing discs that lack *tkv* function exhibit an absence of Mad phosphorylation and *sal* expression (positive BMP target), indicating that *tkv* is required for mediating BMP signals in the wing disc (Singer et al., 1997; Tanimoto et al., 2000). Furthermore, hypomorphic *tkv* alleles whether in homozygous/homoallelic or heteroallelic combinations can result in a “thickened” veins phenotype or loss of L4 vein tissue in adult wings (de Celis, 1997; Terracol and Lengyel, 1994). These observations are consistent with *tkv*’s role in BMP signaling during wing development.

The requirement for *sax* during wing development however, differs from *tkv*. In contrast to *tkv* mutants, adults that are mutant for *sax* display less severe wing phenotypes. For instance, wings from adults that are homozygous for the hypomorphic allele sax^{P} display a loss of the PCV and minor ectopic vein material at the distal tips of L2 and L5, whereas transheterozygous sax^{1}/sax^{2} (GOF/antimorphic alleles) adults display largely wild-type wings (Letsou et al., 1995; Nellen et al., 1994; Twombly et al., 2009). Furthermore, large *sax* loss-of-function clones that encompass the entire wing have normal wing vein patterns, suggesting that *sax* is not completely required for wing development (Ray and Wharton, 2001). Therefore, the developing wing must have compensatory mechanisms to deal with the complete loss of *sax*.

sax, however, does affect formation of the pMad gradient in the wing disc. For instance, *sax* null clones in the wing disc exhibit a dramatic loss of pMad and *sal* expression, indicating that *sax* facilitates BMP signaling (Bangi and Wharton, 2006b; Singer et al., 1997). Additionally, the pMad gradient is sensitive to the dosage of *sax,* as reducing *sax* copy number expands pMad distribution along the anterior-posterior axis of the wing disc (Bangi and Wharton, 2006b). In contrast to large *sax* null clones that span the entire wing, loss of *sax* in some smaller *sax* null clones induced ectopic vein tissue, depending on their position in the wing. This result suggests that small *sax* clones create discontinuities in the pMad gradient in the larval wing disc that result in the specification of ectopic veins (Ray and Wharton, 2001).

Much like in the embryo, genetic experiments indicate that Sax and Tkv differentially affect signaling induced by BMP ligands in the wing disc. For instance, wing defects associated with Gbb or Dpp overexpression are preferentially suppressed by coexpressing the dominant negative forms of Sax or Tkv, respectively (Haerry et al., 1998; Nguyen et al., 1998). These results suggest that Sax is the high affinity receptor for Gbb, whereas Tkv is the high affinity receptor for Dpp. Furthermore, these observations

have been interpreted to indicate that Gbb and Dpp signal primarily through their respective "high affinity" receptors.

Intriguingly, results from a number of experiments do not comport with Sax being the primary facilitator of Gbb signaling during wing development despite their purported high affinity for each other. For example, wing phenotypes associated with *sax* loss of function do not phenocopy *gbb* loss of function phenotypes as would be expected if Gbb were signaling through Sax (Khalsa et al., 1998; Ray and Wharton, 2001; Singer et al., 1997). Additionally, the observation that removing a copy of *sax* does not enhance wing phenotypes in a *gbb* mutant background is inconsistent with Sax being the mediator of Gbb signals (Khalsa et al., 1998). In contrast, reducing *tkv* function exacerbated wing phenotypes associated with *gbb* hypomorphs indicating that *tkv* can mediate Gbb signaling. But perhaps the greatest discrepancy was the observation that wing phenotypes associated with *gbb* overexpression are suppressed by coexpressing wild-type *sax* (Bangi and Wharton, 2006b). This result indicated that wild-type *sax*, much like its dominant negative form, can actually inhibit Gbb-induced BMP signaling rather than mediate it. Furthermore, this ability to inhibit BMP signaling is an endogenous behavior of *sax,* since reducing the gene dosage of *sax* enhanced *gbb*-overexpression phenotypes. This lay in stark contrast to the corresponding experiment in which *gbb* overexpression wing phenotypes were suppressed by reducing the gene dosage of *tkv*—consistent with *tkv* being a type I receptor that facilitates BMP signaling. Therefore, the contribution of Sax to BMP signaling during wing development can involve both facilitating and antagonizing functions with regard to BMP signaling, depending on the molecular context, gene dosage, or signaling component stoichiometry.

A proposed model reconciles these disparate behaviors by predicating Sax's activity on the identity of the other type I receptor in the signaling complex (**Fig. 1.3)** (Bangi and Wharton, 2006b). In this model, the combination of Sax with Tkv forms a

complex that is competent for signaling. In contrast, a Sax:Sax complex would bind ligand but be incompetent for transducing signals (i.e. phosphorylating Mad). This inability of Sax to transduce signals alone is also apparent at the NMJ, where the intracellular domain of Sax was not sufficient to propagate Wit signals (McCabe et al., 2004). Moreover, it has been suggested that the antagonistic behavior of Sax serves to regulate the diffusion range and availability of its preferred ligand Gbb and thereby acts as a buffering mechanism that contributes to the robustness of the pMad gradient (Bangi and Wharton, 2006b).

It is unclear, however, whether the kinase activity of Sax is activated in the presence of Tkv or if it remains latent within a Sax:Tkv complex. Although the observed synergy displayed by Tkv and Sax in the embryo and developing wing suggests the former scenario, these experiments relied on using constitutively-active forms of Tkv and Sax (Haerry et al., 1998; Neul and Ferguson, 1998; Nguyen et al., 1998). Additionally, if the latter case is true, Sax might be acting as a "silent" co-receptor to accommodate binding of Gbb:Dpp heterodimers.

The functions of Sax and Tkv are demonstrably different in the well-characterized developmental processes of embryonic dorsal-ventral axis specification, NMJ development, and wing patterning. Tkv and its constitutively-active form display greater signaling capacity than its Sax counterparts particularly in the embryo and the developing wing. The role Sax, on the other hand, seems to mainly augment Tkv-mediated signaling. This augmentation may serve to modulate signaling intensity or to determine which BMP ligands initiate signaling. For instance, Sax may cooperate with Tkv to discriminate Gbb:Dpp and Scw:Dpp heterodimers from the corresponding homodimers. And while Sax and Tkv have different affinities for Dpp, Gbb, and Scw, these binding preferences alone cannot account for the differences in the signaling activity of Sax and Tkv. For instance, Tkv facilitates Gbb signaling in the developing

wing, whereas Sax antagonizes Gbb signals despite being the preferred receptor for Gbb. Furthermore, overexpression of constitutively-active *tkv* is sufficient to rescue embryos mutant for *dpp*—the preferred ligand for Tkv. In contrast, overexpression of constitutively-active *sax* cannot rescue *dpp* mutant embryos suggesting that despite uncoupling the requirement for ligand the "activity" of constitutively active *sax* is not enough. These results suggest that the signaling/kinase activities of Sax and Tkv are intrinsically different with Sax displaying a dual behavior in its ability to facilitate and inhibit BMP signaling depending on the developmental context. The molecular underpinning of Sax's disparate behavior is unknown, but mechanistic insights may lie in the differences between the sequence and structure of Sax and Tkv.

Protein domains of the type I receptor

Like all TGF-β/BMP type I receptors Sax and Tkv contain sequences that correspond to a cysteine-rich, extracellular ligand-binding domain, a single-pass transmembrane domain, and a well-conserved, intracellular kinase domain characterized by a glycine-serine rich domain (GS domain) required for activation of the type I receptor kinase (Wrana et al., 1994a; Franzén et al., 1995; Huse et al., 2001; Massagué, 1998; Brummel et al., 1994) (**Fig. 1.11 & 1.12**). Despite the overall sequence conservation of the kinase domain, differences in particular regulatory regions such as the L45 loop and the E6 loop can impact signaling specificity and activity. The L45 loop determines R-Smad substrate specificity such that BMP type I receptors target Smad1/5/8 and TGF-β/Activing type I receptors target Smad2/3 (Feng and Derynck, 1997; Persson et al., 1998). The E6 loop plays a critical role in activation of the type I receptor as indicated by an E6 loop mutation that negatively impacts transphosphorylation by the type II receptor (Weis-Garcia and Massagué, 1996). Additionally, the E6 loop has been implicated in type I receptor dimerization (Huse et

al., 1999; Weis-Garcia and Massagué, 1996). Therefore, differences between the sequence of Sax and Tkv in particular protein domains may account for their distinct behaviors.

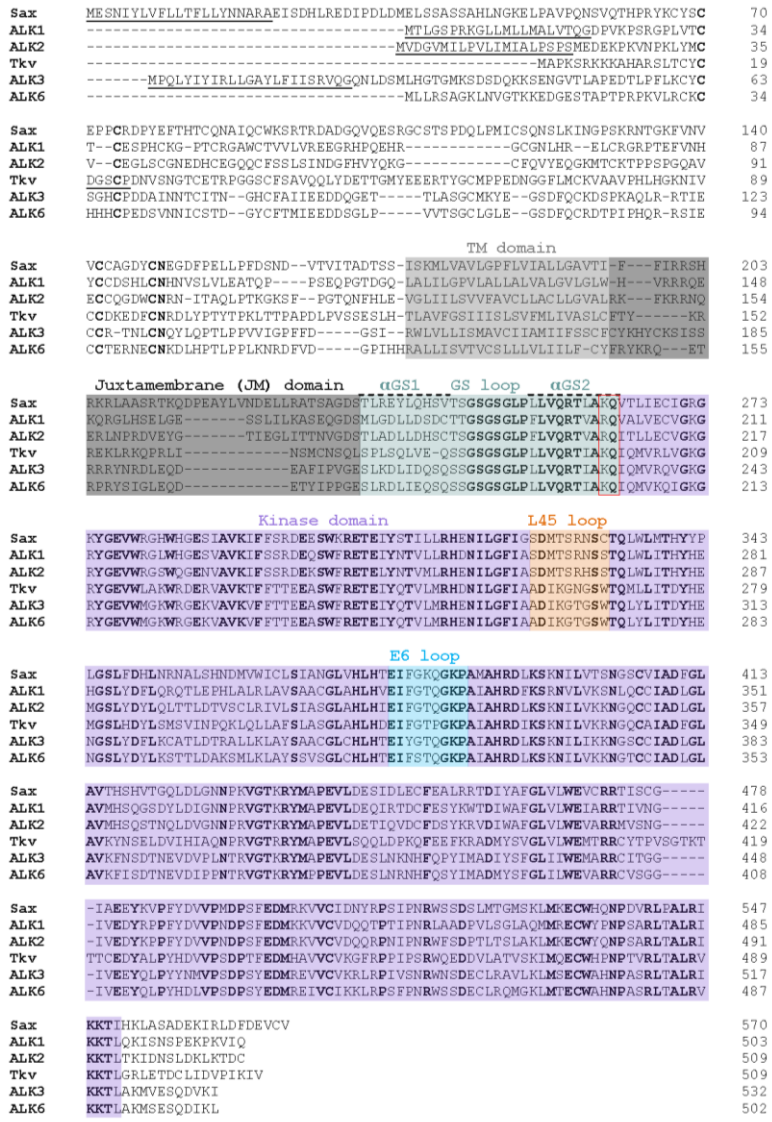


Figure 1.11. Protein alignment of BMP type I receptors. *Drosophila* Sax, Tkv, human ALK1, ALK2, ALK3 and ALK6 proteins were aligned. Underlined sequences in extracellular domain (region upstream of TM) indicate putative signal peptides predicted by SignalP 4.0 (Petersen et al., 2011). No signal peptides were predicted for Tkv or ALK6. TM, transmembrane (light gray). JM, juxtamembrane domain (dark gray). The GS domain (teal) is composed of the αGS1 helix, GS loop, and αGS2 helix. Dashed lines below αGS1 and αGS2 indicate alpha helices. Kinase domain (purple). L45 loop (orange). E6 loop (cyan). Boxed (red) sequences in the αGS2 helix highlight the conserved residues that confer constitutive activity when mutated. A QD mutation is routinely used to generate constitutively active type I receptors. R206H in ALK2 also confers hyperactivity and is responsible for the heterotopic bone disease fibrodysplasia ossificans progressiva (FOP).

Extracellular domain

The extracellular domain of all TGF-β superfamily type I receptors is marked by a cysteine-rich domain (10 cysteines). Structural studies indicate that disulfide bonds formed by these conserved cysteines stabilize the extracellular domains into the characteristic "three-finger toxin fold" found in all type I receptors of the TGF-β superfamily (Greenwald et al., 1999; Groppe et al., 2008; Kirsch et al., 2000b; Kotzsch et al., 2009; Mahlawat et al., 2012). Biochemical experiments from these same studies have also mapped residues important for ligand binding within this cysteine-rich region. The sequences of the extracellular domains are also the most divergent between type I receptors and most likely reflect their previously mentioned ligand-specific binding affinities (**Fig. 1.11**).

Kinase Domain

The kinase domains of type I receptors are highly conserved, Ser/Thr kinases that share sequence similarities to tyrosine kinases (Hanks and Hunter, 1995; Manning et al., 2002). Thus, it has been proposed that type I receptors of the TGF-β superfamily may represent an evolutionary transition between Tyr and Ser/Thr kinases (Huse et al., 1999). Consistent with being Ser/Thr kinases, type I receptors canonically phosphorylate serines at the C-terminus of R-Smads in response to TGF-β/BMP signaling (Abdollah et al., 1997b; Kretzschmar et al., 1997; Souchelnytskyi et al., 1997).

Type I receptors have also been implicated in non-canonical signaling. For instance BMP type I receptors can activate the MAPK/p38 cascade through interactions with XIAP1, TAB1, and the TGF-β-activated kinase 1 TAK1 (Adachi-Yamada et al., 1999; Derynck and Zhang, 2003; Moustakas and Heldin, 2005; Shibuya et al., 1998; Yamaguchi et al., 1999). Whether the BMP type I receptors act merely as scaffolding for these components or directly phosphorylates them remains unclear. Intriguingly, two

studies also suggest that TAK1 can mediate C-terminal phosphorylation of Smad1/5/8 thereby further integrating BMP and MAPK signaling pathways (Greenblatt et al., 2010; Shim et al., 2009).

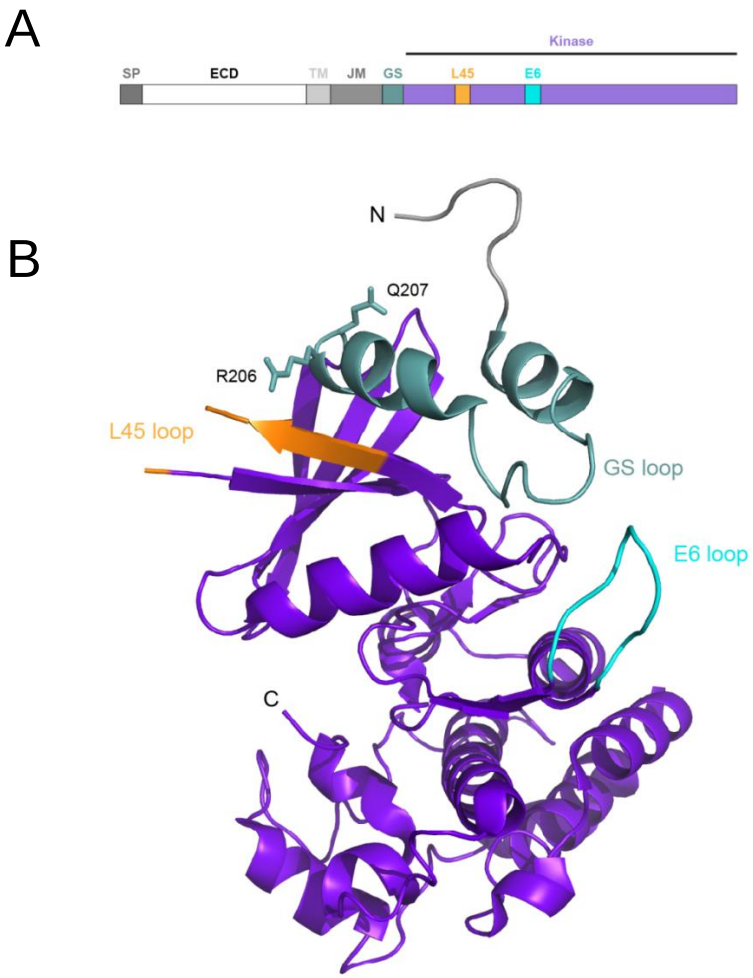


Figure 1.12. Structure of the ALK2 intracellular domain. A. Diagram of the protein domains in a BMP type I receptor. SP, signal peptide (dark gray). ECD, extracellular domain (white). TM, transmembrane domain (light gray). JM, juxtamembrane domain (gray). GS domain (teal). KD, kinase domain (purple). L45, L45 loop (orange). E6, E6 loop (cyan). **B**. Structure of the ALK2 intracellular domain indicating the same protein domains as in (A) as well as the two conserved residues R206 and Q207 in the αGS2 helix. Respectively mutating these residues to a histidine and an aspartic acid confers constitutive activity. N, N-terminus. C, C-terminus. Color scheme based on (**Fig. 1.1 and 1.11**).

Like most protein kinases, type I receptor kinase domains consist of an N-terminal and C-terminal lobe with the ATP binding site situated in a groove between the N and C lobes (Chaikuad et al., 2012; Huse et al., 1999) **(Fig. 1.12)**. The crystal structure of TβRI indicates that the N and C lobes of type I receptors are slightly rotated with respect to other protein kinases (Huse et al., 1999). Type I receptors are therefore considered to natively adopt an autoinhibited conformation because this rotation "shears" the groove such that ATP would be prevented from binding (Chaikuad et al., 2012; Huse et al., 1999). Further intramolecular interactions mediated by structural elements such as the GS domain serve to stabilize this conformation (Chaikuad et al., 2012; Huse et al., 1999).

GS domain

The highly conserved GS domain of TGF-β superfamily type I receptors autoregulates type I receptor kinase activity. This intracellular domain situated between the juxtamembrane domain and the kinase domain consists of a glycine-serine (GS) rich region—the serines of which are targets of type II receptor transphosphorylation (**Fig. 1.13**) (Attisano et al., 1996; Wieser et al., 1995; Wrana et al., 1994a; Wrana et al., 1994b). Flanking either side of the GS repeats are α-helices designated α-GS1 and α-GS2. In its unphosphorylated state, the GS domain acts to keep the type I receptor kinase domain locked in an autoinhibited conformation (Chaikuad et al., 2012; Huse et al., 1999). Upon ligand-induced receptor complex formation, the type II receptor transphosphorylates the GS domain inducing a conformational change that releases autoinhibition (Huse et al., 2001). The activated type I receptor in turn phosphorylates intracellular effectors known as the receptor-mediated Smads (R-Smads)(Derynck and Zhang, 2003; Massagué et al., 2005). Furthermore, the phosphorylated GS domain may contribute to R-Smad binding

as the resulting negative charge of the phosphorylated GS domain has been proposed to interact with a patch of highly basic residues found on R-Smads (Huse et al., 2001).

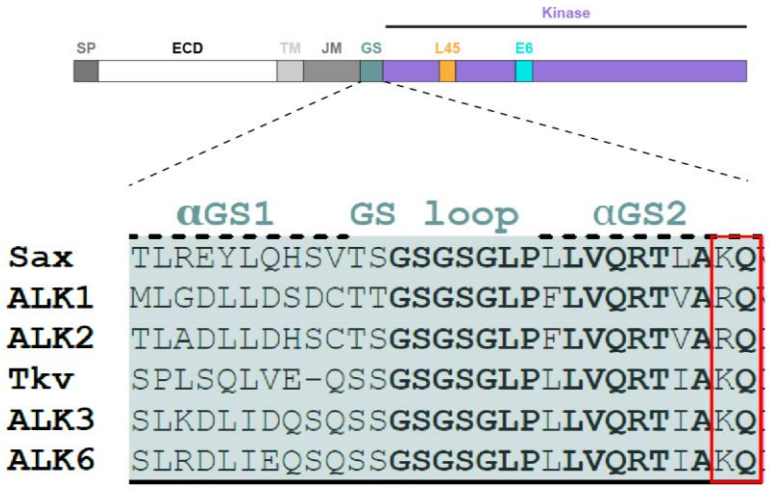


Figure 1.13. Sequence alignment of BMP type I receptor GS domains. *Drosophila* Sax, Tkv, human ALK1, ALK2, ALK3 and ALK6 proteins were aligned. The GS domain (teal) is composed of the αGS1 helix, GS loop, and αGS2 helix. Dashed lines below αGS1 and αGS2 indicate alpha helices. Ser/thr residues in the GS loop are the targets of type II receptor transphosphorylation. Boxed sequence in the αGS2 helix highlight the conserved residues that when mutated confer constitutive activity. A QD mutation is routinely used to generate constitutively active type I receptors. R206H in ALK2 also confers hyperactivity an is responsible for the heterotopic bone disease fibrodysplasia ossificans progressiva (FOP). SP, signal peptide; ECD extracellular domain; TM, transmembrane (light gray); JM, juxtamembrane domain (dark gray). Kinase domain (purple). L45 loop (orange). E6 loop (cyan).

Additionally, FK506-binding protein 1A (FKBP1A/FKBP12) can inhibit type I receptor kinase activity through interaction with the GS domain (Chaikuad et al., 2012; Chen et al., 1997; Gruendler et al., 2001; Huse et al., 1999; Huse et al., 2001; Kugimiya et al., 2005; Wang et al., 1996; Wang and Donahoe, 2004). This small, 12kDa inhibitor binds to a Leucine-Proline motif at the C-terminal end of the GS loop (**Fig. 1.13**) and is thought to simultaneously stabilize the autoinhibited comformation of type I receptors and mask the serines of the GS domain (Chen et al., 1997; Huse et al., 1999; Wang et al.,

1996). In doing so, FKBP1A is thought to guard against leaky, ligand-independent type I receptor signaling (Chen et al., 1997; Gruendler et al., 2001; Huse et al., 2001; Wang et al., 1996). FKBP1A is ultimately displaced from the type I receptor upon ligand binding and phosphorylation of the GS domain (Chen et al., 1997; Huse et al., 2001; Wang et al., 1996) . It has thus been suggested that the GS domain affects receptor activation on two levels. Phosphorylation converts the GS domain from an inhibitor-binding site to a Smad substrate-binding site while releasing the type I receptor from its autoinhibited conformation (Huse et al., 2001).

Mutations in the αGS2 helix underscore the importance of the GS domain in autoregulating type I receptor kinase activity. Mutating the conserved glutamine/threonine at the C-terminal end of the αGS2 to an aspartic acid confers constitutive kinase activity to type I receptors of the TGF-β superfamily (**Fig. 1.13**) (Akiyama et al., 1997; Attisano et al., 1996; Chen and Massagué, 1999; Haerry et al., 1998; Macías-Silva et al., 1998; Wieser et al., 1995). These mutant receptors can phosphorylate their Smad substrates independent of ligand. Interestingly, α-GS2 domain mutations can also disrupt binding of FKBP1A, which indicates that these constitutively active type I receptors are no longer inhibited by FKBP1A (Chen et al., 1997; Groppe et al., 2007; Groppe et al., 2011; Shen et al., 2009; Song et al., 2010).

GS domain mutation results in the heterotopic bone disease Fibrodysplasia Ossficans Progressiva (FOP)

Of clinical relevance, mutation of the conserved, basic residue arginine 206 to histidine in the human BMP type I receptor ALK2 is the genetic basis for the classic form of fibrodysplasia ossificans progressiva (**Fig. 1.13**) (Kaplan et al., 2009; Shore et al., 2006). This rare genetic disorder is characterized by the episodic deposition of heteropic bone in the place of muscle and connective tissues. Due to the progressive nature of the

disorder, heterotopic bone growth can be extensive—essentially encasing and immobilizing patients in a "second skeleton." This formation of extraskeletal bone is triggered by spontaneous flareups or injury (Kaplan et al., 2008). Removing bone growths by surgery is thus rendered counterproductive as invasive procedures initiate more ossification at the site. Given that FOP is so rare, patients are at risk of being misdiagnosed with other conditions, such as bone cancer, and undergo unnecessary biopsies or invasive surgeries that further compound their symptoms (Kitterman et al., 2005; Pignolo et al., 2011). Curiously, malformations of the great toe are the only consistent hallmark of FOP patients at birth. The first episodes of heterotopic ossification do not occur until later in childhood suggesting that the onset of FOP is developmentally regulated.

Work done in various model systems all indicate that the R206H mutation confers ligand-independent hyperactivity to the ALK2 receptor (Billings et al., 2008; van Dinther et al., 2010; Le and Wharton, 2012; Shen et al., 2009; Song et al., 2010). Structural studies and models predict that the R206H mutation disrupts binding of the intracellular inhibitor FKBP1A to ALK2 as a molecular mechanism resulting in dysregulated kinase activity (Chaikuad et al., 2012; Groppe et al., 2007; Shore et al., 2006). Consistent with this hypothesis, both *in vitro* and cell culture analyses indicate that the R206H destabilizes the interaction between FKBP1A and ALK2 (Groppe et al., 2011; Shen et al., 2009; Song et al., 2010). However, loss of FKBP1A-mediated inhibition may not be the basis for dysregulated BMP signaling in all cases of FOP since other ALK2 mutations related to atypical forms of FOP have been identified that are not expected to affect FKBP1A binding (Kaplan et al., 2009).

Although FOP mutant receptors appear to act as constitutively active receptors it is unknown whether other factors can regulate their signaling activity. Given that the location of the R206H mutation is situated near the GS domain could its kinase activity

still require transphosphorylation by the type II receptor? Does the signaling activity of ALK2^{R206H} receptor require receptor complex formation? Are there genetic modifiers that repress or enhance signaling from FOP mutant receptors? These questions remain unanswered.

L45 loop

Despite the similarity in signaling pathway architecture, members of the TGF-β superfamily signal through different receptors that culminate in the phosphorylation of distinct Smads. For example, TGF-β's and Activins induce the phosphorylation of Smad2/3, while BMP's primarily induce the phosphorylation of the Smad1/5/8 class (Derynck and Zhang, 2003; Massagué et al., 2005; Schmierer and Hill, 2007). This partitioning of substrate phosphorylation, in part, allows each branch of the TGF-β ligand superfamily to affect different cellular and developmental processes because phosphorylation of Smad2/3 versus Smad1/5/8 can elicit distinct transcriptional responses.

Smad substrate specificity is determined by the L45 loop of the type I receptor—a nine residue motif located downstream of the GS domain and situated between the β4 and β5 sheets (**Fig. 1.13A**). Chimeric studies indicate that Smad specificity can be exchanged between type I receptors by swapping L45 loops of different sequence classes (Armes et al., 1999; Chen et al., 1998b; Chen and Massagué, 1999; Feng and Derynck, 1997; Persson et al., 1998). For instance, a chimeric construct of BMPR-IB with the L45 loop from TβRI interacts with and phosphorylates Smad2 rather than its normal substrate Smad1 (Chen et al., 1998b; Persson et al., 1998). Furthermore, the chimeric construct activates TGF-β-specific transcriptional responses instead of BMP-specific responses(Chen et al., 1998b; Persson et al., 1998).

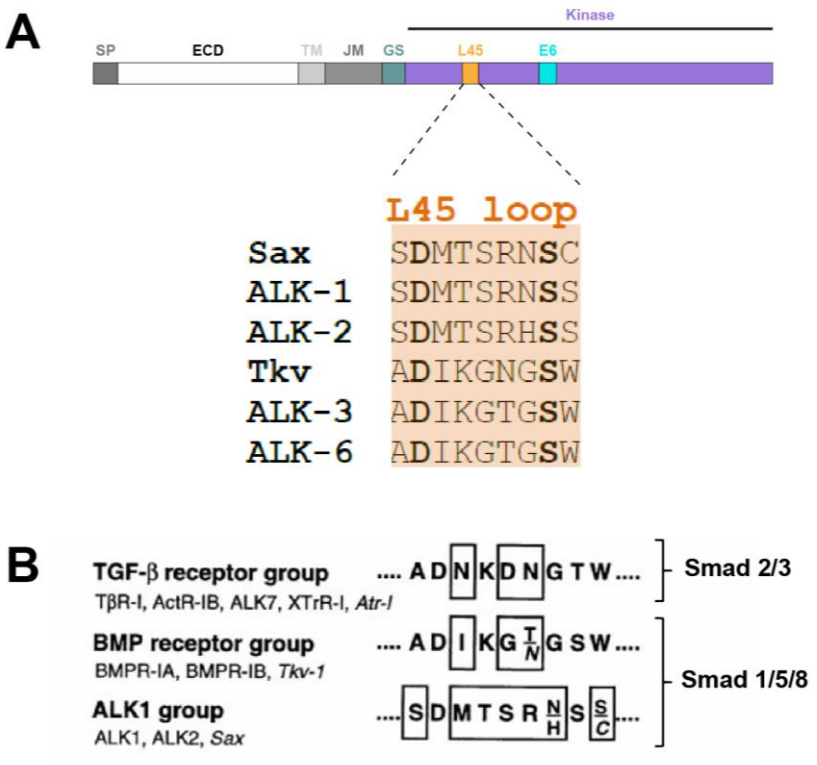


Figure 1.14 Sequence alignment of BMP type I receptor L45 loops. A. Sequence alignment of the L45 loops from *Drosophila* Sax, Tkv, and human ALK1, ALK2, ALK3 and ALK6 proteins. SP, signal peptide; ECD extracellular domain; TM, transmembrane (light gray); JM, juxtamembrane domain (dark gray). Kinase domain (purple). L45 loop (orange). E6 loop (cyan) **B.** L45 loops fall into three categories. The TGF-β receptor group, which specifies Smad2/3 as a substrate, includes ALK4 (ActR-IB) ALK5 (TβRI), ALK7 , XTrR-1 (*Xenopus* TβRI), and Atr-I (*Drosophila* Baboon). The BMP receptor group includes ALK3 (BMPR-IA), ALK6 (BMPR-IB) and Tkv. The ALK1 group includes ALK1, ALK2, and Sax. The BMP receptor and ALK1 receptor groups specifiy Smad1/5/8 as substrates. Adapted from (Chen and Massagué, 1999)

The L45 loops of the different type I receptors fall into three distinct categories based on amino acid sequence conservation: 1) the BMP receptor group, 2) the ALK1 group, and 3) the TGF-β receptor group (**Fig. 1.14B**) (Chen and Massagué, 1999). The BMP receptor group is composed of ALK3, ALK6, and the *Drosophila* ortholog Thick veins (Tkv), whereas the ALK1 group consists of ALK1, ALK2, and the *Drosophila*

ortholog Saxophone (Sax). The L45 loop from either of these groups is sufficient to specify Smad1/5/8 binding. On the other hand, the TGF-β group L45 loop that is conserved within the TGF-β group dictates Smad2/3 binding. The TGF-β group includes TβRI, ALK5, ALK7, and the *Drosophila* type I receptor Baboon (Babo), which mediates Activin-like signaling in *Drosophila*.

E6 loop

Although the type II receptor is responsible for transphosphorylating the type I receptor GS domain, the E6 loop of the type I receptor can influence GS domain phosphorylation (Weis-Garcia and Massagué, 1996). The E6 loop is a short, but highly conserved region downstream of the GS domain **(Fig. 1.15)** nested between the αE helix and the β6 strand (Chaikuad et al., 2012; Huse et al., 1999). Mutation of the conserved glycine in the E6 loop of TβR-I to an aspartic acid (G322D) reduces type II receptor-mediated phosphorylation of the TβR-I^{G322D} mutant. Consequently, TβRI^{G322D} is not activated and thus cannot transduce signals induced by the TGF-β1 ligand (Weis-Garcia and Massagué, 1996). Importantly, the TβRI^{G322D} mutant receptor and type II receptor TβRII coimmunopurify, which suggests that the G322D mutation interferes with transphosphorylation, but not the interaction between type I and type II receptors (Weis-Garcia and Massagué, 1996).

Cotransfection of a TβRI construct that harbors a "kinase-inactivating" mutation can rescue signaling activity of the "activation-deficient" TβRI^{G322D} mutant as well as restore type II receptor transphosphorylation (Weis-Garcia and Massagué, 1996). This complementation suggests that the G322D mutation on one type I receptor acts in *trans* within the receptor complex to prevent transphosphorylation of the other type I receptor's GS domain. Furthermore, it points to the possibility that type I receptors can affect each other's activity within a receptor complex. Interestingly, a proposed model for

the structure of a TβRI:TβRI homodimer places the G322 residue that is at the tip of the E6 loop of one TβRI monomer in position to form a hydrogen bond with the acidic residue, D257, on the other TβRI monomer (Huse et al., 1999). It is conceivable that the G322D mutation alters the configuration of the TβRI:TβRI homodimer such that the GS domain would no longer be accessible to the type II receptor for transphosphorylation.

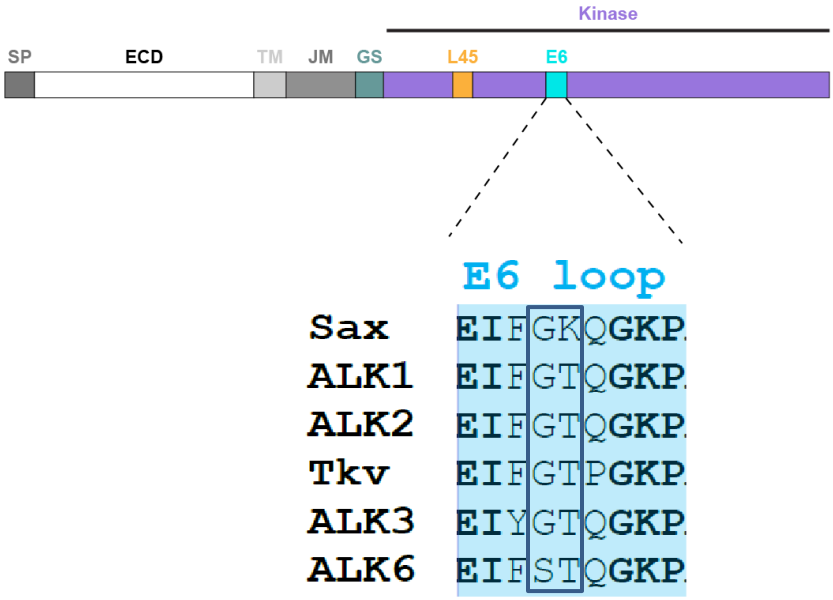


Figure 1.15. Sequence alignment of BMP type I receptor E6 loops. The E6 loop is a 9 amino acid residuce implicated in type I receptor activation and dimerization (Haerry, 2010; Huse et al., 1999; Weis-Garcia and Massagué, 1996). Sequence alignment of the E6 loops from *Drosophila* Sax, Tkv, and human ALK1, ALK2, ALK3 and ALK6 proteins. SP, signal peptide; ECD extracellular domain; TM, transmembrane (light gray); JM, juxtamembrane domain (dark gray). Kinase domain (purple). L45 loop (orange). E6 loop (cyan). Boxed sequences indicates the highly conserved glycine residue and the adjacent lysine in Sax that is divergent from the other BMP type I receptors.

The E6 loop can also affect type I receptor kinase activity. For instance, the ability of Sax^{QD}, a *Drosophila* BMP type I receptor harboring the constitutively activating QD mutation, to phosphorylate Mad was reportedly increased by a second site mutation in the Sax sequence (K382T) (Haerry, 2010). This K382T mutation is located

immediately downstream of a conserved glycine (corresponding to G322 in TβRI) in the E6 loop of Sax and converts lysine 382 in Sax to a threonine, which is the conserved residue found at that position in all other type I receptors (Fig. 1.15).

Concluding remarks

The doctoral research described herein characterizes the dual behavior of Sax. Both *in vivo* and cell culture experiments indicate that Gbb is not sufficient to activate Sax kinase activity. Mutations in the GS domain, however, render Sax kinase activity sensitive to stimulation by Gbb. Furthermore, overexpressing either of the *Drosophila* BMP type II receptors Wit or Punt can induce Sax signaling activity. These results suggest that an additional mechanism acting at the level of type II receptor-mediated activation negatively regulates Sax kinase activity.

Through detailed molecular analyses of different Sax-Tkv chimeric receptors, we have identified two domains in the type I receptor, the GS domain and the E6 loop, that are critical for determining the signaling behaviors of Sax and Tkv. Importantly, full "Tkv-like" signaling activity requires the pairing of the Tkv GS domain with the Tkv E6 loop. Full "Sax-like" inhibition of BMP signaling also appears to require pairing of the Sax GS domain with the Sax E6 loop. However, the signaling activity of Sax-Tkv chimeric receptors containing either of the Sax determinants is dramatically reduced indicating that the Sax GS domain and E6 loop are incompatible with the Tkv determinants. Taken together, these results further underscore the importance of the GS domain in determining type I receptor signaling activity. We propose a model in which the activation of Sax in a Sax:Sax-containing complex is hindered either by an inability to interact with type II receptors or steric hindrance that renders the GS domain inaccessible to the type II receptor.

In addition, I have established a *Drosophila* model for the heterotopic bone disease fibrodysplasia ossificans progressiva (FOP) which is caused by a constitutively activating R206H mutation in the GS domain of the human Sax orthologs ALK2. Using this model I have demonstrated that hyperactive signaling activity displayed by $ALK2^{R206H}$ requires type II receptor activity. This observation further underscores the importance of the type II receptor in signal transduction and implicates the requirement of a receptor complex for signal transduction even for constitutively active type I receptors. Our work also suggests a new avenue in designing therapies for the treatment of FOP that focuses on disrupting the interaction between $ALK2^{R206H}$ and BMP type II receptors.

Chapter 2

BMP type II receptor activity and mutations in the GS domain uncover the kinase activity of the *Drosophila* BMP type I receptor Saxophone

I performed all of the experiments presented in this chapter.

ABSTRACT

In *Drosophila*, BMP signals are transduced by the ALK3/6 ortholog Tkv and the ALK1/2 ortholog Sax. In the larval wing imaginal disc, BMP ligands Dpp and Gbb signaling through Tkv and Sax establishes a BMP activity gradient critical for wing vein patterning. The behaviors of Tkv and Sax in this tissue, however, are dramatically different. Whereas Tkv transduces BMP signals in the wing disc, Sax was observed to both facilitate and antagonize BMP signaling. To explain this dual behavior of Sax, a model was proposed wherein Sax could mediate BMP signals when complexed with Tkv (Sax:Tkv) in the signaling complex. In contrast, a complex that consists of two Sax molecules (Sax:Sax) cannot transduce signals. Instead, Sax:Sax complexes antagonize signaling by titrating BMP ligand into non-signaling active complexes. The mechanism that keeps Sax:Sax complexes inactive, however, has not been identified. Furthermore, it is not known whether Sax transduces signals in the context of a Sax:Tkv receptor complex or if Sax behaves as a "silent" co-receptor. It is therefore possible that Sax is a "dead kinase" that does not directly phosphorylate its substrate.

The experiments described herein demonstrate that Sax retains kinase activity. Two mutations in the regulatory GS domain of Sax were characterized that uncovers Sax kinase activity. Furthermore, we demonstrate that both *Drosophila* BMP type II receptors, Punt and Wit, can stimulate Sax-mediated signaling. Intriguingly, Sax signaling stimulated by Punt or Wit could not be enhanced by Gbb, Sax's preferred ligand. This result calls into question the ability of Gbb to coordinate functional receptor signaling complexes composed of Sax and either type II receptor. We propose that the kinase activity of Sax is kept inactive by a regulatory mechanism that hinders transphosphorylation of the Sax GS domain.

INTRODUCTION

Bone Morphogenetic Protein (BMP) signaling is mediated by BMP type I receptors, which couple extracellular communication to the intracellular responses that regulate diverse biological processes ranging from dorsal-ventral axis specification, limb patterning, cell growth and proliferation, and apoptosis(Wu and Hill, 2009). In humans, there are four BMP type I receptors: the highly similar ALK1/2 proteins and the highly similar ALK3/6 proteins. The importance of these receptors is underscored by a number of diseases, such as juvenile polyposis syndrome (JPS) (Howe et al., 2001; Howe et al., 2004; Kim et al., 2003; Zhou et al., 2001), hereditary hemorrhagic telangiectasia type 2 (HHT2) (Abdalla and Letarte, 2006; Bayrak-Toydemir et al., 2006; Gu et al., 2006; Olivieri et al., 2007; Wehner et al., 2006), and fibrodysplasia ossificans progressiva (FOP) (Fukuda et al., 2008; Gregson et al., 2011; Kaplan et al., 2009; Petrie et al., 2009; Shore et al., 2006), that are associated with mutations that dysregulate the activity of the human BMP type I receptors. Therefore, understanding how type I receptors transduce BMP signals and how its activity is regulated can provide not only insight into a number of developmental processes, but also potential therapies for diseases associated with type I receptor dysregulation.

BMP signaling is initiated by secreted BMPs, dimeric ligands, that bind to a receptor complex composed of two type I receptors and two type II receptors (Lin et al., 2006; Schmierer and Hill, 2007). Type I and type II receptors are distantly related proteins characterized by a cysteine-rich extracellular ligand binding domain, single-pass transmembrane domain, and an intracellular serine/threonine kinase domain (Hinck, 2012; Mueller and Nickel, 2012). The presence of a glycine-serine rich region,

termed the GS domain, differentiates the type I receptor from the type II receptor. Upon receptor complex formation, the type II receptor kinase phosphorylates the GS domain of the type I receptor to activate its kinase (Franzén et al., 1995; Wrana et al., 1994a). It has been proposed that phosphorylation of the GS domain uncovers type I receptor kinase activity by relieving an autoinhibitory conformation adopted by the type I receptor (Huse et al., 2001). The activated type I receptor kinase, in turn, phosphorylates intracellular effectors of the pathway called R-Smads(Abdollah et al., 1997a; Kretzschmar et al., 1997; Souchelnytskyi et al., 1997). Phosphorylated R-Smads interact with the closely related co-Smad, forming heteromeric complexes that accumulate in the nucleus. These heteromeric Smad complexes in conjunction with other transcription factors regulate target gene expression to elicit a multitude of cellular and developmental responses (Feng and Derynck, 2005; Massagué et al., 2005).

The presence of two type I receptors within a signaling complex raises multiple questions. Do both type I receptors contribute to signaling output of the complex by each phosphorylating Smads? Can the presence of one type I receptor influence the activity of the other? Furthermore, in nearly every species in which the BMP signaling pathway is conserved multiple BMP type I receptors have been identified. Can the identity of the type I receptors within a complex affect the final signaling output? Several studies have begun to address these questions. For instance, BMP signaling during dorsal-ventral axis specification in zebrafish embryos appears to occur through a requisite heteromeric receptor complex that contains two distinct type I receptors, either ALK3 or 6 with ALK8 (zebrafish ortholog of ALK2) (Little and Mullins, 2009). In certain contexts, the human BMP type I receptor ALK1 requires ALK5, a type I receptor from the closely-related TGF-β signaling pathway, for full kinase activity. Perhaps the most extreme example of how type I receptor composition affects signaling output, however, is the apparent ability of the *Drosophila* ALK2 ortholog, Saxophone (Sax) to facilitate or antagonize BMP

signaling depending on identity of the other type I receptor in the complex. A current model posits that Sax can facilitate BMP signaling when paired with the other *Drosophila* BMP type I receptor Tkv. In contrast, Sax cannot transduce BMP signals when paired with another Sax molecule in the receptor complex. Instead, Sax:Sax complexes inhibit the pathway by titrating BMP ligand. The precise molecular mechanisms governing each of these scenarios, however, remains poorly understood.

In *Drosophila,* BMP signaling is mediated by two BMP type I receptors encoded by the genes *saxophone (sax)* and *thickveins (tkv)*. Additionally, two type II receptors are encoded by *punt* and *wishful thinking (wit)*. Biochemical and genetic experiments suggest that Tkv is the high affinity receptor for the *Drosophila* BMP ligand Decapentaplegic (Dpp), whereas Sax binds to Glass bottom boat (Gbb) and Screw (Scw) with high affinity (Haerry et al., 1998; Haerry, 2010; Nguyen et al., 1998). Both *sax* and *tkv* are required for mediating BMP signaling during stem cell maintenance, embryonic dorsal-ventral patterning, wing development, and neuromuscular junction (NMJ) development (Bangi and Wharton, 2006b; Brummel et al., 1994; Casanueva and Ferguson, 2004; Haerry et al., 1998; Kawase et al., 2004; McCabe et al., 2004; Nellen et al., 1994; Penton et al., 1994; Rawson et al., 2003; Ray and Wharton, 2001; Singer et al., 1997; Tanimoto et al., 2000; Terracol and Lengyel, 1994). However, it is in the well-characterized process of wing development that the dual behavior of Sax has been observed (Bangi and Wharton, 2006b).

BMP signaling plays a critical role in patterning the larval wing imaginal disc, which gives rise to an adult wing characterized by an invariant pattern of five longitudinal wing veins (Fig. 1.9 & 1.10; reviewed in Blair, 2007). Antibody staining against phosphorylated Mad serves as a readout of BMP signaling and reveals a gradient of BMP signaling activity across the anterior/posterior (A/P) axis of the wing imaginal disc (Tanimoto et al., 2000). This gradient is characterized by a sharp peak of pMad

intensity in the posterior compartment that drops rapidly into a "trough" over the A/P boundary, and then rises gradually again into a broader peak of intensity in the anterior compartment.

Both *sax* and *tkv* are required to establish this gradient of BMP signaling activity in the wing disc. Loss-of-function clones for either gene in this tissue exhibit loss of pMad and loss of expression of the target gene *sal,* consistent with the role of type I receptors in facilitating BMP signaling (Bangi and Wharton, 2006b; Singer et al., 1997; Tanimoto et al., 2000). Yet several observations call into question Sax's role in mediating BMP signaling during wing development. Despite being the high affinity receptor for Gbb, loss of *sax* function does not phenocopy *gbb* loss of function (Khalsa et al., 1998; Ray and Wharton, 2001; Singer et al., 1997). Furthermore, wing phenotypes associated with *gbb* loss of function can be enhanced by removing one copy of *tkv* but not *sax* (Khalsa et al., 1998).

Further genetic analysis reveals a more complex picture where Sax can both facilitate and antagonize BMP signaling during wing development (Bangi and Wharton, 2006b). Overexpression of Gbb in the developing larval wing disc results in adult wings characterized by ectopic vein tissue, consistent with misregulated BMP signaling. Coexpression of wild-type Sax, however, suppresses these Gbb-associated wing phenotypes indicating that Sax can inhibit Gbb signaling. Furthermore, overexpressing g*bb* in a *sax* null heterozygous background enhanced the wing phenotypes associated with *gbb* overexpression, indicating that the antagonisitc behavior of Sax is an endogenous function and is not an artifact of overexpression. In contrast, coexpression of Tkv enhances *gbb*-overexpression phenotypes, whereas reducing *tkv* copy number suppresses these wing phenotypes. These results are consistent with Tkv's role as a type I receptor in mediating BMP signaling.

To explain the dual behavior of Sax, a model has been proposed in which the signaling activity of a BMP receptor complex is largely determined by the combination of type I receptors in the complex (**Fig. 1.3;** Bangi and Wharton, 2006b). In this model, receptor complexes in which the type I receptors are Sax:Tkv or Tkv:Tkv can promote signaling. In contrast, complexes in which both type I receptors are Sax cannot transduce BMP signals. The molecular basis for the inability of Sax:Sax-containing complexes remains to be determined. Furthermore, whether Sax can phosphorylate Mad in the context of a Sax:Tkv containing complex is unknown. One possibility is that Sax is a "dead" kinase and consequently does not phosphorylate Mad in either Sax:Sax or Sax:Tkv complexes. In this scenario Sax functions simply as a co-receptor in Sax:Tkv complexes. Alternatively, Sax's kinase activity may be regulated in such a way that it remains silent in Sax:Sax complexes, but is activated in Sax:Tkv complexes—perhaps by the very presence of Tkv. In particular, it would be important to investigate if type II receptors are able to interact with and activate Sax in either context.

In this report, we further characterized Sax function in BMP signaling *in vivo* and in cell culture to better understand the dual behavior of Sax at the molecular level. We found that Gbb does not stimulate Sax kinase activity and therefore, cannot signal through Sax alone. However, we also identified conditions under which Sax can mediate BMP signaling. Two GS domain mutations, Q263D and K262H, were characterized that uncovered the kinase activity of Sax. Both Sax^{Q263D} and Sax^{K262H} enhanced rather than inhibited Gbb-signaling *in vivo* and in cell culture. Q263D is an aspartic acid substitution of a conserved glutamine residue in the αGS2 helix that is routinely used to generate constitutively active type I receptors (**Fig. 1.13**). K262H is a histidine mutation of the lysine residue in Sax that corresponds to the R206H mutation in ALK2 responsible for the classic form of the heterotopic bone disease fibrodysplasia ossificans progressiva (FOP). The R206H mutation has also been shown to confer ALK2 with constitutive

activity. However, rather than generating constitutively active Sax receptors, the Sax^{K262H} and Sax^{Q263D} mutant receptors displayed Gbb-ligand dependent signaling activity on par with wild-type Tkv.

We also identified a role for the type II receptor in stimulating Sax signaling activity since overexpression of either *Drosophila* type II receptors, Wit or Punt, could cooperate with Sax to activate BMP signaling. Stimulation of Sax signaling was also more sensitive to the concentration of Wit which may indicate that Sax preferentially interacts with Wit. Interestingly, we found that Wit can enhance Gbb signaling, whereas Punt cannot, which may reflect a higher affinity interaction between Gbb and Wit. Intriguingly, the cooperative signaling between Sax and type II receptors could not be further enhanced by Gbb.

These results indicate that Sax can transduce BMP signals under certain non-physiological conditions. The location of the "activating" mutations in the GS domain, which is targeted by the type II receptor, coupled with the observation that overexpression of either type II receptor can stimulate Sax activity suggests that the nature of type I-type II interaction is critical for determining Sax's behavior. We propose that under endogenous conditions, Sax:Sax dimers interact with type II receptors either with low affinity or in a configuration that is not conducive for signaling. However, dramatically increasing the concentration of type II receptors or mutating the Sax GS domain allows Sax:Sax dimers to now interact with type II receptors in such a way to produce signaling competent complexes.

MATERIALS AND METHODS

Clonal analysis

sax mutant clones were generated in the following genotypes: 1) *yw hsFLP122*; FRT^{G13} sax^{4} *shaIN* /FRT^{G13} *M ubi-GFPx2*, and 2) *yw hsFLP122*; FRT^{G13} sax^{5} *shaIN* /FRT^{G13} *M ubi-GFPx2*. Clones were induced by heat-shock (37°C) for 2 hours. Large clones were generated by heat-shocking first-instar larvae, whereas smaller clones were generated by heat-shocking second instar larvae.

Type I Receptor and *gbb* Overexpression

Receptors and *gbb* were overexpressed using the GAL4-UAS system (Brand and Perrimon, 1993). The *ap-GAL4* driver was used to drive UAS-transgene expression in the dorsal compartment of the wing imaginal disc. *w; ap-Gal4, UAS-GFP/T2;3* virgins were crossed to males of the following genotypes:

Figure 2.2	**Figure 2.7**
yw; ; UAS-gbb9.1	*yw; ; UAS-gbb9.1*
yw; UAS-tkv 1-3B	*yw; UAS-tkvA B23 (Q199D)*
yw; UAS-tkv1-3B, UAS-gbb9.1	*yw; UAS-saxA 4B2 (Q263D)*
yw; UAS-sax-3xFLAG (1-1MA)	*yw; UAS-*sax^{K262H}*-3xFLAG*
yw; UAS-sax-3xFLAG (1-1MA); UAS-gbb9.1	*yw; UAS-saxA 4B2 (Q263D); UAS-gbb9.1*
	yw; UAS-sax^{K262H}*-3xFLAG; UAS-gbb9.1*

Drosophila melanogaster Strains and Crosses

All fly strains were cultured using standard sucrose, yeast extract agar food at 25°C. All fly strains are described in Flybase and obtained from Bloomington Stock

Center except for: *UAS-gbb9.1* (Khalsa et al., 1998), *UAS-tkvQD* (Haerry et al., 1998), and *UAS-sax-3xFLAG(1-1M-A)*,was a germline transformant derived from constructs described below.

Plasmid Constructs and Mutagenesis

Gateway cloning (Invitrogen) was used to clone all cDNAs into the following *Drosophila* Gateway Vectors: pTWF for GAL4-UAS driven expression in transgenic animals, pAWF (C-terminal 3xFLAG), or pAWH (C-terminal 3xHA). pAWF and pAWH contain a Actin5C promoter for constitutive expression in cell culture. *ALK2 and* $ALK2^{R206H}$ cDNAs were a generous gift from Eileen Shore. *punt* and *wit* cDNAs were a gift from Michael O'Connor. *pAW gbbHA, pAWF-*sax^{K262H}*, and pAWF-*sax^{Q263D} were constructed by Takuya Akiyama. Quikchange Site-directed Mutagenesis (Stratagene) was used to generate all mutants and deletions.

Ligand-binding domain deletions. Ligand-binding domain deletion mutants were generated by site-directed mutagenesis. For Sax^{Q263D} and Sax^{K262H}, sequences corresponding to Cys67 to Cys 148 were removed using the following primers:

Sax delta LBD rev (prKW81)

5'- GGAAAGTCTCCCTCATTTTTTATCTGGGATGCG-3'

Sax delta LBD fwd (prKW82)

5'- CGCATCCCAGATACAAAAATGAGGGAGACTTTCC-3'

sax^{5} (Sax^{A289D}) dominant-negative mutant. The following primer was used:

sax[5] A289D (prKW9) 5'- GGC GAA AGC ATC GAC GTG AAG ATA T-3'

Immunohistochemistry

Everted late third instar larvae were dissected and fixed in 4% paraformaldehyde/phosphate buffered saline (PBS; v/v) for 20 min at room temperature

followed by 5 washes in PBST (0.3% Triton X-100). Fixed tissues were then incubated overnight in blocking solution (10% normal goat serum in PBST) at 4°C. After blocking, the cuticles were incubated overnight with anti-PS3 (Epitomics, 1880- 1) primary antibody diluted 1:1,000 in blocking solution. Cuticles were then washed 5 times with PBST and incubated overnight with GαRb Alexa Fluor568 secondary antibody diluted 1:1,000 in blocking solution. Following 5 additional washes of PBST, wing discs were removed and mounted in 80% glycerol/0.5% N-propyl gallate. Confocal images were collected using a Zeiss LSM510 Meta confocal laser scanning microscope.

Image Analysis pMad intensity profiles.

Intensity profiles of pMad distribution were measured in confocal Z stacks of wing discs using the Fiji Image Processing Package (http://fiji.sc/wiki/index.php/Fiji). The profiles
shown are the average intensity plots measured in the dorsal compartments of five wing discs and aligned by the posterior and anterior peaks of pMad distribution.

***Drosophila* Schneider 2 (S2) Cell Maintenance and Transfections**

S2 cells were cultured in Shields and Sang M3 Insect Medium (Sigma S8398) pH 6.5 containing 10% Insect Medium Supplement (Sigma I7267) and 2% fetal bovine serum (F3018). Transient transfections were carried out using Effectene Transfection Reagent (Qiagen 301427).

Quantitative Cell-Based BMP Signaling Assay (*brkSE-lacZ*)

An adapted protocol based on a previously described assay was used to measure BMP signaling activity (Bangi and Wharton, 2006b; Müller et al., 2003; Twombly et al.,

2009). This assay makes use of a reporter construct expressing *lacZ* under the control of a Su(H) transcriptional activation response element as well as a *brk* transcriptional silencer element (Su(H)/*brkSE-lacZ*). Cotransfection of the reporter construct with plasmids encoding *Su(H)* and an activated formof Notch (N*) lead to *lacZ* transcription while the activation of BMP signaling leads to a repression of *lacZ* expression by virtue of the BMP-responsive brk silencer element. BMP signaling can thus be measured as a loss of β-galactosidase activity. 2.8 x10^6 cells were cotransfected with Su(H), N*, Su(H)/*brkSE-lacZ*, and luciferase plasmids, all under the control of the Actin 5C promoter.

Constructs and their concentrations used in this assay are indicated in the figure legends. Cells were harvested and lysed 3 days post-transfection and β-galactosidase activity of cleared lysate was measured using the dual luciferase assay system (Dual-Light, Applied Biosystems) and normalized to luciferase activity, which served as a transfection control for each sample. The normalized value obtained from the cleared lysate of cells cotransfected with only Su(H), N*, Su(H)/ *brkSE-lacZ* and luciferase was set to 100%. Tukey HSD test was performed for multiple pairwise comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; ns, not significant $p > 0.05$).

S2 cell-based Mad phosphorylation assay

BMP signaling activity was also measured by Mad phosphorylation in an S2 cell-based assay. S2 cells (8.0 x 10^6) were either transfected with 700ng of *pAC FLAG-Mad* (a gift of M. O'Connor) alone or cotransfected with 700ng of *pAC FLAG-Mad* in combination with 300 ng of *pAWF sax*, *pAWF tkv*, or *pAWF STT*. Five days post-transfection, transfected cells were centrifuged at 2000 rpm for 5 min at room temperature. Cleared cell pellets were resuspended in conditioned media (preparation described below) from untransfected S2 cells or S2 cells stably -transfected with pAW

gbb1xHA. Cells were incubated in conditioned media for 3 hours at room temperature. After incubation, cells were collected by centrifugation at 2000 rpm for 5 min at room temperature and lysed in SDS sample buffer. Protein extracts were analyzed by western blot (described below) to measure the extent of Mad phosphorylation. The level of pMad (band intensity) for each sample was measured using ImageJ and normalized against the expression level of FLAG-Mad (as measured by anti-FLAG staining).

Preparation of conditioned media.

Starter cultures (concentration: 5.0 x 10^6 cells/mL) of untransfected S2 cells and S2 cells stably-transfected with *pAW gbb1xHA* were grown for 5 days at 25°C in serum (FBS) free M3 media supplemented with 10% insect media supplement and PenStrep (1:400 dilution). After incubation, cells were centrifuged at 2000 rpm for 5 min at room temperature. The supernatant was recovered and centrifuged at 13,000 rpm for 5 min at room temperature to clear residual debris. The cleared supernatants from untransfected S2 cells and S2 cells stably-transfected with *pAW gbb1xHa* were used as conditioned media for S2 cell-based Mad phosphorylation assays.

Western blotting

Protein samples were analyzed by western blot using standard protocols. Monoclonal antibody 3D6-24 against Gbb peptide 424-440 was generated at the UAB Epitope Recognition and Immunoreagent Core Facility. Anti-Gbb (1:1000), anti-Flag (M2, 1:1000, Sigma), and anti-pSmad3 [EP823Y, 1:1000, Epitomics (51)], and horseradish peroxidase–conjugated secondary antibodies (1:10,000; Jackson ImmunoResearch) were used for western blot analyses. ECL-Plus (GE Healthcare Life Sciences) was used for chemiluminescent detection.

RESULTS

sax is required for BMP signaling in the wing pouch of the *Drosophila* wing disc

To test the requirement of *sax* in establishing the activity gradient of BMP signaling in the larval wing disc, we generated loss-of-function sax^4 and sax^5 mutant clones. sax^4 is a null mutation that results in an early stop codon in the extracellular domain of Sax (**Fig. 2.1)**. sax^5 is a loss-of-function allele in which alanine 289 is replaced with an aspartic acid. The A289D amino acid substitution is located in the kinase domain of Sax just downstream of the ATP binding site. Large and small clones were recovered by inducing clones in first or second instar larva, respectively. Clones were also found in all compartments (anterior, posterior, dorsal, and ventral) of the wing disc. Compared to wild-type control clones, pMad was dramatically reduced in both sax^4 null clones and sax^5 loss-of-function clones (**Fig. 2.1 B-C' vs A**). Loss of pMad occurred in all *sax* clones independent of their location, indicating a general requirement for *sax* in all compartments of the wing pouch. The loss of pMad in large clones of either allele indicate that loss of *sax* activity as early as the first instar larval stage negatively impacts BMP signaling in third instar larval wing discs. These results indicate that *sax* facilitates BMP signaling in the wing pouch.

Sax does not mediate Gbb-induced signaling

Although our clonal analysis indicates that *sax* is required for BMP signaling in the wing disc, previous work suggested that *sax* can antagonize BMP signaling induced by the *Drosophila* ligand *gbb*. This, however, was based on interpreting wing phenotypes rather than directly observing changes in pMad staining.

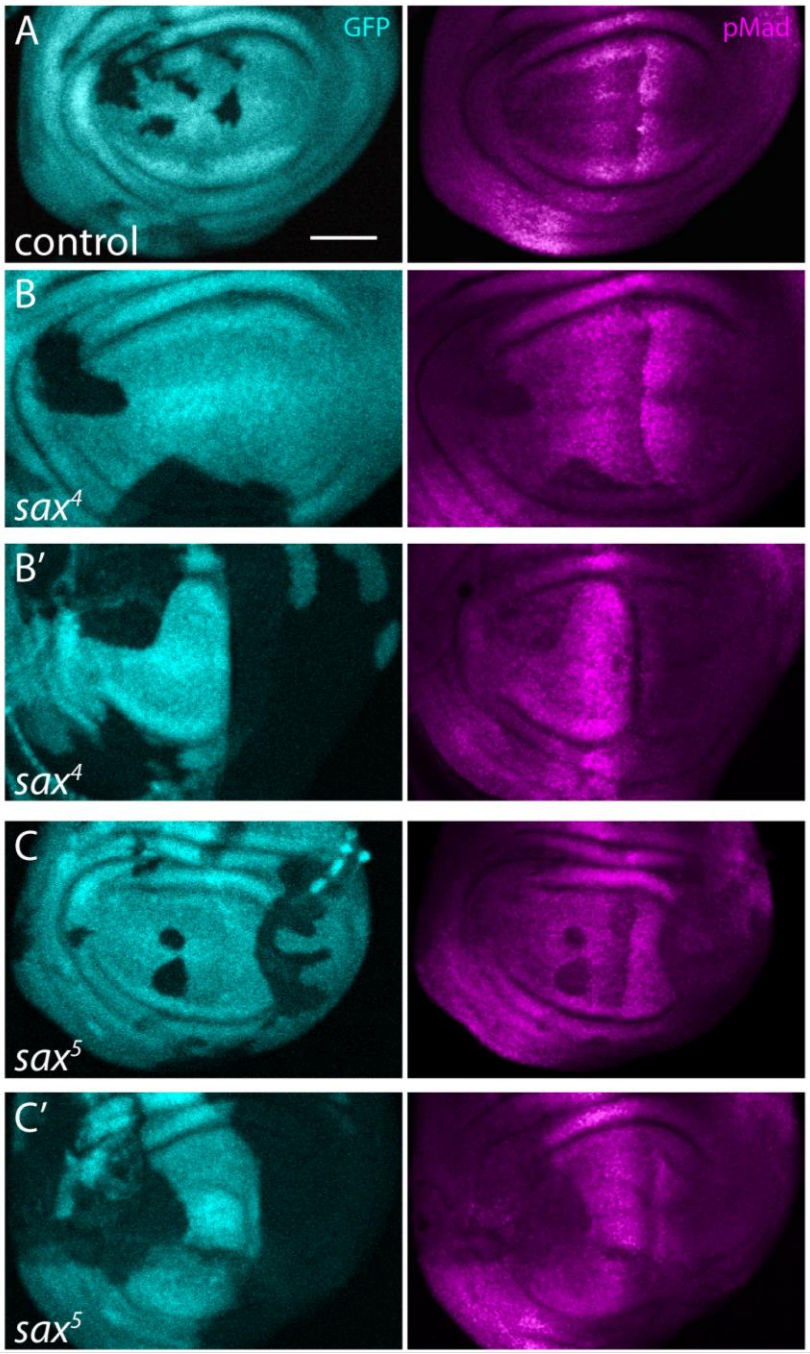


Figure 2.1. Loss of *sax* function reduces pMad in the larval wing disc. Wing pouch of third instar discs (dorsal is up, anterior left) stained for pMad (magenta, right panels). GFP signal (cyan, left panels). All wing discs are shown to scale (scale bar = 50 mm) **A**. pMad staining is unperturbed in *sha*^{IN} control clones. Wild-type pMad staining is characterized by a sharp, posterior peak of intensity and a broad shoulder in the anterior compartment separated by a trough of low pMad staining. **B, B'.** pMad is reduced in both small (**B**) and large (**B'**) *sax*^{4} null clones. The mutation responsible for *sax*^{4} results in an early-stop codon in the extracellular domain. **C, C'.** pMad is reduced in both small (**C**) and large (**C'**) *sax*^{5} loss of function clones. The mutation associated with *sax*^{5} is an alanine-to-aspartic acid substitution at position position 289, which is situated in the kinase domain of the Sax receptor.

Namely, the coexpression of *sax* with *gbb* rescued wing phenotypes induced by *gbb*-overexpression alone. This is in stark contrast to the behavior of *tkv*, which when coexpressed with *gbb* enhances *gbb*-overexpression wing phenotypes.

We sought to verify the difference in the ability of *sax* and *tkv* to mediate *gbb*-induced signaling by examining the effects of overexpression of these components on the pMad gradient. Expression of the *sax, tkv*, and *gbb* UAS-transgenes were directed to the dorsal compartment of the wing pouch using the *ap-Gal4* driver. Overexpression of each BMP signaling component in the dorsal compartment of the wing pouch had varying effects on the shape of the pMad gradient.

Overexpression of *gbb* had differing effects in certain domains of the wing disc (**Figure 2.2B**). Unexpectedly, overexpression of *gbb* depressed pMad levels in the center of the disc such that the amplitude of both the anterior and posterior peaks of pMad staining decreased--giving the appearance of a widened "trough." The opposite effect, however, was observed in the peripheral wing disc where *gbb* overexpression increased pMad levels. These results suggest that the overexpression of *gbb* inhibits BMP signaling in the center of the disc while increasing signaling activity in the periphery.

Overexpression of the type I receptor *tkv* collapses the pMad gradient into one steep peak, consistent with previous observations (**Figure 2.2 C,** & (Tanimoto et al., 2000). The width of the dorsal compartment in discs expressing *tkv* was also markedly reduced. The pMad gradient in wing discs overexpressing *sax* was also narrower, however, distinct anterior and posterior peaks of pMad intensities were maintained (**Figure 2.2 E**). The gradient in the anterior portion of *ap>sax* wing discs became much steeper while the posterior peak was moderately reduced from its wild-type level. Overexpression of *sax* also mildly decreased the width of the posterior compartment of these wing discs. While overexpressing either *sax* or *tkv* results in different effects on the

pMad gradient, overall overexpression of either type I receptor inhibits BMP signaling, perhaps through a dominant mechanism such as ligand titration and sequestration.

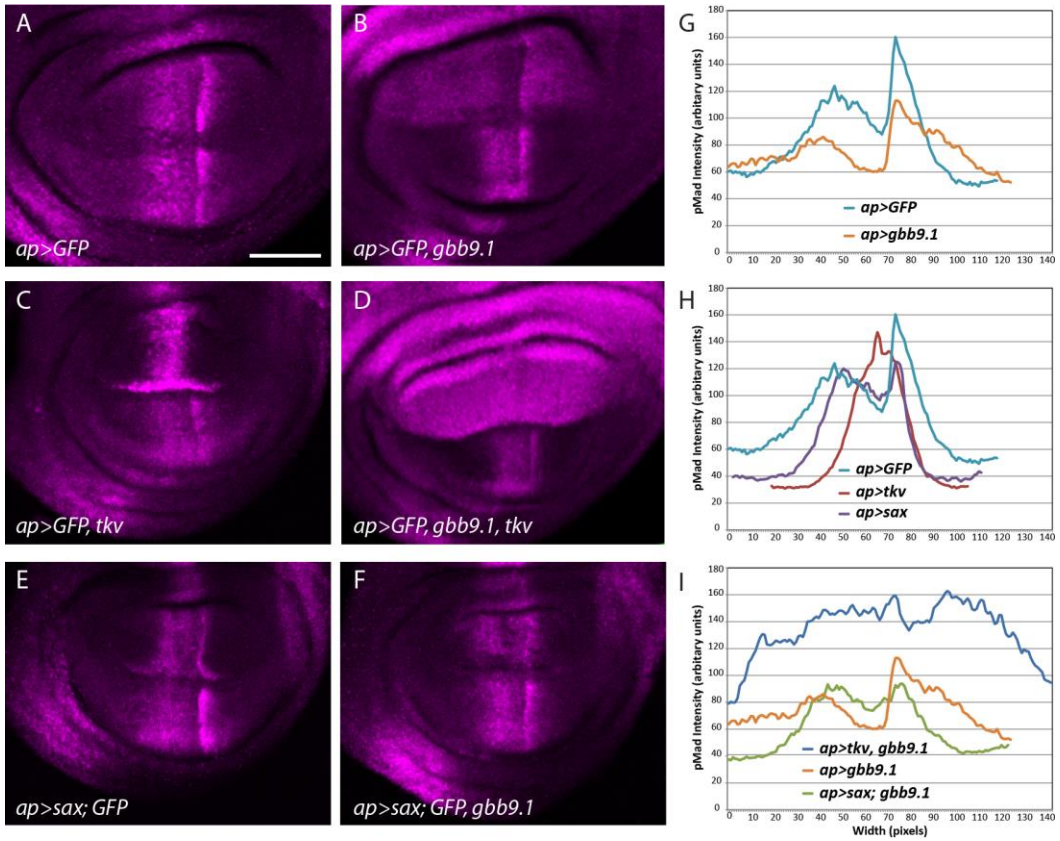


Figure 2.2. Gbb signals are mediated through Tkv and not Sax. Confocal images of pMad (magenta) staining in the wing pouch (dorsal is up, anterior left) of third instar larval wing discs overexpressing **A.** *GFP*, **B.** *GFP* and *gbb*, **C.** *GFP* and *tkv*, **D.** *GFP*, *gbb,* and *tkv,* **E.** *sax* and *GFP*, and **F.** *sax*, *GFP*, and *gbb*. Expression of UAS transgenes were driven by the dorsal compartment driver *ap-Gal4*. Scale bar = 50 μm. **G-I**. Comparison of average pMad intensity plots (n= 5 wing discs) measured across the dorsal compartment at its midway point. **G.** Effect of *gbb* overexpression (orange line) on the pMad gradient **H.** Effect of *tkv* (red line) or *sax* (purple line) overexpression on the pMad gradient. **I.** Effect of coexpressing *gbb* with either *tkv* (dark blue line) or *sax* (green line) on the pMad gradient. Overexpression of *tkv* and *gbb* exhibit synergistic increase in pMad staining. In contrast, co-overexpression of *sax* and *gbb* did not display synergistic signaling. Light blue line in **G** and **H** represents the wild-type pMad gradient in *ap>GFP* wing discs.

The difference in the behavior of *sax* and *tkv* becomes apparent when either receptor is coexpressed with *gbb*. Coexpression of *tkv* with *gbb* results in a dramatic increase in pMad staining across the entire dorsal compartment of the wing pouch, indicating that *tkv* can mediate *gbb* signals. In contrast, coexpression of *sax* with *gbb* does not dramatically increase pMad levels. Instead, pMad levels are strikingly reduced throughout the posterior compartment and the peripheral anterior compartment while slightly increased in the center of the disc. Similarly, this ability of *sax* to antagonize BMP signaling is also observed when *sax* is coexpressed with *gbb* using the posterior compartment-specific *hedgehog-GAL4 (hh-GAL4)* driver (**Appendix 6.1**). These results suggest that while *tkv* can phosphorylate Mad efficiently in response to *gbb* signals, *sax* cannot and in some cases inhibits *gbb*-induced phosphorylation of Mad.

This disparate behavior of *sax* and *tkv* can also be observed in S2 cell culture. In this study, we made use of a *Drosophila* S2 cell-based BMP signaling assay to investigate the differential behavior of Sax and Tkv in mediating signaling. The BMP signaling assay exploits a BMP-sensitive *lacZ* reporter that contains the *brinker* (*brk*) silencer element previously shown to mediate the transcriptional repression of *brk* in response to activation of BMP signaling (**Fig. 2.3** &(Müller et al., 2003). *lacZ* transcription is activated through *Suppressor of Hairless* (*Su(H)*) binding sites in response to a constitutively active Notch construct. Activation of the BMP signaling pathway mediates the repression of *lacZ* transcription through the adjacent *brk* silencer element on the *lacZ* reporter construct. The reduction in *lacZ* expression mediated by the *brk* silencer element has been shown to occur in a quantitative manner and thus, the reduction in β-galactosidase activity serves as a quantitative measure of BMP signaling output (Müller et al., 2003; Pyrowolakis et al., 2004).

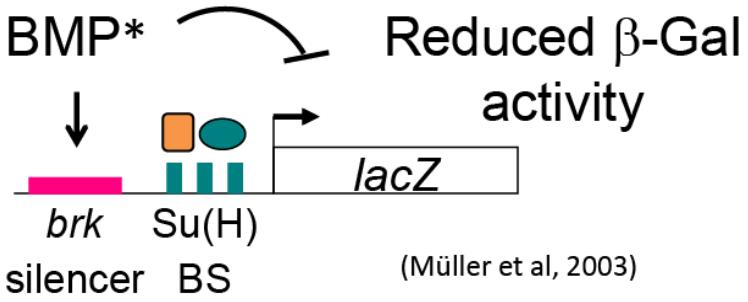


Figure 2.3 BMP signaling represses expression of the *brkSE-lacZ* reporter construct. Changes in BMP signaling in cell culture can be quantitatively measured by exploiting the *brkSE-lacZ* reporter construct, which consists of the *lacZ* gene fused to a BMP-responsive silencer element from the regulatory sequence of *brk* (magenta). *lacZ* expression is activated by exogenous Notch^{ICD} (orange rectangle) and exogenous Su(H) (teal oval) via upstream *Su(H)* binding sites (teal rectangles). BMP signaling represses *lacZ* transcription via the *brk* silencer element. Therefore, a reduction in β-gal activity serves as a quantitative measure of BMP signaling output.

β–Gal activity in S2 cells transfected with only the *brkSE-lacZ* construct is interpreted to represent background level of BMP signaling (**Fig. 2.4A,** NR). Transfection of 100ng of *gbb* represses *brkSE-lacZ* reporter activity by roughly 40%, indicating that transfection of *gbb* is sufficient to activate BMP signaling in S2 cells (**Fig. 2.4B,** NR). *gbb*-induced repression of *brkSE-lacZ* is enhanced by *tkv,* consistent with our observation that *tkv* can mediate *gbb*-signals in the wing disc (**Fig. 2.4B,** Tkv vs NR). In contrast, cotransfection of 50ng of *sax* with *gbb* opposes *gbb*-induced repression of *brkSE-lacZ* (**Fig. 2.4B,** Sax vs NR). Surprisingly, this derepression by *sax* increases *brkSE-lacZ* expression such that it's higher than background. This result indicates that *sax* is unable to mediate *gbb* signals. Cotransfection of *sax*^{5}, a dominant-negative allele predicted to have no kinase activity, also alleviates *gbb*-induced repression of reporter activity (**Fig. 2.4B,** Sax^{A289D} vs NR). The fact that the respective abilities of *sax* and *sax*^{5}

to antagonize *gbb*-induced signaling are indistinguishable suggests that, in this context, the kinase activity of wild-type *sax* is inactive.

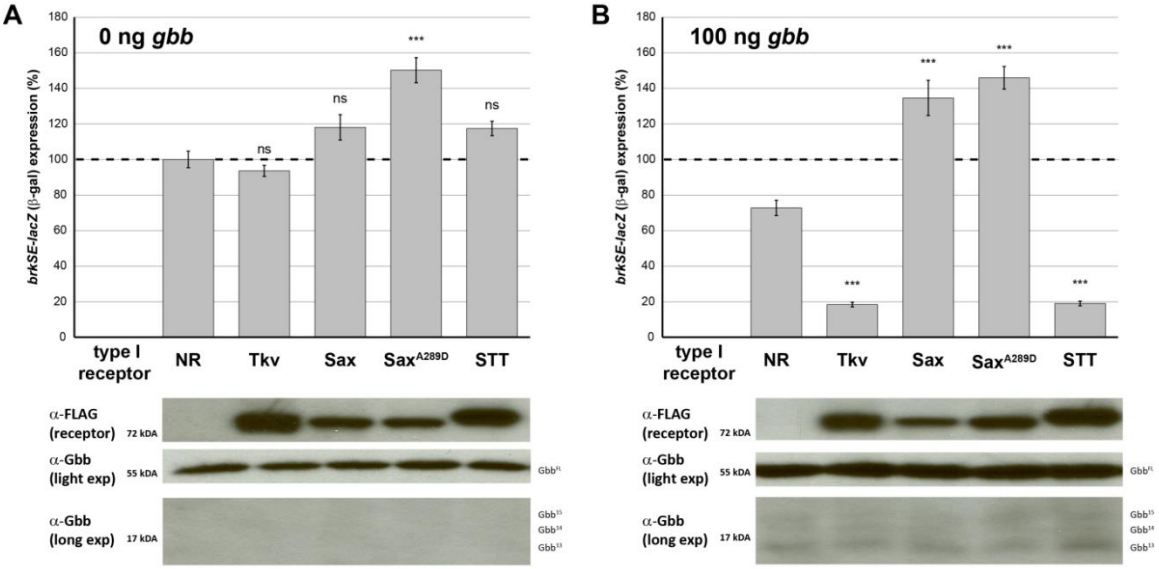


Figure 2.4. Sax inhibits Gbb-induced BMP signaling in S2 cells. A. Tkv, Sax, and the chimeric receptor did not affect *brkSE-lacZ* expression.The dominant-negative Sax^{A289D} (*sax^{5}* allele), however, increased *lacZ* expression. S2 cells transfected with 50 ng of the indicated receptor. **B.** Tkv and the chimeric receptor STT enhanced Gbb-mediated repression of *brkSE-lacZ* expression. In contrast, Sax and the dominant negative Sax^{A289D} mutant receptor (*sax^{5})* inhibited Gbb-mediated repression of *brkSE-lacZ*. S2 cells were cotransfected with 50 ng of the indicated receptor and 100 ng of *gbb*. *brkSE-lacZ* expression is repressed by BMP signaling. Therefore, loss of β-gal activity serves as a quantitative measure of BMP signaling. Data represent the mean ±SEM (n=4) of experiments performed in quadruplicate. Tukey HSD test was performed for multiple pairwise comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; ns, not significant $p > 0.05$).

Although previous studies indicate that Gbb preferentially binds to Sax (Bangi and Wharton, 2006b; Haerry et al., 1998; Haerry, 2010), we considered the possibility that inhibition of Gbb signaling was being mediated through interaction of Gbb with the Sax extracellular domain. To test this scenario, we made use of a chimeric receptor in which the extracellular and transmembrane domains of Sax were fused to the Tkv intracellular kinase domain (STT, **Fig. 3.3A**) to determine whether the antagonistic

behavior of Sax resides in its extracellular domain. Using the *brkSE-lacZ,* we measured STT's effect on Gbb-signaling. Cotransfection of the *STT* chimeric construct enhanced *gbb*-induced repression of *brkSE-lacZ* to an extent that was equivalent to *tkv* (**Fig.2.4B**, STT compared to NR and Tkv). This implies that the inability of Sax to transduce Gbb signaling resides in the Sax intracellular kinase domain.

Similarly, the opposite behaviors of *sax* and *tkv* on Gbb-signaling in S2 cells were observed by directly measuring phosphorylation of Mad (Fig. 2.5). When S2 cells that overexpress *FLAG-Mad* were treated with Gbb-conditioned media, we observed mild phosphorylation of Mad (pMad) (**Fig. 2.5B,** NR, light gray vs gray bar). The level of Mad phosphorylation in cells transfected with Tkv-3xFLAG and STT-3xFLAG is enhanced 4.6 and 4.3 fold, respectively, by Gbb-HA conditioned media treatment (**Fig. 2.5 B**, compare Tkv and STT vs NR). It should be noted that both Tkv and STT are capable of inducing pMad without added Gbb-HA (**Fig. 2.5B**, Tkv and STT, light gray bars.). We interpret this to indicate that transfected Tkv and STT can respond to endogenous BMP ligands expressed by S2 cells.

In contrast, Gbb-induced Mad phosphorylation is suppressed in cells overexpressing both *FLAG-Mad* and *sax-3xFLAG* (**Fig. 2.5B,** Sax vs NR). Whereas Tkv and STT are capable of enhancing Mad phosphorylation in response to Gbb signals, Sax cannot. Instead, Sax acts to inhibit Gbb-induced phosphorylation of Mad. Taken together, the inability of Sax to mediate Mad phosphorylation in response to Gbb signals, both *in vivo* and in cell culture, suggests that the kinase activity of Sax is inactive even when confronted with Gbb.

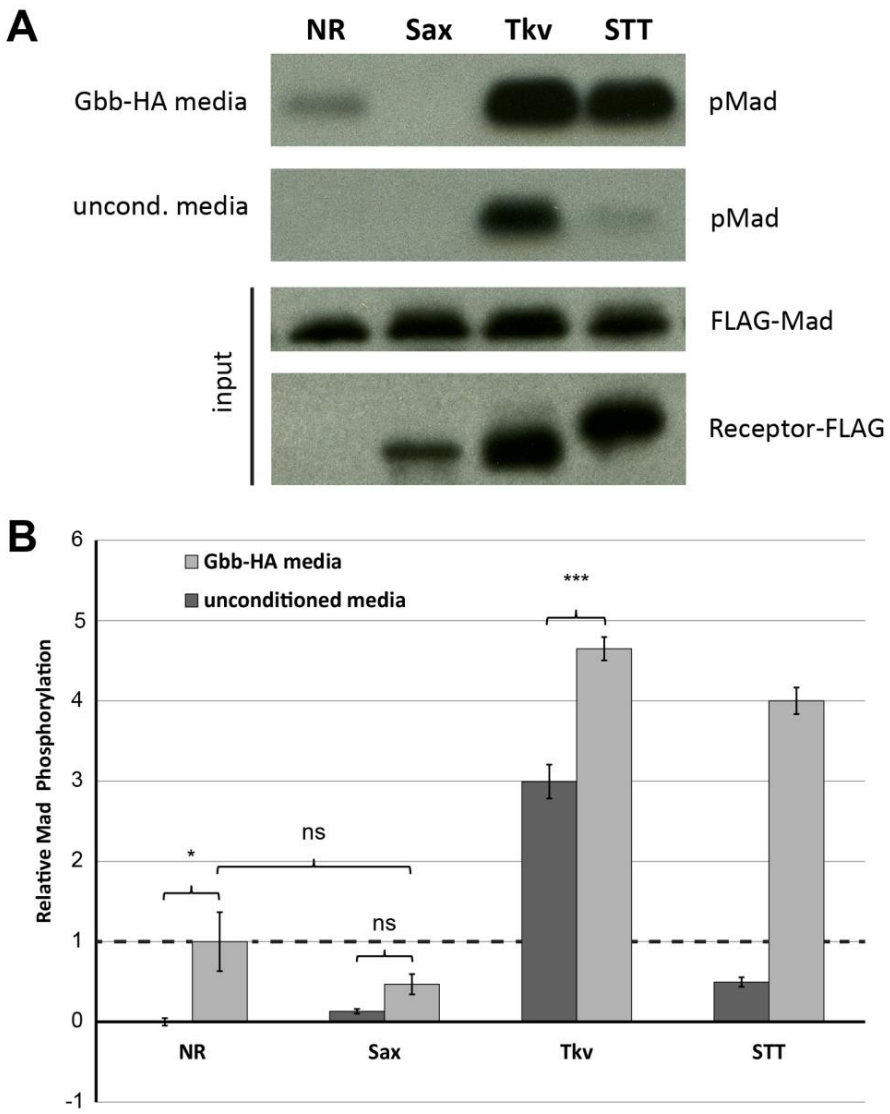


Figure 2.5. Sax inhibits Mad phosphorylation in S2 cells. Tkv and STT enhanced, whereas Sax inhibited Gbb-induced Mad phosphorylation. **A.** Representative western blot of Mad phosphorylation assay. S2 cells were cotransfected with *pAC FLAG-Mad* and the indicated FLAG-tagged receptors. STT = chimeric protein consisting of Sax extracellular and transmembrane domains fused to Tkv intracellular kinase domain. NR = no receptor transfected. Transfected cells were split and treated with Gbb-HA conditioned cell culture media or unconditioned media. Cell lysates were prepared and western blotted using α-PS3 (pMad) and α-FLAG (M2) antibodies. **B.** Quantification of pMad signal normalized to corresponding FLAG-Mad signal. Data represent mean ±SEM (n=3). pMad signal in untransfected S2 cells treated with Gbb-HA conditioned media was set to 1 (dashed line). Tukey HSD test was performed for multiple pairwise comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; ns, not significant $p > 0.05$).

Mutations in the GS domain of *sax* confer ligand-dependent kinase activity

Activation of the type I receptor kinase requires the type II receptor mediated transphosphorylation of the serine residues in its GS domain . Mutations located just downstream of the GS domain have previously been shown to "constitutively" activate type I receptor kinase activity. For instance, aspartic acid substitution of a well-conserved glutamine/threonine residue has canonically been used to generate constitutively active type I receptor kinases in both the BMP and TGF-B signaling pathways.

Of clinical relevance, a histidine substitution of the arginine (R206) residue adjacent to the conserved Glu/Thr residue confers hyperactivity to ALK2 (human ortholog of Sax) and is responsible for the progressive human bone disease, fibrodysplasia ossificans progressiva (FOP). While previous work has demonstrated that the QD mutation confers constitutive signaling activity onto Tkv (Tkv^{Q199D}), its effect on Sax activity is less clear. For instance, overexpression of sax^{Q263} *in vivo* results in milder adult wing phenotypes than overexpressing tkv^{Q199D} (Haerry et al., 1998; Khalsa et al., 1998) Furthermore, transfection of sax^{Q263D} in S2 cells induces the phosphorylation of Mad, albeit to a lesser extent than transfecting tkv^{Q199D} (Haerry, 2010). These results suggest that if sax^{Q263} does indeed have constitutive activity, it must be weaker than the constitutive activity exhibited by $tkv^{Q199D.}$

To better understand the effect of GS mutations on type I receptor activity, we measured the ability of sax^{Q263D}, sax^{K262H}, tkv^{Q199D}, and $ALK2^{R206H}$ to activate the BMP signaling pathway in S2 cells and *in vivo*. Transfection of either tkv^{Q199D} or $ALK2^{R206H}$ was sufficient to dramatically repress *brkSE-lacZ* expression consistent with constitutive activity **(Fig. 2.6)**. However, transfection of either sax^{Q263D} or sax^{K262H} did not significantly repress *brkSE-lacZ* expression compared to background (**Fig. 2.6,** Sax^{Q263D} and Sax^{K262H} vs NR)

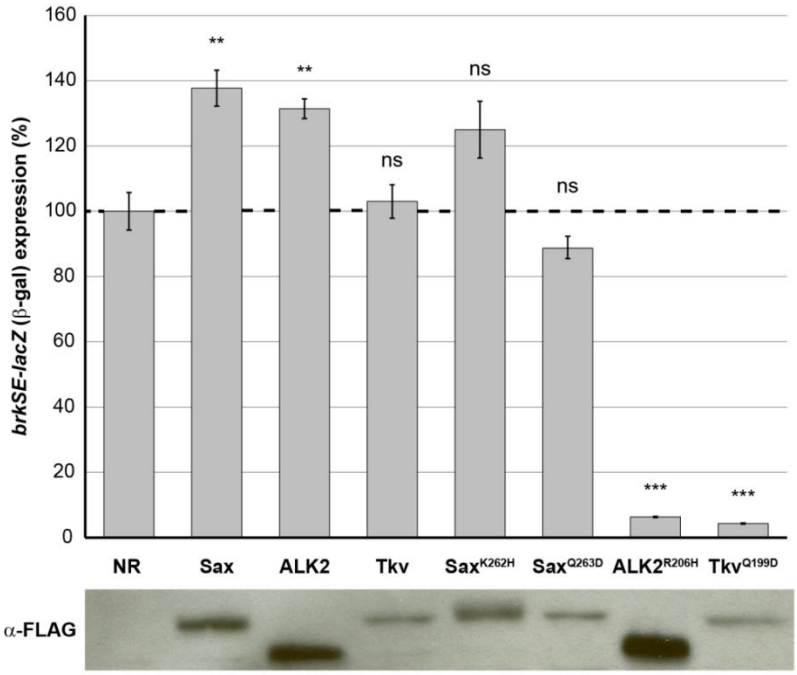


Figure 2.6 Constitutive signaling by *sax*^{Q263D} or *sax*^{K262H} is not observed in S2 cells. β-gal activity in S2 cells transfected with 50 ng of the indicated type I receptors. BMP signaling respresses *brkSE-lacZ* expression. Therefore, loss of β-gal activity serves as a quantitative measure of BMP signaling. β-gal activity in S2 cells transfected with *brkSE-lacZ* was set to 100% (indicated by dashed line). Data represent the mean ±SEM (n=3). Tukey HSD test was performed for multiple pairwise comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; ns, not significant $p > 0.05$). Representative western blot indicating expression level of the FLAG-tagged type I receptors (below chart). Sax^{K262H}, Sax^{Q263D}, ALK2^{R206H}, and Tkv^{Q199D} = GS domain mutants.

Consistent with what was observed in the *brkSE-lacZ* assays, driving expression of the constitutively-active *tkv*^{Q199D} mutant in the dorsal compartment of larval wing discs was sufficient to induce dramatically high levels of ectopic pMad **(Fig. 2-7B)**. Expression of either *sax*^{Q263D} or *sax*^{K262H}, however, collapses the pMad gradient into a single peak in the center of the wing disc--reminiscent of the pattern of pMad observed in wing discs overexpressing wild-type *tkv* **(Fig. 2-7 C & D compared to Fig. 2.2 C)**. These results suggest that the mutations downstream of the GS domain do not confer constitutive activity to the Sax receptor, but do alter Sax's activity. Given that the pMad gradient in *ap>sax*^{Q263D} and *ap>sax*^{K262H} wing discs resembled the pattern observed in

ap>tkv, we asked whether Sax^{Q263D} or Sax^{K262H} could now behave like Tkv and transduce BMP signals rather than inhibit like wild-type Sax does.

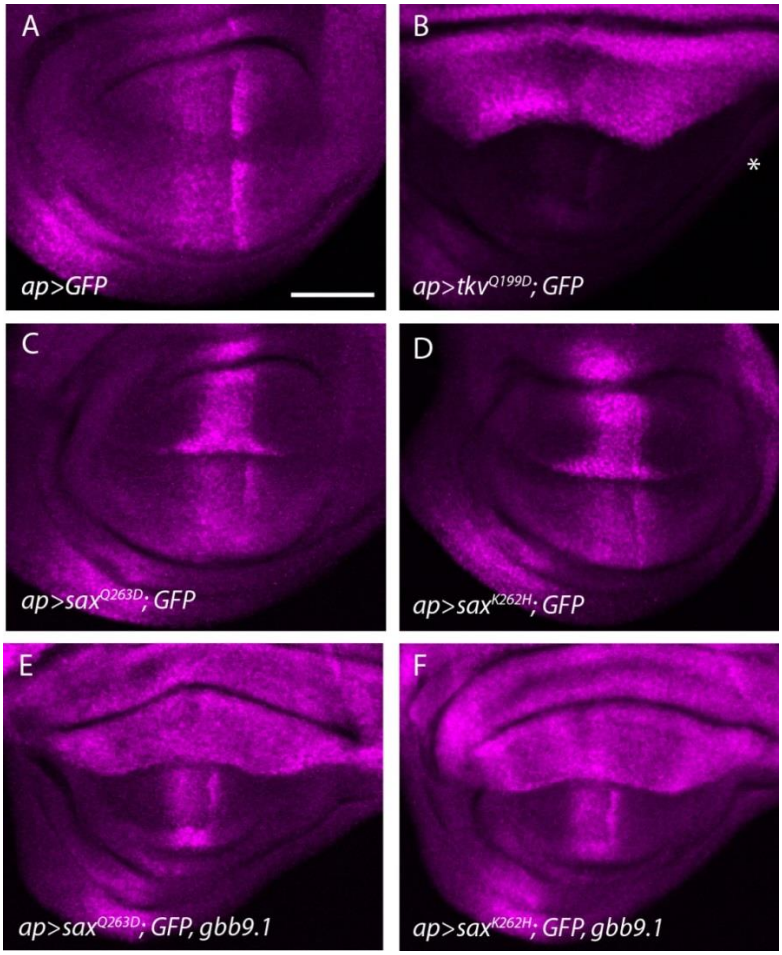


Figure 2.7. Sax GS mutants can mediate *Gbb* signals. Confocal images of pMad (magenta) staining in the wing pouch (dorsal is up, anterior left) of third instar larval wing discs overexpressing **A.** *GFP*, **B.** *tkv^{Q199D}* and *GFP*, **C.** *sax^{Q263D}* and *GFP*, **D.** *sax^{K262H}* and *GFP,* **E.** *sax^{Q263D}*, *GFP,* and *gbb* **F.** *sax^{K262H}*, *GFP,* and *gbb*. Co-overexpression of *gbb* with either *sax^{K262H}* or *sax^{Q263D}* results increased pMad levels. This synergistic effect is reminiscent of co-overexpression *gbb* with *tkv* (**Fig.2.2D**) Expression of UAS transgenes were driven by the dorsal compartment driver *ap-Gal4*. Scale bar = 50 μm. * pMad staining intensity in the ventral compartment of *ap>tkv^{Q199D}, GFP* wing discs (B) appears dramatically reduced because confocal images were collected at lower gain settings than what was used for the other genotypes. This was to avoid oversaturation of the pMad intensity that was very high in the dorsal compartment due to *tkv^{Q199D}* overexpression.

Interestingly, coexpression of *gbb* with either the sax^{Q263D} or sax^{K262H} mutant induced Mad phosphorylation across the dorsal compartment comparable to wing discs coexpressing wild-type *tkv* and *gbb*. A high level of ectopic Mad phosphorylation was also observed when *hh-GAL4* was used to coexpress *gbb* and sax^{K262H} in the posterior compartment—indicating that this effect was not compartment-specific (**Fig. 6.1'**). This is in stark contrast to the behavior of wild-type *sax*, which inhibits *gbb*-mediated BMP signaling. These results indicate that the QD and RH mutation does not confer constitutive signaling activity in *sax* as it does for *tkv* and *ALK2,* respectively. Instead, these mutations confer *gbb*-dependent signaling activity to *sax*.

To test whether the signaling activity of sax^{Q263D} and sax^{K262H} are dependent on ligand-binding, I generated constructs that lacked the ligand-binding domain (ΔLBD) of *sax* for each respective *sax* mutant. Using the *brkSE-lacZ* reporter assay, we tested the ability of *gbb* to repress reporter activity in the presence of wild-type, mutant, and mutant ΔLBD receptors. Consistent with earlier results, cotransfection of *STT* enhanced *gbb*-induced *lacZ* reporter repression whereas *sax* antagonized it (**Fig. 2.8C & 2.9C** . In contrast, both sax^{Q263D} and sax^{K262H} enhanced *gbb*-induced repression of *brkSE-lacZ* (**Fig. 2.8C & 2.9C**). These results support the *in vivo* observation that both *sax* mutants induced ectopic pMad in response to *gbb* overexpression. Importantly, the ability of both *sax* mutants to enhance *gbb* signaling requires the LBD since neither $sax^{Q263D\text{-}\Delta LBD}$ nor $sax^{K262H\text{-}\Delta LBD}$ could enhance *gbb*-induced *lacZ* reporter repression, strongly suggesting that the kinase activity of both mutants is dependent on Gbb-binding to the receptor (**Fig. 2.8C & 2.9C**). Furthermore, a $sax^{K262H\ A289D}$ double mutant, which harbors the A289D mutation corresponding to the loss-of-function sax^{5} allele, is unable to enhance *gbb*-induced repression of *brkSE-lacZ* in S2 cells, suggesting that the ability of sax^{K262H} to enhance BMP signaling is dependent on its kinase activity (**Fig. 6.2**)

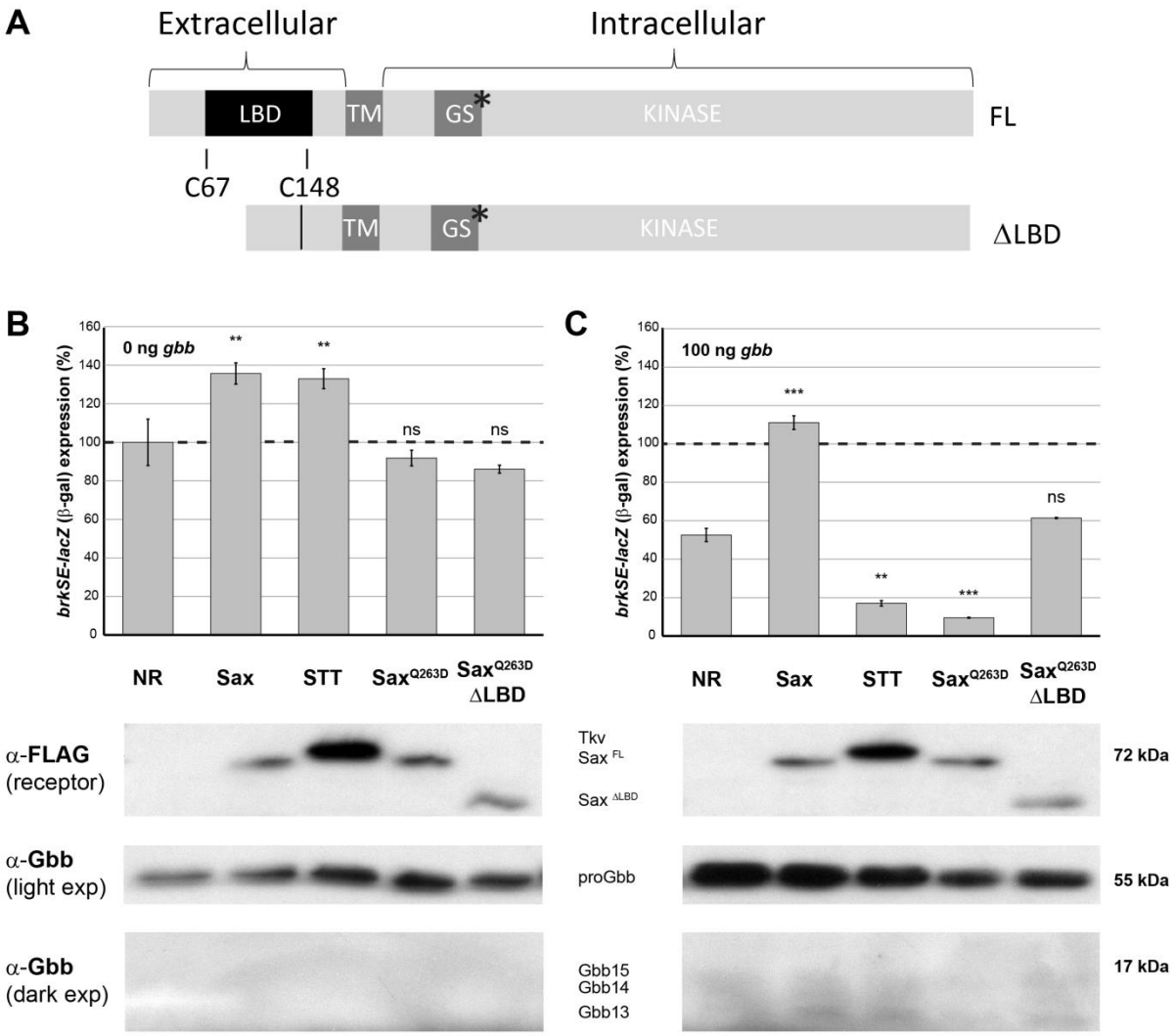


Figure 2.8. Signaling activity of Sax^{Q263D} requires the type I receptor ligand-binding domain. A. Diagram comparing full-length Sax (FL) and the ligand-binding domain deletion mutant Sax$^{\Delta LBD}$ (ΔLBD). * indicates the location of the Q263D mutation. LBD= ligand-binding domain. TM = transmembrane domain. GS = GS (Glycine-Serine Rich) domain. **B.** In the absence of exogenous Gbb, Sax^{Q263D} and Sax^{Q263D} did not affect BMP signaling. Sax and STT moderately inhibited background BMP signaling. S2 cells transfected with 50 ng of the indicated receptor. **C.** Deleting the ligand-binding domain abrogates the ability of *sax^{Q263D}* to mediate Gbb signals. S2 cells cotransfected with 50 ng of the indicated receptor and 100 ng *gbb*. *brkSE-lacZ* expression is repressed by BMP signaling. Therefore, loss of β-gal activity serves as a quantitative measure of BMP signaling. NR indicates that no additional type I receptor DNA was transfected in these conditions. Data represent the mean ±SEM (n=3) of experiments performed in quadruplicate. Multiple pairwise comparisons were performed in R using the Tukey HSD test. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

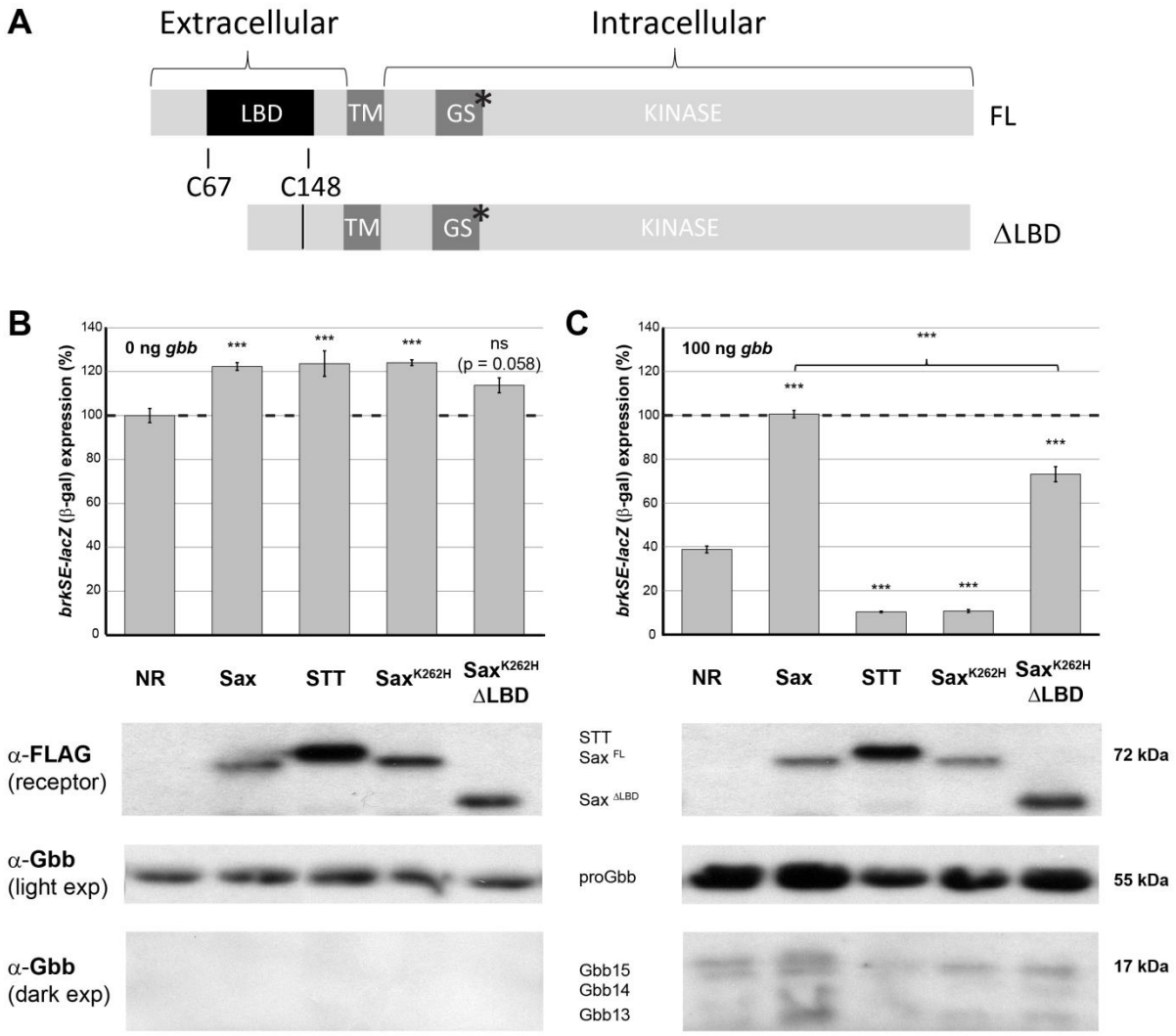


Figure 2.9. Signaling activity of Sax^{K262H} requires the type I receptor ligand-binding domain. A. Diagram comparing full-length Sax (FL) and the ligand-binding domain deletion mutant Sax$^{\Delta LBD}$ (ΔLBD). * indicates the location of the K262H mutation. LBD= ligand-binding domain. TM = transmembrane domain. GS = GS (Glycine-Serine Rich) domain. **B.** In the absence of exogenous Gbb, all type I receptors mildly inhibited background signaling. S2 cells transfected with 50 ng of the indicated receptor. **C.** Deleting the ligand-binding domain abrogates the ability of *sax^{K262H}* to mediate Gbb signals. S2 cells cotransfected with 50 ng of the indicated receptor and 100 ng *gbb*. *brkSE-lacZ* expression is repressed by BMP signaling. Therefore, loss of β-gal activity serves as a quantitative measure of BMP signaling. NR indicates that no additional type I receptor DNA was transfected in these conditions. Data represent the mean ±SEM (n=3) of experiments performed in quadruplicate. Multiple pairwise comparisons were performed in R using the Tukey HSD test. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Drosophila BMP type II receptors Punt and Wit can induce Sax-mediated signaling

Our results thus far indicate that the signaling abilities of Tkv and Sax are different--namely, Tkv is capable of mediating Gbb-induced BMP signaling, whereas Sax cannot. Mutations in the regulatory GS domain (αGS2 helix), however, can uncover ligand-dependent signaling activity in Sax, suggesting that the Sax kinase domain retains activity. In contrast, the corresponding GS domain mutations in Tkv and ALK2 confer constitutive activity. This difference in the signaling requirements of Sax GS mutants versus Tkv GS mutants further underscores how the signaling activities between Sax and Tkv are dissimilar. Taken together, these observations suggest that the signaling activity of Sax is regulated differently from other BMP type I receptors and that this regulation could be occurring at the level of type II phosphorylation.

The observation that the signaling activities of Sax^{Q263D} and Sax^{K262H} were ligand-dependent also suggests a requirement for the type II receptor since the interaction between type I and type II receptors in a signaling complex is mediated by BMP ligands. We therefore tested the ability of the *Drosophila* type II receptors, Punt and Wit, to stimulate the signaling activities of Sax^{Q263D} and Sax^{K262H} using the *brkSE-lacZ* assay. Transfection of *tkv* or sax^{K262H} alone was not sufficient to activate the pathway, whereas a mild activation of the pathway was detected in cells transfected with sax^{Q263D} (**Fig. 2.10 A & B**, Tkv, Sax^{Q263D}, and Sax^{K262H} vs NR, dark gray bars). Transfecting *punt* alone mildly inhibited the pathway, whereas transfection of *wit* alone had no effect on BMP signaling (**Fig. 2.10 A**, Punt, dark gray bar; **Fig. 2.10 B**, Wit, dark gray bar, respectively). However, cotransfecting *tkv,* sax^{Q263D}*, or* sax^{K262H} with either *punt* (**Fig. 2.10 A**, Tkv, Sax^{Q263D}, and $Sax^{K262H,}$ light vs dark gray bars) or *wit* (**Fig. 2.10 B**, Tkv, Sax^{Q263D}, and $Sax^{K262H,}$ light vs dark gray bars) resulted in synergistic activation of BMP signaling. These results suggest that high enough concentrations of type II receptors can

circumvent the requirement for ligand and stimulate the signaling activity of Tkv, Sax^{Q263D}, Sax^{K262H}.

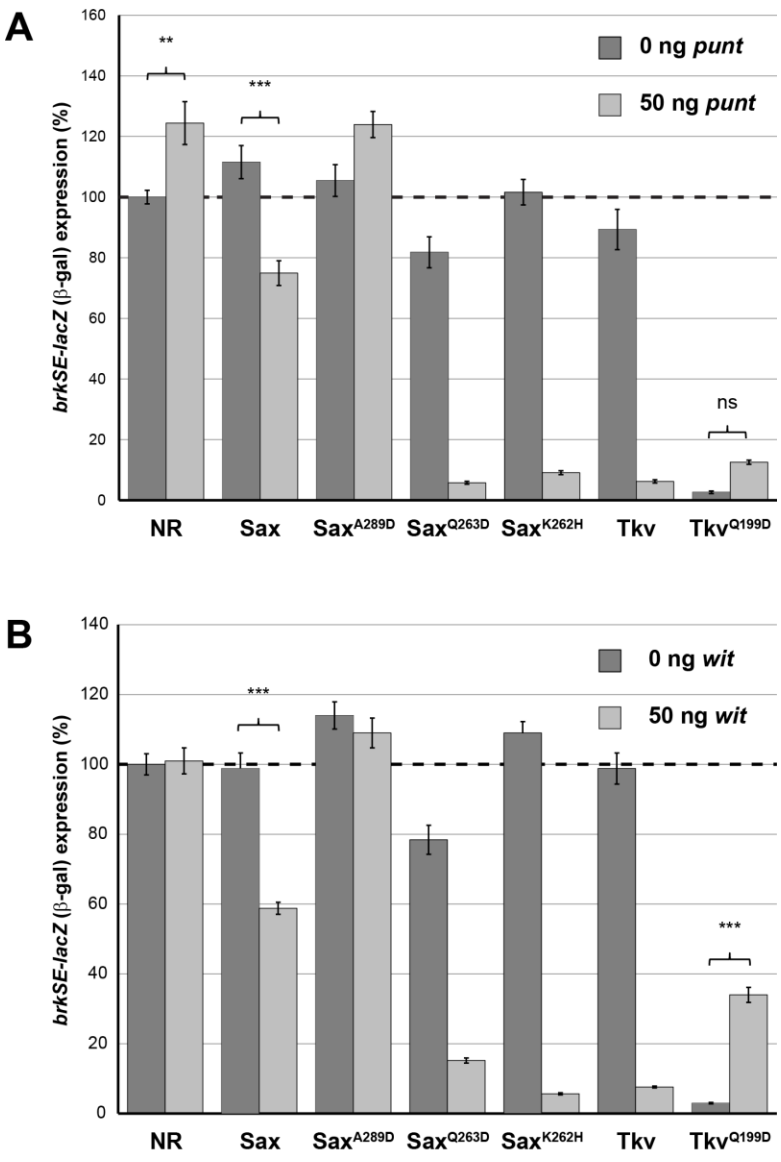


Figure 2.10. Effect of Punt and Wit on signaling activity of different type I receptors. Sax can cooperate with either type II receptor to moderately repress *brkSE-lacZ* expression. **A.** S2 cells transfected with 50 ng of the indicated type I receptors alone (dark gray bars) or cotransfected with 50 ng of *punt* (light gray bars). **B.** S2 cells transfected with 50 ng of the indicated type I receptors alone (dark gray bars) or cotransfected with 50 ng of *wit* (light gray bars). Loss of β-gal activity indicates repression of the BMP-responsive *brkSE-lacZ* reporter construct and is a measure of BMP signaling. β-gal activity in S2 cells transfected with *brkSE-lacZ* (NR) was set to 100% indicated by dashed line). Data represent the mean ±SEM (n=3). Tukey HSD test was performed for multiple pairwise comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; ns, not significant $p > 0.05$). Sax^{A289D} = dominant-negative mutant encoded by *sax[5] allele*. Sax^{Q263D}, Sax^{K262H}, Tkv^{Q199D} = GS domain mutants.

We also tested the effect of Punt and Wit activity on the ability of Tkv^{Q199D} to signal. Cotransfection of *punt* with *tkv^{Q199D}* did not alter Tkv^{Q199D} signaling activity. Curiously, cotransfection of *wit* antagonized the reporter repression mediated by *tkv^{Q199D}* (**Fig. 2.10 B** Tkv^{Q199D}, light vs dark gray bar). This result was unexpected given that data from our lab indicates that the signaling activity of *tkv^{Q199D}* is type II receptor-dependent **(Fig. 4.2 G, LE)**. These results are difficult to interpret, however, because the level of *brkSE-lacZ* repression mediated by Tkv^{Q199D} may have reached saturation considering the dramatic reduction in β-gal activity. Therefore, the concentration of t*kv^{Q199D}* that is transfected should be reduced in future experiments.

Importantly, we also observed the ability of Punt and Wit to stimulate wild-type Sax signaling. Cotransfection of *sax* with either *punt* or *wit* resulted in moderate activation of the pathway (**Fig. 2.10 A & B**, Sax light vs gray bars) albeit to a lesser extent than the observed cooperative signaling between the type II receptors and Tkv, Sax^{Q263D}, or Sax^{K262H}. These results are noteworthy for several reasons. First, these observations represent conditions under which the wild-type Sax protein can mediate signaling, which until this point had not yet been identified using the *brkSE-lacZ* assay. Furthermore, it is highly likely that the type II receptors are specifically stimulating Sax *kinase* activity since neither Punt nor Wit could cooperate with the dominant-negative Sax^{A289D} mutant receptor to activate signaling. Thus, these results are inconsistent with Sax being a dead kinase.

Moreover, the cooperative signaling exhibited by the type II receptors and Sax was markedly weaker than observed for the Sax GS domains. This observation is consistent with a model wherein Sax^{Q263D} and Sax^{K262H} are more prone/sensitive to type II receptor activation by virtue of their GS domain mutations. Therefore, in this scenario the barrier to activating the kinase activity of the Sax GS domain mutants can be thought

of as being lower than wild-type Sax.If the activation of wild-type Sax and the Sax GS domain mutants (or Tkv for that matter) are indeed quantitatively different, then it may be reflected in the extent to which each receptor's GS domain is phosphorylated.
It may be possible to use quantitative mass spectrometry methods such as SILAC to detect differences in phosphorylation. Our first trial run using standard mass spectromety recovered phosphopeptides that correspond to the GS domain in both wild-type Sax and Sax^{K262H}, although over half of the hits were low scoring (**Table 6**). In subsequent trials, we will enrich for phosphoserine peptides to see if the data improve.

Although both type II receptors stimulated Sax kinase activity, we wanted to investigate whether Sax was preferentially activated by Punt or Wit. We performed a titration experiment to determine the minimal amount of cotransfected *put* or *wit* that was required to stimulate Sax signaling activity. We observed that Punt stimulated Sax only at higher concentrations of transfected *punt,* and that the effect on BMP signaling was not statistically significant when the amount of cotransfected *punt* was increased from 50 ng to 100 ng (**Fig. 2.11 A'**). In contrast to *punt,* as little as 10 ng of cotransfected *wit* was sufficient to stimulate Sax activity (**Fig. 2.11 B'**). Furthermore, cotransfection of *wit* clearly induced Sax signaling activity in a dose-dependent manner. These results indicate that Sax signaling activity is more sensitive to the amount of transfected *wit* than *punt*, and may reflect a preference for activation by Wit. Importantly, the transfection of either *punt* or *wit* alone at all concentrations tested was not sufficient to repress *brkSE-lacZ* expression (**Fig. 2.11 A & B**), reinforcing the cooperative nature between type II receptors and Sax in the activation of BMP signaling.

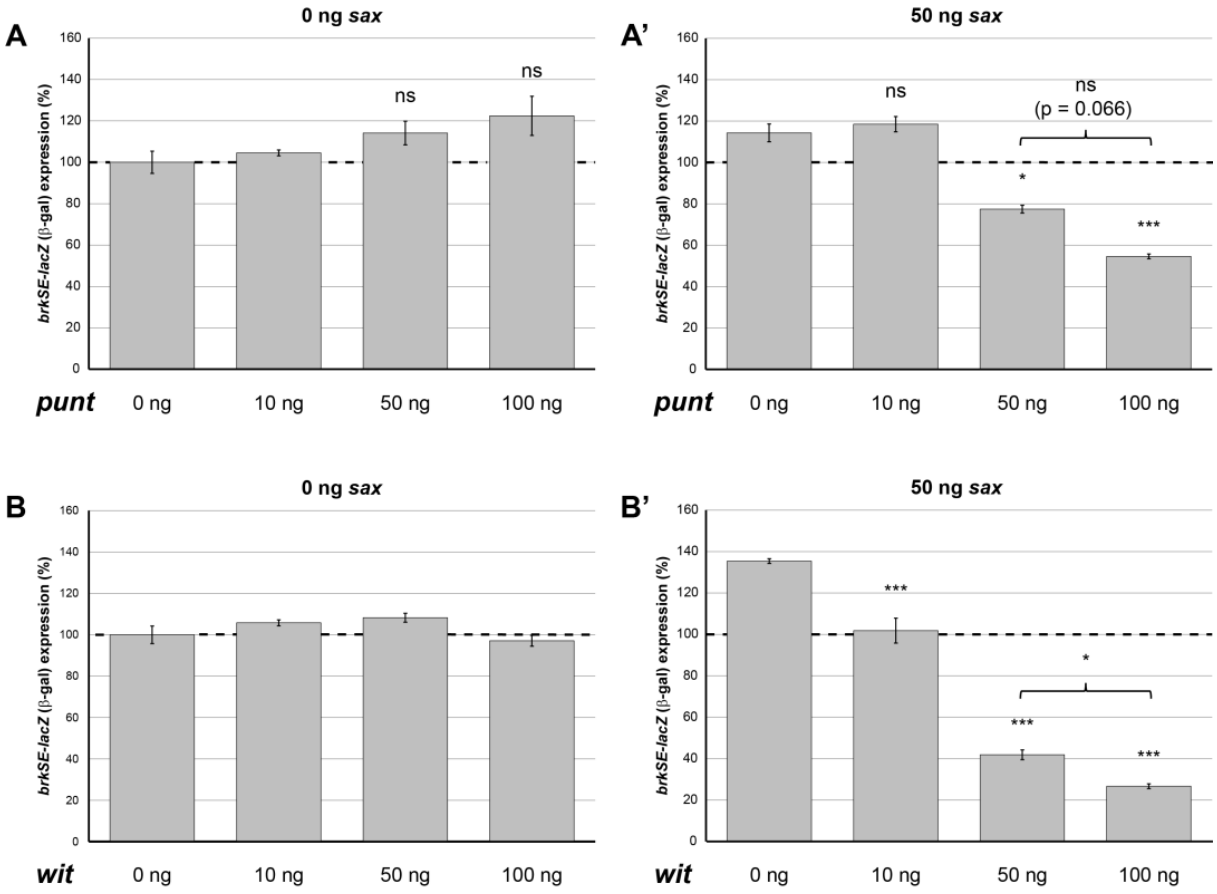


Figure 2.11. Effect of type II receptor concentration on Sax signaling. Sax-mediated repression of the *brkSE-lacZ* reporter is more sensitive to the concentration of cotransfected *wit* than *punt*. **A, A'.** β-gal activity measured in S2 cells transfected with the indicated amounts of *punt* alone (**A**) or cotransfected with 50 ng *sax* (**A'**). **B)** β-gal activity measured in S2 cells transfected with the indicated amounts of *wit* alone (**B**) or cotransfected with 50 ng *sax* (**B'**). Loss of β-gal activity indicates repression of the BMP-responsive *brkSE-lacZ* reporter construct and is a measure of BMP signaling. β-gal activity in S2 cells transfected with *brkSE-lacZ* was set to 100% (indicated by dashed line). Data represent the mean ±SEM (n=3). Tukey HSD test was performed for multiple pairwise comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; ns, not significant $p > 0.05$).

Gbb does not enhance type II receptor-Sax cooperative signaling.

The ability of Sax to cooperatively signal with either of the type II receptors contrasts with Sax's antagonistic behavior toward Gbb . This raised the possibility that Sax inhibited Gbb-induced signaling in our assays because the concentration of type II receptors was too low in the wing disc and in S2 cells to stimulate Sax signaling activity.

We hypothesized that cotransfection of *gbb* with *sax* and either type II receptor would enhance the cooperative signaling produced by Sax and a type II receptor (**Fig. 2.12**).

Consistent with previous results, transfection of *gbb* repressed *brkSE-lacZ* expression by ~40% (**Fig. 2.12,** NR light vs dark gray bar). Cotransfection of *punt* did not affect Gbb signaling, whereas *wit* enhanced Gbb signaling. These results suggest that Gbb can coordinate receptor complexes consisting of endogenously expressed type I receptors and exogenous Wit, but not exogenous Punt (**Fig. 2.12,** Punt vs Wit). As expected, Sax inhibited Gbb-induced signaling, but cooperated with either type II receptor to activate BMP signaling (**Fig. 2.12,** Sax compared to Punt/Sax and Wit/Sax). However, cotransfection of *gbb* did not enhance the BMP signaling produced by Punt:Sax or Wit:Sax (**Fig. 2.12,** Punt/Sax vs Wit/Sax). The inability of Gbb to enhance Wit:Sax cooperative signaling was particularly surprising given data that suggests Gbb signals through Wit and Sax at the NMJ (Aberle et al., 2002; Marqués et al., 2002; Marqués et al., 2003; McCabe et al., 2003; McCabe et al., 2004; Rawson et al., 2003)**.** These results may indicate that Gbb is not mediating the formation of signaling complexes between Sax and either type II receptor.

Several experiments can be done to gain more insight on the inability of Gbb to enhance type II receptor-Sax cooperative signaling. One possibility is that the interaction between Sax and either type II receptor is saturated at the cotransfection amounts tested (50ng), such that Gbb cannot facilitate the formation of additional signaling complexes. We may, however, be able to detect *gbb* enhancement of cooperative signaling by reducing the amount of *punt* and *wit* that is cotransfected with *sax*. Additionally, the question remains as to whether this inability of Gbb to enhance cooperative signaling is specific to Sax. Thus, it will be important to investigate if and how Gbb affects the cooperative signaling exbhibited by type II receptors and the type I receptors capable of mediating ligand-induced signals such as Tkv, Sax^{Q263D}, and Sax^{K262H}. Furthermore,

coimmunoprecipitation experiments should be employed to determine if Gbb can simultaneously interact with Sax and type II receptors within a signaling complexes. First, this would involve crosslinking Gbb to Sax, followed by sequential immunopurification of Gbb and then Sax to enrich for ligand-bound complexes. If type II receptors (either Punt or Wit) coimmunoprecipitate, then it would be strong evidence that Gbb can interact with Sax and a type II receptor simultaneously.

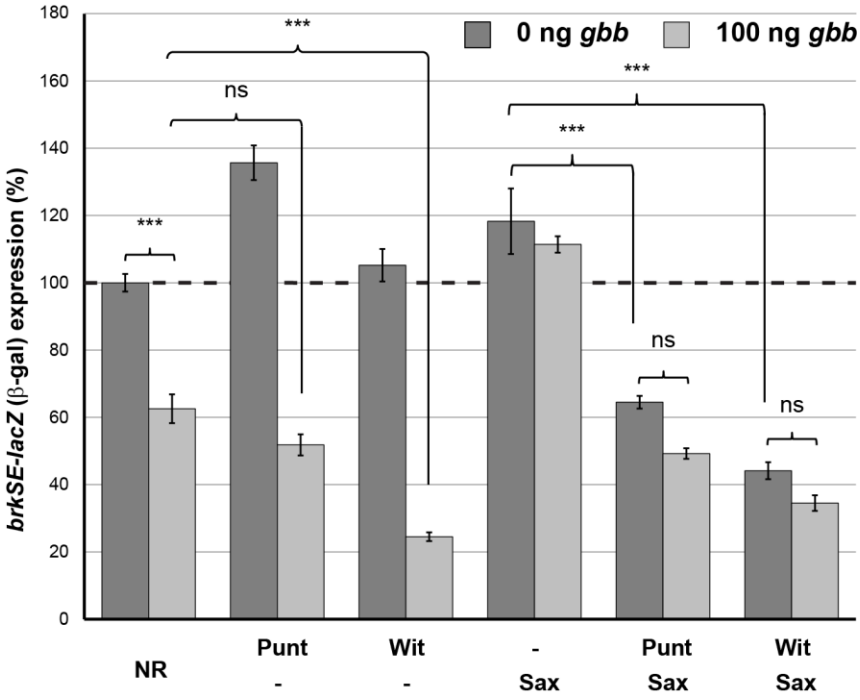


Figure 2.12. Gbb does not enhance Sax:Type II receptor cooperative BMP signaling. Sax mediated repression of the *brkSE-lacZ* reporter can be stimulated by either Punt or Wit, but this cooperative effect is not enhanced by Gbb. β-gal activity measured in S2 cells cotransfected with combinations of 50 ng of *sax, punt,* or *wit* as indicated without *gbb* (dark gray bars) or with 100 ng of *gbb* (light gray bars). Loss of β-gal activity indicates repression of the BMP-responsive *brkSE-lacZ* reporter construct and is a measure of BMP signaling. β-gal activity in S2 cells transfected with *brkSE-lacZ* was set to 100% (indicated by dashed line). Data represent the mean ±SEM (n=3). Tukey HSD test was performed for multiple pairwise comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; ns, not significant $p > 0.05$).

DISCUSSION

Previous genetic experiments have demonstrated the requirement for *sax* in BMP signaling during wing development (Bangi and Wharton, 2006b; Singer et al., 1997). However, the observation that Sax can both promote and inhibit BMP signaling led to the development of model in which the dual behavior of Sax is determined by the combination of type I receptors in the signaling complex (Bangi and Wharton, 2006b). In this model, BMP signaling is antagonized by complexes in which both type I receptors are Sax. Presumably, Sax:Sax complexes are unable to phosphorylate Mad and therefore, inhibit BMP signaling by titrating away available ligand. In contrast, Sax:Tkv- and Tkv:Tkv-containing complexes transduce BMP signals. Importantly, Sax:Tkv complexes must be contributing to BMP signaling to satisfy the requirement for *sax* in establishing the pMad gradient in the wing disc. Consistent with this proposal, our results demonstrate that Sax cannot phosphorylate Mad in response to Gbb in cell culture and *in vivo*, whereas Tkv can (**Fig. 2.2-2.5**).

This model, however, also raises other questions regarding the nature of Sax within in signaling complexes. For instance, is the Sax receptor simply a "dead" kinase? In which case, Sax:Sax complexes would completely lack type I receptor kinase activity and thus, be unable to phosphorylate Mad. Furthermore, a kinase dead Sax would necessitate that Tkv be the sole kinase in a Sax:Tkv complex, whereas Sax would act as a "silent co-receptor." In this context, *sax* may be required in the wing disc solely to form Sax:Tkv complexes and accommodate binding of Gbb:Dpp heterodimers. BMP heterodimer signaling through a requisite hetero-type I receptor complex has been observed in zebrafish (Little and Mullins, 2009), but has not been conclusively demonstrated in *Drosophila*. However, the observed synergistic activation of BMP signaling by Tkv and Sax in the embryo and wing hints at a heteromeric complex.

Furthermore, experiments performed in S2 cells demonstrate that purified Gbb:Dpp and Scw:Dpp heterodimers require the presence of both Sax and Tkv to activate BMP signaling since dsRNAi knockdown of either receptor interferes with heterodimer-induced Mad phosphorylation (Shimmi et al., 2005a; Shimmi et al., 2005b). This result would be consistent with BMP heterodimers signaling through an obligate hetero-type I receptor complex.

Alternatively, Sax kinase activity may be under an additional level of regulation that is present in a Sax:Sax complex, but is either absent or overcome in a Sax:Tkv complex. It may be the case that the presence of Tkv in the complex stimulates Sax kinase activity either through direct activation or indirectly through a factor in *trans*. Certainly, there is precedent for type I receptors influencing each other's activity within a complex. For instance, the full kinase activity of the BMP type I receptor ALK1 requires cooperation with ALK5. Intriguingly, ALK5 is a type I receptor belonging to the closely related TGF-b signaling pathway and represents an instance where the pathways interact/crosstalk to affect signaling outcomes.

Sax possesses a functional kinase domain

Several pieces of data suggest that Sax is not simply a dead kinase nor a "silent co-receptor." Although the results from the *sax* mutant clone experiments confirm the requirement for *sax* in establishing the BMP activity gradient during wing development, the different alleles of *sax* used in the experiment help specify the nature of the requirement (**Fig. 2.1**). For instance, sax^{4} is a null allele that arises from an early stop codon mutation (Q114X) in the extracellular domain of Sax (Bangi, 2005; Twombly et al., 2009). Thus, the loss of Mad phosphorylation in sax^{4} null clones indicates that Sax function is absolutely required for BMP signaling in the wing disc.

In contrast, genetic and sequencing experiments reveal that *sax*^{5} is a dominant-negative allele resulting from an A289D mutation, which is located in a region of the kinase domain involved in binding ATP. The A289D mutation is therefore predicted to disrupt ATP-binding and thereby interferes with the abilility of the Sax kinase domain to phosphorylate Mad. (Bangi, 2005; Hanks and Hunter, 1995; Twombly et al., 2009). Furthermore, the s*ax*^{5} cDNA can also produce a full-length Sax^{A289D} protein as detected by western blot in cell culture (**Fig. 2.4**). Thus, the loss of Mad phosphorylation in *sax*^{5} clones suggests, more specifically, that it is the kinase activity of Sax that is required for normal BMP signaling.

If Sax were only required to act as a silent co-receptor, then we would expect BMP signaling in the wing disc to tolerate Sax mutations that presumably affect type I receptor kinase activity as long as a receptor is still produced. The loss of pMad in *sax*^{5} mitotic clones, however, argues that the BMP signaling requires input from Sax kinase activity. However, it is also possible that Sax^{A289D} might display dominant-negative behavior in *trans* and block Tkv-signaling, for which there is some evidence in cell culture (Twombly et al., 2009).

In addition to the genetic clone experiments, we identified conditions under which Sax kinase activity could be stimulated. Our investigation revealed that wild-type Sax kinase activity can be stimulated by exogenously expressed type II receptors, which further argues against Sax being a dead-kinase. Cotransfection of either *punt* or *wit* with *sax* in S2 cells resulted in activation of Sax signaling as indicated by synergistic repression of the *brkSE-lacZ* reporter. It should be noted, however, that the level of cooperative signaling produced by type II receptor-stimulation of Sax was mild compared to that of Tkv, highlighting again how these two receptors behave differently. This disparity may reflect additional steps that are required in the activation of the Sax kinase or that Sax interacts with type II receptors with lower affinity than Tkv.

Sax is regulated by an additional mechanism acting at the level of the type II receptor and GS domain

If Sax is not a dead kinase, then the question as to how Sax activity is kept inactive in Sax:Sax complexes remains. Our results suggest that Sax:Sax complexes are under an additional level of regulation that prevents them from signaling. Additionally, our results indicate that the Sax GS domain as well as interactions between Sax and the ty pe II receptors may mediate this regulation. In this study, we demonstrated that Sax kinase activity of can be uncovered by two mutations in the GS domain. This region of the receptor contains the serine/threonine residues that are transphosphorylated by the type II receptor. Phosphorylation of these residues activates the type I receptor kinase by destabilizing the autoinhibitory conformation of the type I receptor. We characterized two GS domain mutations, K262H and Q263D, and found that both were sufficient to alter Sax's response to Gbb. Rather than inhibiting BMP signaling, Sax^{K262H} and Sax^{Q263D} transduced Gbb signal as indicated by increased Mad phosphorylation *in vivo* and repression of the *brkSE-lacZ* (**Fig. 2.7-2.9**). Furthermore, both Sax^{K262H} and Sax^{Q263D} mutants exhibited increased sensitivity to stimulation by Punt or Wit as evidenced by the higher cooperative signaling observed between type II receptor and either of the Sax GS domain mutants in S2 cells (**Fig. 2.10**). Based on these results, the Sax GS domain appears to keep Sax kinase inactive even in response to BMP ligand. Mutations in the GS domain, however, can unravel this regulation thereby lowering the threshold for type II receptor transphosphorylation and kinase activation.

Our experiments involving Sax GS domains mutants, however, further underscores how Sax differs from other type I receptors. In contrast to how the GS mutations behave in Sax, the corresponding mutations in other type I receptors actually confer constitutive activity. For instance, the mutation corresponding to Q263D is routinely used to generate constitutively active type I receptors of not only the BMP

signaling pathway (represented by Tkv^{Q199D} in this study) but also the closely-related TGF-β and Activin pathways. The K262H mutation corresponds to a histidine substitution of arginine 206 in the human Sax ortholog ALK2 and is responsible for the heterotopic bone disease fibrodysplasia ossificans progressiva. A number of studies have demonstrated the constitutively-active nature of ALK^{R206H} (Billings et al., 2008; van Dinther et al., 2010; Le and Wharton, 2012; Shen et al., 2009; Song et al., 2010). This discrepancy between how the FOP mutation behaves in Sax and ALK2 may indicate that Sax and ALK2 cannot functional substitute for one another despite their classification as orthologs. If anything, the GS domains mutations appear to abolish Sax's ability to antagonize BMP signaling and "convert" its behavior to reflect typical type I receptor function in transducing signals.

It has been proposed that mutations in the GS domain confer constitutive activity by disrupting interactions that stabilize the autoinhibited conformation of type I receptors. Alternatively, the GS domain mutations may lower the threshold for kinase activation by altering local conformation such that the GS domain serines are exposed for transphosphorylation by the type II receptor. Furthermore, these possibilities are not mutually exclusive. However, the requirement for either exogenous ligand or type II receptor to activate Sax GS mutant signaling suggests that GS mutations may not sufficiently destabilize the autoinhibitory interactions in Sax as they do in other type I receptors. Therefore, the activation of the Sax kinase may involve an additional step or factor.

Model for how Sax kinase is kept inactive

Taken together, these results can be incorporated to expand the model in which Sax:Sax complexes do not transduce signals. We propose that Sax:Sax complexes prohibit proper transphosphorylation of the Sax GS domain by the type II receptor. This

block to transphosphorylation may arise if the Sax GS domain is not accessible to type II receptors within Sax:Sax complexes. Alternatively, Sax:Sax complexes may not readily interact with type II receptors (i.e. low affinity)—a scenario supported by the observation that increasing type II receptor concentration can stimulate Sax signaling activity. The GS mutations that uncover Sax activity may serve to either increase the affinity of Sax:Sax complexes for type II receptors or expose the GS domain for transphosphorylation. Moreover, we expect that Tkv would elicit similar effects in a Sax:Tkv complex to enable the activation of Sax kinase activity. That said, our efforts to detect Tkv-mediated activation of Sax kinase activity, however, remain inconclusive and will require new methods to adequately determine if the presence of Tkv can stimulate Sax signaling (**Fig. 6.6**).

The observation that Sax GS mutants are not constitutively active suggests that Sax requires an additional step for kinase activation that is not required by other type I receptors. This additional layer of regulation may be intrinsic to the Sax receptor. For instance, the specific sequence of the Sax GS domain may stabilize the autoinhibitory conformation to a greater extent than other type I receptors. Another equally possible scenario is that sequences outside of the Sax GS domain may prevent type II receptor interactions.

On the other hand, a regulator may act in *trans* to keep Sax:Sax complexes silent. One potential factor that might mediate this regulation is FKBP1A, which binds to the GS domain to further stabilize the autoinhibitory conformation while also masking the GS domain from transphosphorylation. The presence of FKBP1A could also interfere with the interaction of Sax with type II receptors. Normally, ligand-binding induces the dissociation of FKBP1A from the type I receptor and activation of type I kinase subsequently follows. However, in the case of Sax, FKBP1A might not dissociate from Sax:Sax complexes in response to ligand-binding, thereby keeping Sax:Sax complexes

inactive. In contrast, the Sax GS domain mutations may destabilize the interaction with FKBP1A, but would still require BMP ligand for complete dissociation of FKBP1A and subsequent activation of the Sax^{K262H} or Sax^{Q263D} kinase.

Lastly, we do not expect this additional level of regulation to be present for Tkv given that wild-type Tkv is sufficient to transduce BMP signals. Therefore, differences in the amino acid sequence between Tkv and Sax should account for their disparate behaviors. A molecular dissection of Tkv and Sax will provide critical insight into the determinants of Sax's dual behavior. More specifically, exploiting chimeric receptors in which different domains of Tkv and Sax are substituted for each other should allow us to identify the sequences that are required for the signaling activity observed in Tkv as well as sequences that might mediate the additional layer of regulation that keeps Sax inactive in Sax:Sax complexes. Furthermore, the similar degree to which Tkv, Sax^{Q263D}, and Sax^{K262H} signaling activities were stimulated by the type II receptors suggests that the behavior of the Sax mutants is consistent with behaving like a "pure" type I receptor—with no antagonistic behavior.

Chapter 3

Identifying the molecular determinants responsible for the antagonistic behavior of the *Drosophila* BMP type I receptor Saxophone

The work in this chapter represents a collaboration with Dr. Takuya Akiyama and Robin Brese. My contribution to this chapter includes Figures 3.2, 3.7, 3. 8, & 3.10-3.12 as well as experiments referenced herein that appear in the Appendix.

ABSTRACT

BMP signaling plays critical roles in many aspects of development. The misregulation of BMP signaling is associated with many developmental abnormalities and diseases. Studies have shown that BMP signaling is tightly regulated at a number of different points along the transduction of the signal. Previous work from our laboratory has shown that during normal development the *Drosophila* BMP type I receptor Sax can either promote or antagonize BMP signaling depending on the context. In contrast, Tkv can only facilitate BMP signaling.

Here, we report the identification of several protein domains that are critical for specific behavior of type I receptors. By analyzing the signaling activity of chimeric receptors generated by fusing different domains of Sax and Tkv, we found that the pairing of two determinants dictates type I receptor behavior. One determinant resides in the region encompassing the juxtamembrane and GS domains (JM-GS), whereas the other determinant is located in the kinase domain (KD).

Refinement of these domains suggests that Sax's inhibitory behavior and Tkv's facilitating behavior is a function of sequences found in their respective juxtamembrane domain-αGS1 helix and E6 loop. Given that these regions have been implicated in type I receptor activation by the type II receptor and type I dimerization, we propose that Sax's JM-αGS1-helix and E6 loop prevent Sax:Sax dimers from adopting a conformation that permits type II transphosphorylation. Therefore, Sax:Sax receptor complexes would remain inactivated and not be able to transduce a signal. In contrast, Tkv's JM-αGS1 helix and E6 loop permit dimerization of Tkv:Tkv or Sax:Tkv into a configuration in which type II transphosphorylation can occur. Our findings shed light on how structural elements of the BMP receptors influence signaling/kinase activity.

INTRODUCTION

Bone Morphogenetic Proteins (BMPs), a subfamily of Transforming Growth Factor-β (TGF-β), are secreted signaling molecules that control many developmental processes including patterning, cell migration and homeostasis. BMP signaling is mediated by a tetrameric complex of receptors consisting of two type I and two type II receptors (Bragdon et al., 2011; Mueller and Nickel, 2012; Wu and Hill, 2009). Upon binding of BMPs to receptor complexes, the type II receptor activates the type I receptor by phosphorylating the glycine-serine activation (GS) domain of the type I receptor. The activated type I receptor recruits and phosphorylates a receptor-mediated Smad (R-Smad, Smad1/5/8). The phosphorylated form of R-Smad (pSmad) forms a complex with the co Smad (Smad4) and accumulates in the nucleus to regulate the transcription of BMP target genes (Massagué and Wotton, 2000).

In mammals, BMP signaling is transduced by four type I receptors: the highly similar ALK1/2 proteins and the highly similar ALK3/6 proteins. In contrast to mammals, the *Drosophila* genome encodes only two BMP type I receptors, Saxophone (Sax, ALK1/2 ortholog) and Thickveins (Tkv, ALK3/6 ortholog) (Affolter et al., 1994; Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994). Both receptors are thought to mediate BMP signaling, and several studies suggest that Tkv and Sax act in concert to synergistically activate the pathway (Haerry et al., 1998; Neul and Ferguson, 1998; Nguyen et al., 1998; Shimmi et al., 2005a; Shimmi et al., 2005b). However, a previous study investigating the role of Sax during wing development revealed an unexpected dual behavior of Sax (Bangi and Wharton, 2006b). In contrast to Tkv, Sax exhibits both facilitating and antagonistic functions.

Our current model proposes that receptor complexes composed solely Sax type I receptor molecules do not transmit a signal, whereas complexes composed of either two

Tkv molecules or one Tkv and one Sax receptor molecule will each transduce a signal. We also propose that signaling incompetent Sax:Sax complexes bind and sequester ligand, thereby affecting the total pool of ligand available for signaling. Sax:Sax complexes could therefore behave as a ligand "sink" to modulate/limit the level and possibly the range of BMP signaling depending on the spatial distribution of the different receptor complexes. The precise mechanism by which receptor complex composition is established is not clear. Furthermore, the means by which Sax:Sax complexes are incapable of signaling while Tkv: Tkv and Sax:Tkv complexes, instead, transduce signals is not understood.

All BMP type I receptors are characterized by a signal peptide, extracellular ligand binding domain, transmembrane (TM) domain, juxtamembrane (JM) domain, GS domain, and kinase domain (**Fig. 1.12, 3.1**, & **3.2A**). Despite a proposed overall similarity in protein structure, differences are apparent between the primary sequences of Tkv and Sax. For instance, the extracellular domains of Tkv and Sax are divergent, most likely reflecting each receptor's preference for specific BMP ligands. Previous work has indicated that Tkv is the high affinity receptor for Decapentaplegic (Dpp), whereas Sax is the high affinity receptor for Glass Bottom Boat (Gbb) and Screw (Scw) (Haerry et al., 1998; Haerry, 2010; McCabe et al., 2003; Neul and Ferguson, 1998; Nguyen et al., 1998). Tkv and Sax also differ in their L45 loop sequence, which dictates R-Smad specificity (Armes et al., 1999; Chen et al., 1998b; Chen and Massagué, 1999; Feng and Derynck, 2005; Itoh et al., 2003). Tkv is characterized by an L45 loop shared by ALK3/6, whereas Sax's L45 loop belongs to the ALK1/2 group of type I receptors (Chen and Massagué, 1999). Both L45 loop sequences, however, are sufficient to dictate signaling through Smad1/5/8 and not the TGF-β/Activin Smad2/3 in vertebrates (Armes et al., 1999; Chen et al., 1998b; Chen and Massagué, 1999; Feng and Derynck, 1997). Differences in the primary sequence of Tkv and Sax also map to the juxtamembrane

domain, a region that has not been characterized, and the E6 loop, which has been implicated in the activation and dimerization of type I receptors (Haerry, 2010; Huse et al., 1999; Weis-Garcia and Massagué, 1996). In the experiments presented below, we investigated whether the differences in these particular domains of Tkv and Sax account for Sax's dual behavior.

In this study, we made use of a *Drosophila* Schneider 2 (S2) cell culture system (Bangi and Wharton, 2006b; Müller et al., 2003) which provides a method to quickly investigate the antagonistic function of Sax in cells. As such, we found that BMP signaling is predominantly inhibited in cells transfected with *sax* alone, whereas BMP signaling is promoted in cells transfected with *tkv*.

Generating chimeric receptors that consist of domains swapped between Sax and Tkv revealed particular regions of Sax critical for antagonizing signaling and regions of Tkv critical for its function in facilitating signaling. In particular, the signaling behavior of type I receptors is dictated by two determinants—one found in the J-GSD region encompassing the juxtamembrane and GS domains (J-GSD) and one in the kinase domain (KD). Furthermore, full Sax-like inhibitory function requires the pairing of these two determinants from the Sax receptor. Similarly, full Tkv-like signaling activity requires the pairing of the Tkv determinants. Further refinement of these domains suggests that one element resides in the JM-αGS1-helix and the other is the E6 loop. Both of these subdomains have been implicated in kinase activation by the type II receptor(Franzén et al., 1995; Huse et al., 2001; Weis-Garcia and Massagué, 1996; Wrana et al., 1994a). Furthermore, the E6 loop has also been suggested to impact type I dimerization(Huse et al., 1999). We propose that these domains affect the configuration of type I receptors within a signaling complex such that Sax:Sax cannot be activated by type II receptors, whereas Sax:tkv and Tkv:Tkv complexes can.

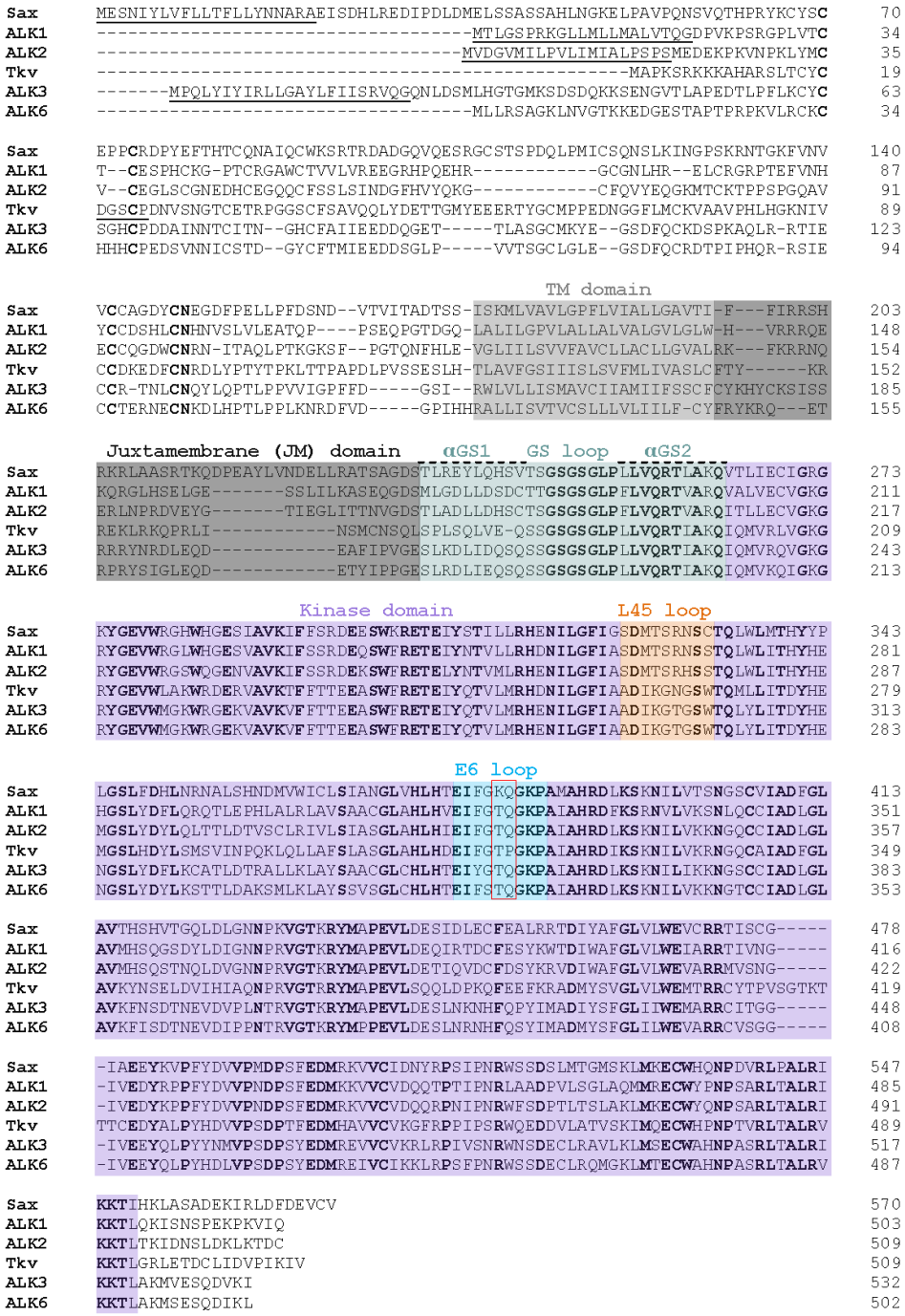


Figure 3.1. Protein alignment of Sax, Tkv, and human BMP type I receptors.
Drosophila Sax, Tkv, human ALK-1, ALK-2, ALK-3 and ALK-6 proteins were aligned. Underlined sequences in extracellular domain (region upstream of TM) indicate putative signal peptides for each protein. TM, transmembrane. Thick underline region indicates sequences included in the J-GSD for chimeric receptor construction. The J-GSD includes the juxtamembrane (JM) domain, αGS1 helix, GS loop, and αGS2 helix.

MATERIALS AND METHODS

DNA constructs

We used *sax-RA* and *tkv-RD* (Flybase: http://flybase.org/) transcripts as templates to produce all DNA constructs used in this study. *sax-tkv* chimeras were generated by PCR. Extracellular-transmembrane (ETD), glycine-serine activation (J-GSD) and kinase (KD) domains of *sax* and *tkv* were amplified by PCR. These regions were connected in different combinations by a second round of PCR. Invitrogen Gateway system (Invitrogen) was used to obtain all DNA constructs in pAW (DGRC, an S2 cell expression vector).

To generate Hemagglutinin (HA)-tagged and FLAG-tagged forms of *sax*, *tkv* and *sax-tkv* chimeras, we used Gateway cloning to recombine *sax, tkv and sax-tkv* chimeric cDNA into *pAWH* (DGRC, an S2 cell expression vector containing an actin promoter and a C-terminal 3x HA tag) and *pAWF* (DGRC, an S2 cell expression vector containing an actin promoter and a C-terminal 3x FLAG tag), respectively.

All primers used in this study are listed in Table S1. Underline indicates *attB1* and *attB2* DNA sequences. Bold characters in *sax* and *tkv* primers contain sequential homology against *tkv* and *sax* cDNAs, respectively. Homology sequences were used to combine each region of *sax* and *tkv* at 2nd PCR.

Genes/PCR products	Primer sequences
sax sense	5’-ggggacaagtttgtacaaaaaagcaggctGATAGGCTCGGACAAATAAC-3’
sax anti-sense	5'-ggggaccactttgtacaagaaagctgggtCTAAACGCAGACCTCGTCGAAG-3'
tkv sense	5'-ggggacaagtttgtacaaaaaagcaggctGTTGTTGTTGCTCTTGTTGTAG-3'
tkv anti-sense	5'-ggggaccactttgtacaagaaagctgggtTTAGACAATCTTAATGGGCAC-3'
sax ETD sense	5’-ggggacaagtttgtacaaaaaagcaggctGATAGGCTCGGACAAATAAC-3’
sax ETD anti-sense	5'-**cgtggctgcttgcgcagcttctcgcgtcgcttgtaggt**AATGAAGAAGATGGTCACGGC-3'
sax GSD sense	5'-CGTCGTAGCCATCGCAAGCGTC-3'
sax GSD anti-sense	5'-GACCTGTTTGGCTAGCGTACGC-3'
sax KD sense	5'-**gattaccattgctggtgcaaagaaccattgccaagcagatt**ACCCTGATCGAGTGCATTG-3'
sax KD anti-sense	5'-ggggaccactttgtacaagaaagctgggtCTAAACGCAGACCTCGTCGAAG-3'
tkv ETD sense	5’-ggggacaagtttgtacaaaaaagcaggctGTTGTTGTTGCTCTTGTTGTAG-3'
tkv ETD anti-sense	5'-**ggttcgcgaggcagccagacgcttgcgatggctacgacg**GAAACATAAGCTAGCCACG-3'
tkv GSD sense	5'-ACCTACAAGCGACGCGAGAAGC-3'
tkv GSD anti-sense	5'-AATCTGCTTGGCAATGGTTCTTTG-3'
tkv KD sense	5'-**gccgctgctagtgcagcgtacgctagccaaacaggtc**CAGATGGTGCGACTGGTGGGC-3'
tkv KD anti-sense	5'-ggggaccactttgtacaagaaagctgggtTTAGACAATCTTAATGGGCAC-3'
sax HA tag sense	5’-ggggacaagtttgtacaaaaaagcaggctGATAGGCTCGGACAAATAAC-3’
sax HA tag anti-sense	5'-ggggaccactttgtacaagaaagctgggtgAACGCAGACCTCGTCGAAGTCC-3'
tkv HA tag sense	5'-ggggacaagtttgtacaaaaaagcaggctGTTGTTGTTGCTCTTGTTGTAG-3'
tkv HA tag anti-sense	5'-ggggaccactttgtacaagaaagctgggtgGACAATCTTAATGGGCACATCGATTAG-3'

Quantitative *Drosophila* S2 cell based BMP signaling assay

Cell-based BMP signaling assays using a *lacZ* reporter were performed as previously described (Müller et al., 2003) with some modifications. To investigate the effects of receptors on the BMP signaling activity, we used 100 ng of receptor DNA constructs and 50 ng of *glass bottom boat (gbb)* encoding a BMP ligand. S2 cells were transfected at a density of 3.5 x 10^6 cells/mL with various combinations of DNA using Effectane kit (QIAGEN) and incubated at 25°C for 60 - 72 hours. After incubation, S2 cells (~ 3 x 10^6 cells) were lysed and β-galactosidase activities were measured by using the Dual-Light system (Applied Biosystems).

Western blot

Protein samples were prepared from S2 cells (~ 2 x 10^6 cells) used in signaling assays and were subjected to 10% SDS-PAGE. HA-tagged proteins were analyzed by western blot analysis. Antibodies used in this research were rat anti-HA (3F10) (1:1,000, Roche), mouse anti-Actin (clone C4) (1:10,000, Millipore), streptavidin-HRP (1:100,000, XX).

Biotinylation

S2 cells (~ 1 x 10^7 cells) were treated with ice-cold sulfo-NHS-Biotin (Pierce) at 4°C for 30 min. After biotinylation, cells were lysed and then HA-tagged proteins were recovered by immunoprecipitation (IP) using Dynabeads-Protein G (Invitrogen) conjugated ratanti-HA antibody (3F10). IP samples were analyzed by western blot analyses.

RESULTS

Sax antagonizes BMP signaling

A previous study revealed that Sax can both facilitate and antagonize BMP signaling *in vivo* (Bangi and Wharton, 2006b). In this study, we made use of a *Drosophila* S2 cell-based BMP signaling assay to investigate the differential behavior of Sax and Tkv in mediating signaling and to identify specific protein domains that may be responsible for their inherent differences. The BMP signaling assay exploits a BMP-sensitive *lacZ* reporter containing the *brinker* (*brk*) silencer element previously shown to mediate the transcriptional repression of *brk* in response to activation of BMP signaling (Müller et al., 2003). The reduction in *lacZ* expression mediated by the *brk* silencer element has been shown to occur in a quantitative manner and thus, the reduction in β-galactosidase activity serves as a quantitative measure of BMP signaling output (Müller et al., 2003; Pyrowolakis et al., 2004). For example, transfection of a *gbb* expression construct reduces β-galactosidase expression as indicated by the loss of β-Gal activity, referred to as "Gbb signaling" (**Fig. 3.2B**).

We first examined how Sax expression in this assay system influences Gbb signaling. When *gbb* and *sax* cDNAs were co-transfected into S2 cells, β-galactosidase expression was increased compared to cells transfected with *gbb* alone, indicating that Sax antagonizes Gbb signaling as previously report (**Fig. 3.2B**; (Bangi and Wharton, 2006b). Since Sax can both facilitate and inhibit the BMP signaling pathway, the effects of Sax and Sax^5 were compared. Sax^5 carries a dominant negative mutation (A289D) in the kinase domain and is expected to lack kinase function (Twombly et al., 2009). Therefore, Sax^5 should antagonize rather than transduce Gbb signaling. We found that the degree to which BMP signaling inhibited by Sax was comparable to that of Sax^5, suggesting that Sax predominantly exhibited antagonistic functions in this S2 cell system

(**Fig. 3.2B**). On the other hand, Tkv expression resulted in enhancement of Gbb signaling (**Fig. 3 .2B**). In summary, Sax and Tkv expression in our system showed precisely the opposite effects on BMP signaling: Sax antagonizes BMP signaling, while Tkv enhances it. This finding motivated us to identify critical protein regions of Sax for antagonistic behaviors.

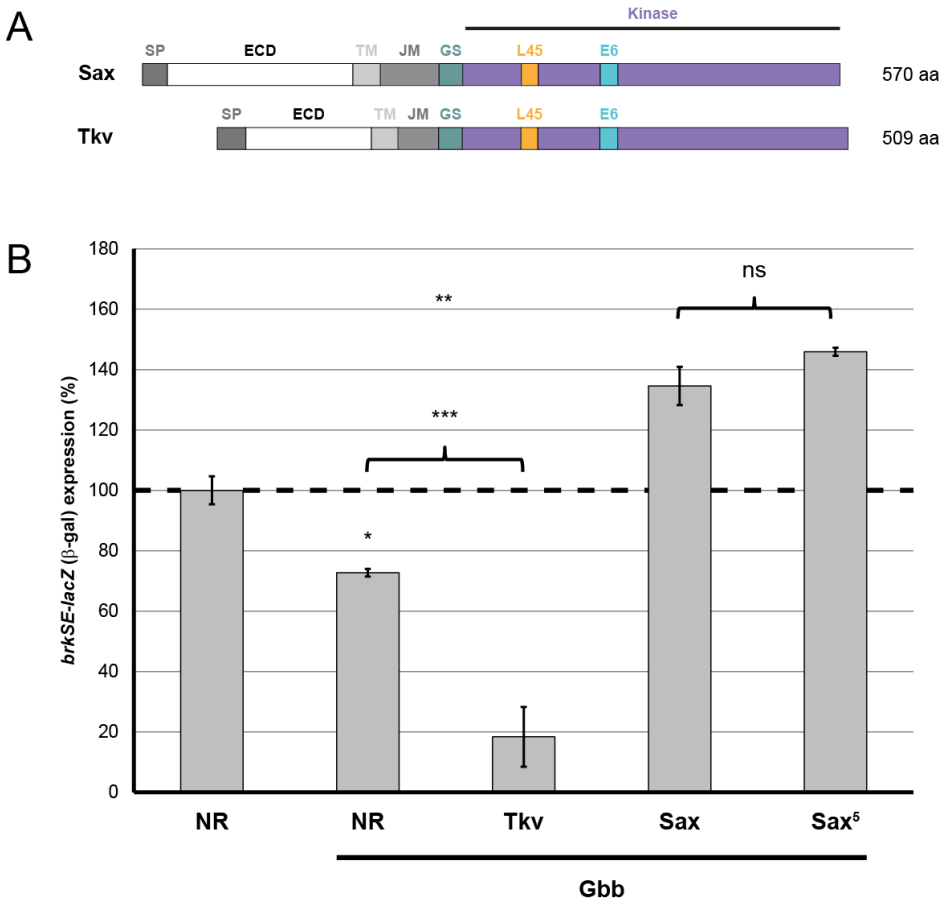


Figure 3.2. Sax inhibits BMP signaling in S2 cells. (**A**) A schematic illustration of BMP type I receptors Sax and Tkv. SP, signal peptides; TM, transmembrane; JM, juxtamembrane; GS, glycine-serine activation; L45, L45 loop; and E6, E6 loop. (**B**) Effect of Tkv, Sax, and Sax^{5} on BMP signaling. Overexpression of Sax repressed BMP signaling activity as does Sax^{5} protein, whereas Tkv over-expression enhanced it. BMP-mediated repression of the *brkSE-lacZ* reporter was measured from β-galactosidase activity. Background BMP signaling measured in S2 cells transfected with the *brkSE-lacZ* alone was set to 100% and is indicated by the dashed line. Data plotted are the mean (± SEM) of experiment performed in triplicate. Multiple pairwise comparisons were performed in R using the Tukey HSD test to determine statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

The J-GSD and kinase domain (KD) of Sax exhibit inhibitory effects

Sequence comparison between Sax and Tkv revealed considerable differences in the extracellular domain which contributes to interactions of BMP ligand, but few distinctions in the intracellular GS and kinase domains (**Figure 3.1A**). Based on sequence homology with other BMP type I receptors, Sax and Tkv proteins were divided into three domains: extracellular-transmembrane domain (ETD), juxtamembrane-GS domain (J-GSD), and kinase domain (KD). Based on these designations, we generated six Sax-Tkv chimeras in which different combinations of these domains were fused and investigated their effects on Gbb signaling (**Fig. 3 3B**).

As described above, Sax antagonized whereas Tkv mediated Gbb signaling (**Fig. 3.3B** and **Table 1**). Our analysis of the chimeric receptors in which only the ETD's were swapped reveals that STT retains Tkv's ability to mediate Gbb signaling and TSS retains Sax's ability to inhibit Gbb signaling. Interestingly, STT displayed stronger Gbb-induced signaling activity than Tkv which probably reflects Gbb's higher affinity for the ligand binding domain of Sax compared to that of Tkv (Bangi and Wharton, 2006b; Haerry et al., 1998; Haerry, 2010). These results indicate that exchanging the extracellular-transmembrane domains was not sufficient to alter the signaling behavior of the Sax or Tkv intracellular (J-GSD + KD) domains, and that the elements underlying the respective behaviors of Sax and Tkv must reside in their intracellular domains.

When either the J-GSD or KD of Sax was replaced with the corresponding Tkv domain, the resulting STS and SST chimeric receptors antagonized Gbb signaling less efficiently than the wild-type Sax receptor. Exchanging either the J-GSD or KD of Tkv for the corresponding domain in Sax resulted in chimeric receptors (TST and TTS) that were unable to facilitate Gbb signaling (**Fig. 3.3B** and **Table 3**). It should be noted that the TTS receptor, which shares the same intracellular domain as STS, inhibited Gbb signaling more efficiently despite the fact that STS should bind to Gbb with higher

affinity. It is possible that the TTS receptor is inhibiting background BMP signaling in addition to Gbb-signaling by binding endogenously expressed Dpp via its Tkv ETD.

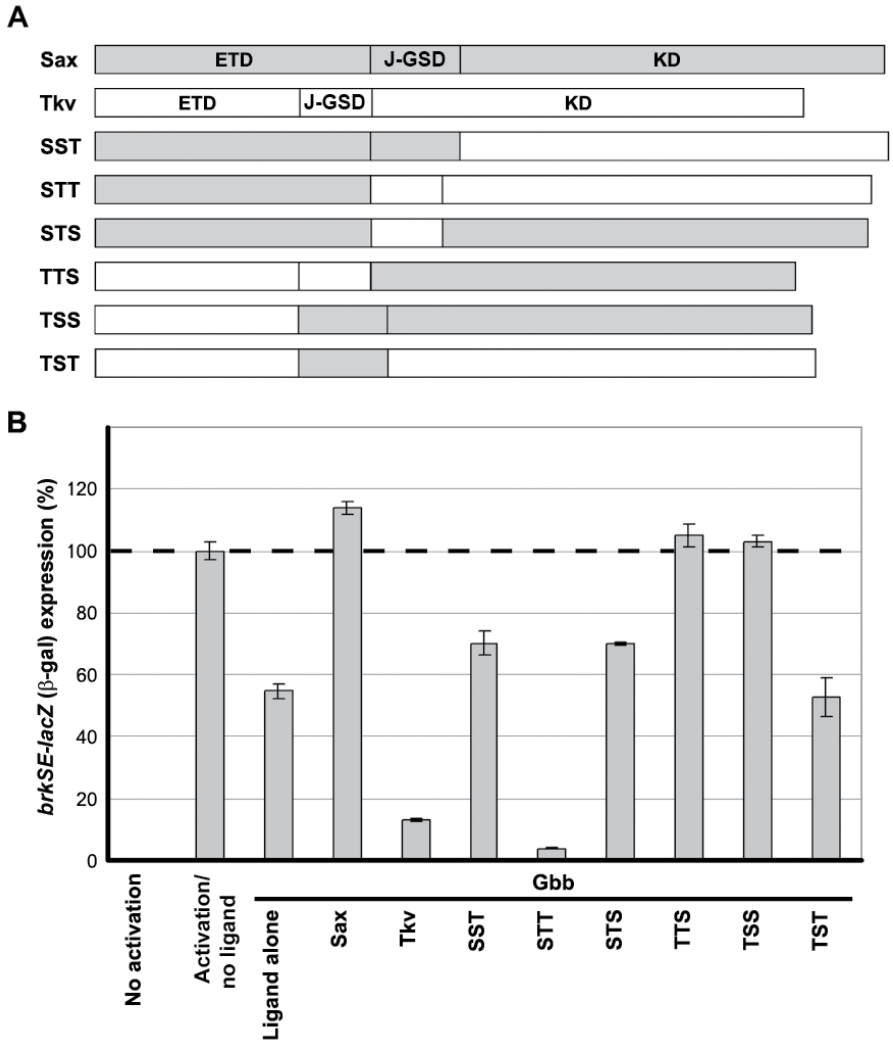


Figure 3.3. Sax J-GSD and kinase domain (KD) possess antagonistic functions. A. Diagrams of Sax, Tkv and Sax-Tkv chimera proteins. Both Sax and Tkv proteins were separated into three domains: ETD (residues 1-199 in Sax, residues 1-148 in Tkv), J-GSD (residues 200-264 in Sax, residues 149-200 in Tkv) and KD (residues 265-570 in Sax, residues 201-509 in Tkv). Gray and white indicate Sax and Tkv, respectively. **B.** Effects of Sax-Tkv chimera proteins on BMP signaling. 50 ng of *gbb* cDNA was co-transfected into *Drosophila* S2 cells with each chimeric construct. After incubation, β-galactosidase activities were measured. Data plotted are the mean (+/- SD) of experiments performed in triplicate.

The chimeric receptors behaved similarly in response to *dpp*, with greater inhibition observed in chimeric constructs with the extracellular domain of Tkv reflecting the high affinity with which Tkv and Dpp bind **(Fig. 6.9B).** Importantly, Sax also inhibited Dpp signaling, suggesting that Sax's inhibitory behavior is not limited to Gbb**.** However, we were unable to determine if Sax could inhibit Scw signaling since transfection of *scw* alone did not activate BMP signaling (**Fig. 6.9'**). This observation is consistent with the inability of purified Scw to induce phosphorylation of Mad in S2 cells (Shimmi et al., 2005b).

Altogether, our data suggest that the J-GSD and KD of Tkv must be paired together to achieve full "Tkv-like" signaling activity. When either the J-GSD or KD of Tkv is replaced with the corresponding Sax domain the signaling activity of the resulting chimeric receptor is negatively impacted. Therefore, in terms of signal transduction, the J-GSD and KD of Tkv are incompatible with the KD and J-GSD of Sax, respectively. These results suggest that the interaction of elements residing in the J-GSD and KD is critical for determining the signaling activity of a type I receptor.

Receptor	Effect on Gbb signaling ("ligand alone")	p-values (compared to "ligand alone")
Tkv	+	** (0.001)
STT	+ +	*** (0.001)
Sax	- -	** (0.002)
SST	-	* (0.04)
STS	-	** (0.004)
TTS	- -	*** (0.0002)
TSS	- -	** (0.002)
TST	**ø**	**ns** (0.7)

Table 3. Effects of Sax, Tkv and Sax-Tkv chimera receptors on Gbb signaling. Tkv and STT enhanced Gbb signaling ("+" strongly enhanced, "+ +" very strongly enhanced). Sax, SST, STS, TTS, and TSS repressed Gbb signaling ("-" mildly repressed, "- -" strongly repressed). TST had no effect on Gbb signaling ("**ø**" no effect). Statistical analyses compared to "ligand alone" were performed and p-values are indicated in parentheses (* p>0.05, ** p>0.01, *** p>0.001, **ns** p≥0.05).

Sax-Tkv domain swap chimeras localize on cell surface

Next, we generated HA-tagged versions of Sax, Tkv, and the chimeric receptors and measured their protein production to rule out the possibility that protein instability/degradation accounts for the observed differences in signaling activity. Overall, the respective antagonistic or promotive functions were conserved in the HA-tagged versions of the chimeras, albeit with subtle differences (**Fig. 3.4**). Protein lysates prepared from cells used in the signaling assays were examined by western blot analyses, which confirmed that all receptor constructs produced equivalent amounts of HA-tagged molecules (**Fig. 3.5A**).

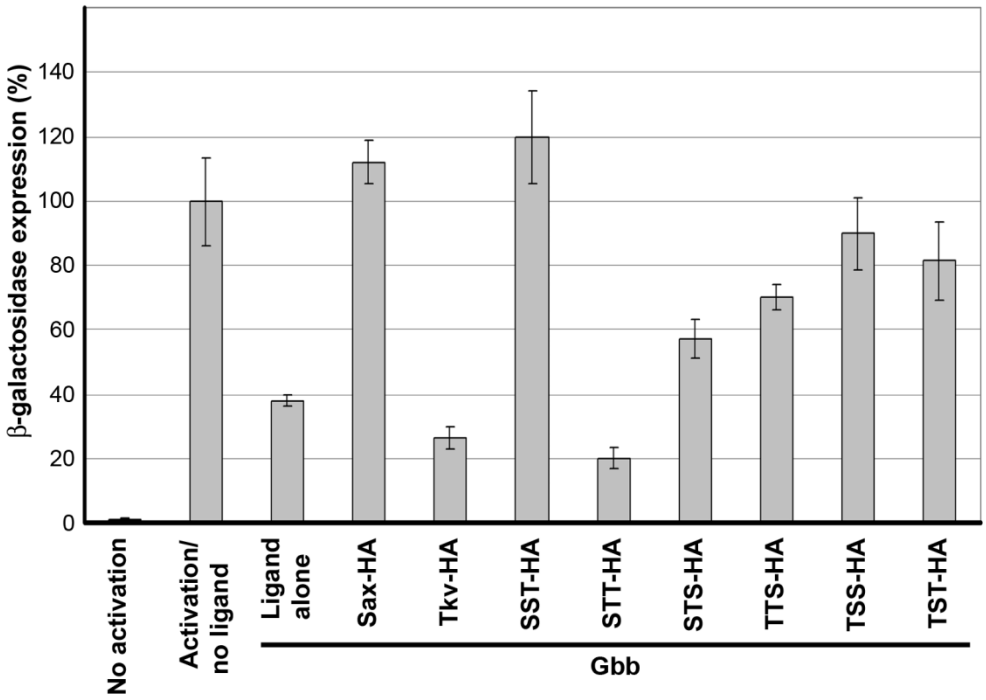


Figure 3.4. HA-tag does not disturb the functions of Sax, Tkv and Sax-Tkv chimeras. Abilities of HA-tagged Sax, Tkv, Sax-Tkv chimeras were assessed by S2 cell based BMP signaling assay with presence of 50 ng of *gbb*. Data plotted are the mean (+/- SD) of experiments performed in triplicate.

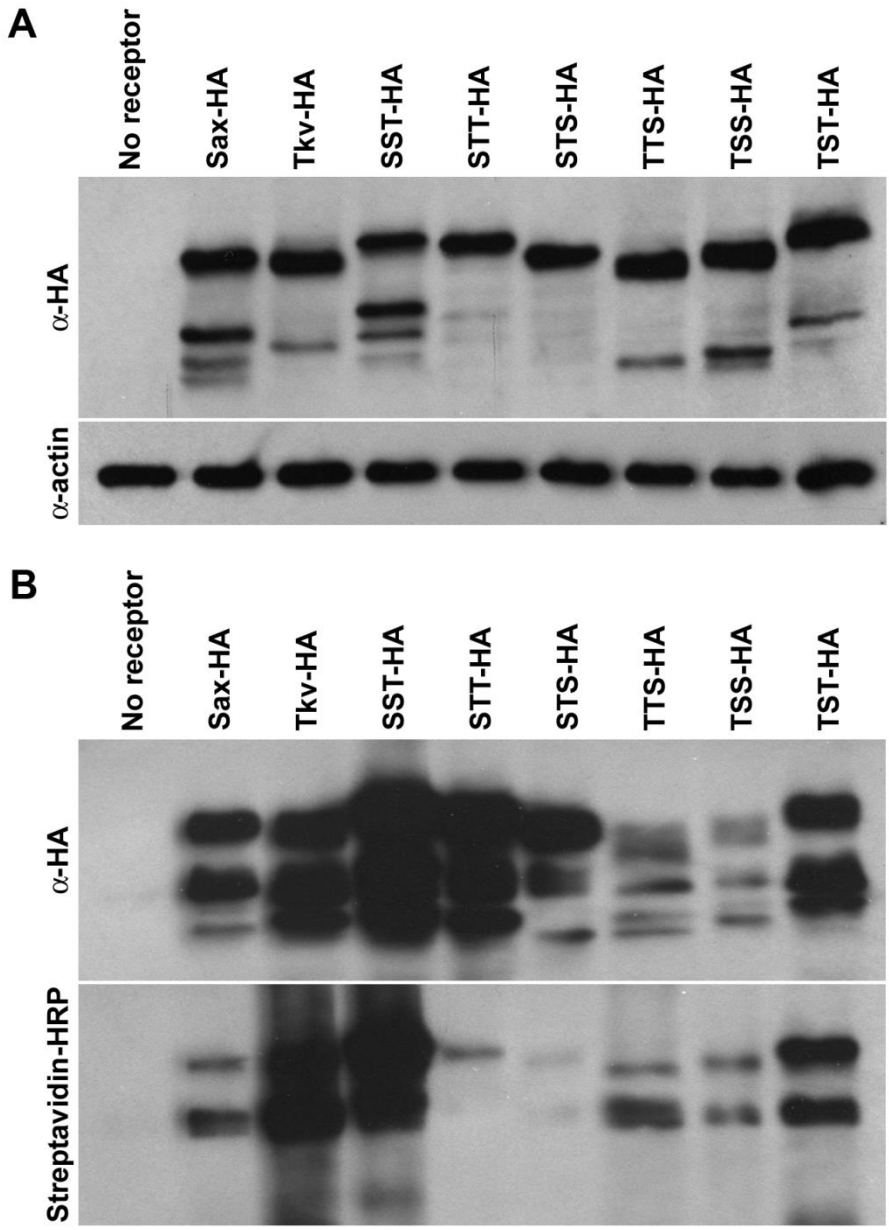


Figure 3.5. Protein characterization of Sax, Tkv and Sax-Tkv chimeras. A. Protein expression of Sax, Tkv and Sax-Tkv chimeras. Protein lysates prepared from signaling assays were subjected to SDS-PAGE. HA-tagged proteins were detected by using anti-HA antibody. Actin was used as an internal control. Expression levels of Sax, Tkv and chimera proteins were comparable. **B.** Biotinylation of cell surface proteins. DNA constructs encoding Sax, Tkv, and chimeras were transfected into S2 cells. After incubation, all proteins on cell surface were labeled by biotin. Then, HA-tagged proteins were recovered by IP using Dynabeads conjugated anti-HA antibody. IP samples were subjected to SDS-PAGE and were analyzed by western blot using anti-HA antibody and streptavidin-HRP.

We next investigated protein localization of wild-type and chimera receptors using a method that labels cell surface proteins with biotin. After biotin labeling, the receptors were immunopurified with HA antibodies and the amount of HA-tagged receptors recovered from the cell surface was visualized by HRP-conjugated streptavidin. We observed that the cell surface localization of Sax-HA, Tkv-HA, and the HA-tagged chimeric receptors varied greatly from one another (**Fig. 3.5B**), despite equivalent expression of the receptors (**Fig. 3.5A**). For instance, Tkv-HA and SST-HA were highly enriched at the cell membrane, whereas very little STT-HA or STS-HA localized to the cell surface. Since BMP receptors bind to ligands at the cell surface, the degree to which each receptor localizes to the cell membrane may in part determine that receptor's specific effect on BMP signaling. Therefore, the different signaling activities of each type I receptor that was tested may reflect differences in the cellular trafficking and localization of these receptors.

Localization of the HA-tagged receptors was also investigated using immunohistochemical approaches. Anti-HA antibody staining revealed that both wild-type and chimeric receptors localized near the cell membrane of S2 cells and were often found in puncta (**Fig. 3.6**). In contrast, the cytosolic protein FKBP12-HA was evenly distributed throughout the cytoplasm. However, without a cell membrane-specific marker, localization of the receptors to the cell surface could not be conclusively determined. Furthermore, the punctate HA staining pattern suggests that subpopulations of the type I receptors were contained in vesicles, but it remains unclear whether these vesicles were endocytic or exocytic.

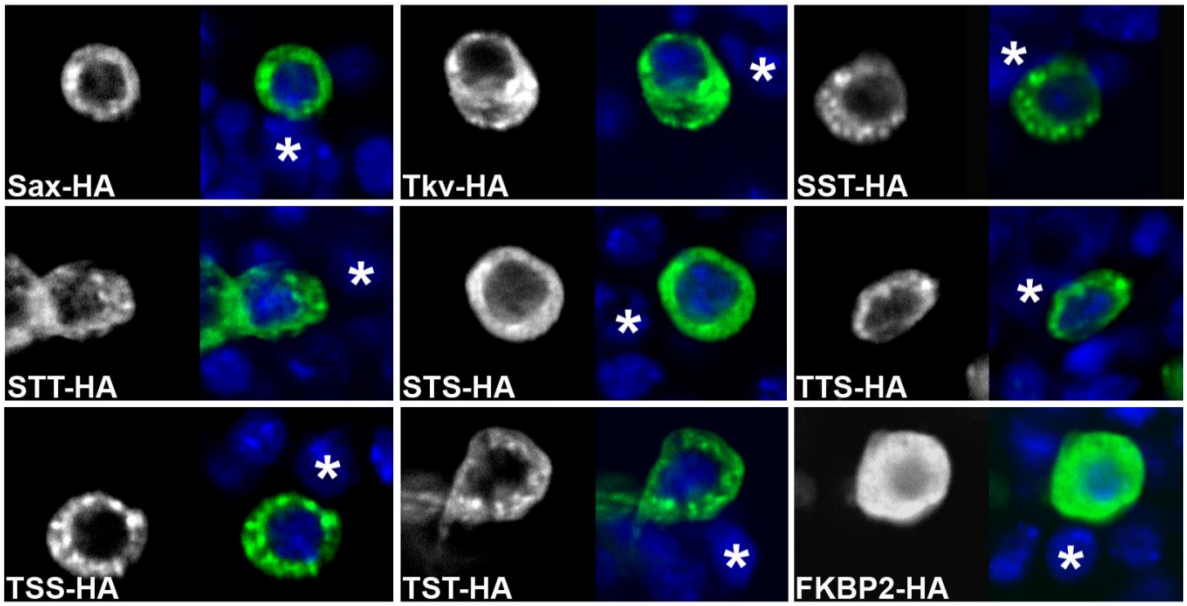


Figure 3.6. Cellular localization of HA-tagged Sax, Tkv and Sax-Tkv chimeras.
S2 cells were transfected with 300 ng of DNAs encoding HA-tagged Sax, Tkv and Sax-Tkv chimeras. After incubation, S2 cells were fixed and stained with anti-HA antibody to visualize localizations of HA-tagged molecules in S2 cells. Nuclei of S2 cells were stained with Hoechst shown by blue. HA signals of wild-type receptors and chimeric receptors were strongly detected in peripheral regions of S2 cells, while a cytosolic protein FKBP2 exhibited uniform signals.
* indicate S2 cells with no DNA transfection as negative controls.

Identifying the kinase domain determinant

Thus far our results indicate that STT can transduce Gbb signals as effectively as Tkv, whereas the ability of STS and SST to transduce Gbb signals was reduced compared to either STT or Tkv. These results suggest that neither the J-GSD nor the KD of Tkv is fully compatible with the Sax protein. Furthermore, full Tkv-like signaling ability must require the Tkv J-GSD and a secondary element found in the Tkv KD.

We identified the E6 loop of the kinase domain as a candidate secondary element because it has been implicated in both the activation of the type I receptor kinase and dimerization of type I receptors (Huse et al., 1999; Weis-Garcia and Massagué, 1996).

Furthermore, a second site mutation in the E6 loop was found to increase the ability of the "constitutively active" Sax^{QD} mutant receptor to phosphorylate Mad (reported as Sax^{A} in Haerry, 2010). This mutation (K382T) converts lysine 382 in Sax to a threonine, which is the conserved residue at this position in all other type I receptors (**Fig. 1.15**). It is therefore possible that diverging E6 loop sequences is in part responsible for Sax's dual/antagonistic behavior.

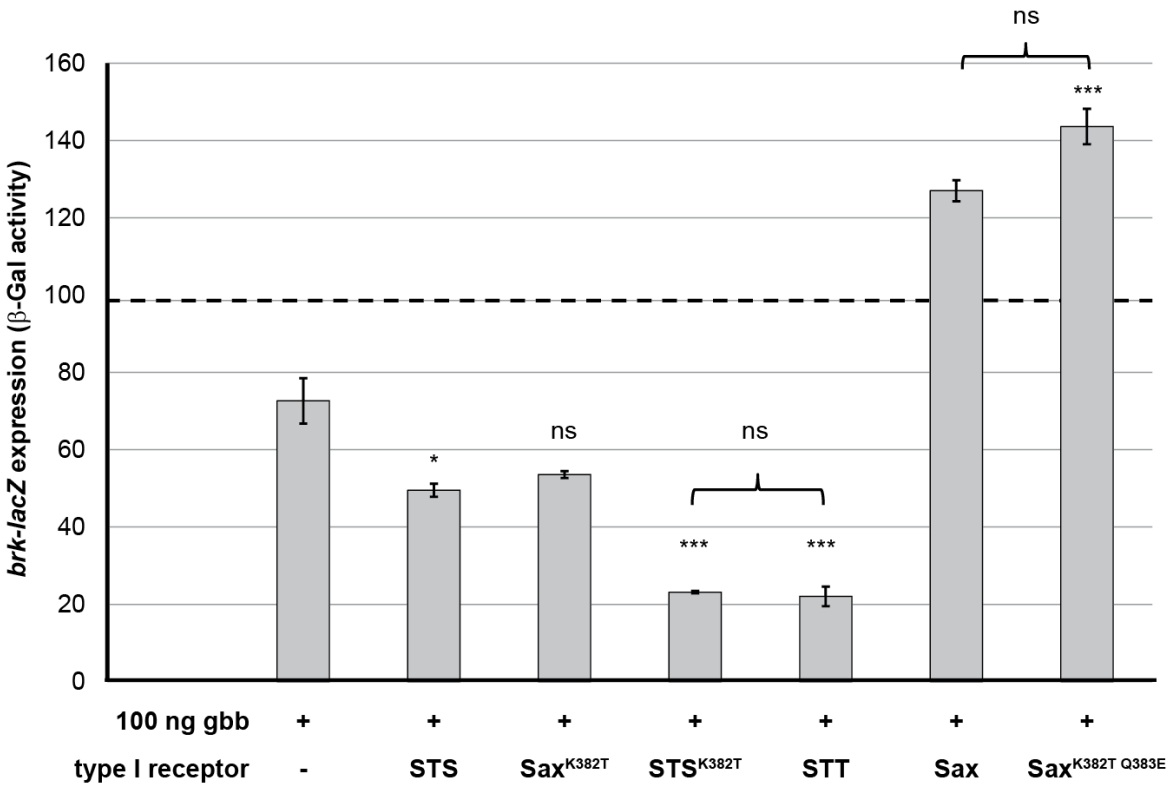
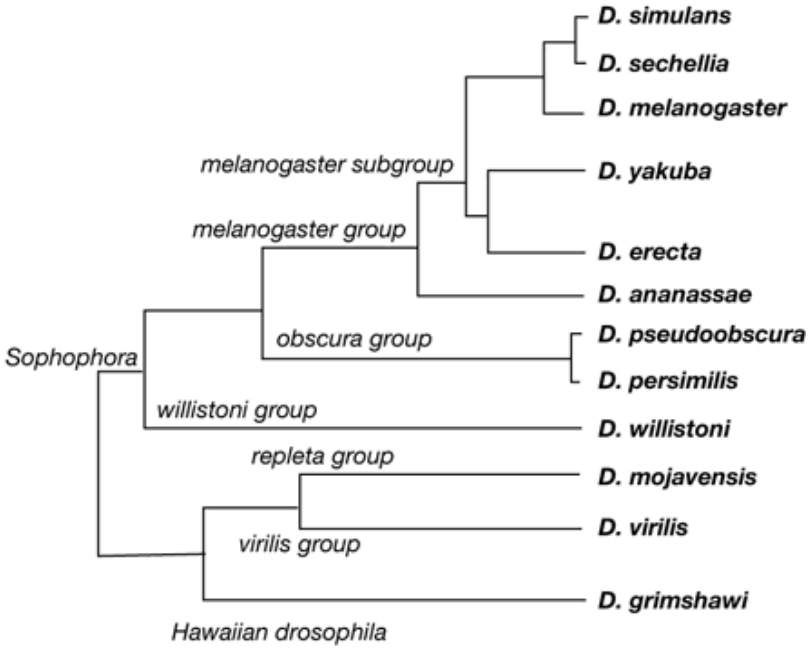


Figure 3.7. Effect of K382T mutation in the E6 loop of STS. The STS^{K382T} mutant and STT enhances *gbb*-induced signaling to the same extent. BMP-mediated repression of the *brkSE-lacZ* reporter was measured from β-galactosidase activity. Background BMP signaling measured in S2 cells transfected with the *brkSE-lacZ* alone was set to 100% and is indicated by the dashed line. Data plotted are the mean (± SEM) of experiment performed in triplicate. Multiple pairwise comparisons were performed in R using the Tukey HSD test to determine statistical significance (* p≤0.05, ** p≤0.01, *** p≤0.001).

We generated K382T mutations in the context of wild-type Sax, STT, and STS to test the E6 loop's impact on the ability of Sax to impact Gbb signaling in our *brk-lacZ* reporter assay (**Fig. 3.7**). Consistent with our previous results, cotransfection of *sax* with *gbb* inhibits *gbb-induced* signaling. STT enhanced *gbb*-induced signaling the strongest (~40% *brk-lacZ* expression), whereas sax^{K382T} and STS displayed moderate enhancement of *gbb*-induced signaling (~60% *brk-lacZ* expression). The STS^{K382T} mutant chimeric construct, however, enhanced *gbb*-induced signaling to the same extent that STT. This result indicates that the Tkv juxtamembrane/GS domain and the K382T mutation in the E6 loop are sufficient for conferring Tkv levels of signaling onto a chimeric type I receptor.

Interestingly, the *Drosophila melanogaster* E6 loop sequence (FG**KQ**GKP) is conserved among the six species of the *melanogaster* group, whereas the other six species (*obscura, willistoni, repleta, & virilis* groups) share a different conserved E6 loop sequence (FG**TE**GKP) (**Fig. 3.8**). In the *melanogaster* group, the E6 loop is characterized by a two amino acid KQ motif (**Fig. 3.8**, represented by *D. melanogaster*), which is replaced by a TE sequence in the other six *Drosophila* species (**Fig. 3.8**, represented by D. pseudoobscura). Given the importance of the E6 loop in type I receptor activation, this difference in E6 loop sequence raised the possibility that Sax activity in *D. melanogaster* might differ from its activity in the other *Drosophila* species. Furthermore, the observation that the Sax^{K382T} mutant, which has a hybrid E6 loop, no longer inhibited Gbb signaling (**Fig. 3.7**) suggests that the antagonistic behavior of Sax observed in *D. melanogaster* might not be shared in the non-*melanogaster* group. However, the activity of Sax from other *Drosophila* species has not yet been characterized. Therefore, it is not known whether Sax's ability to inhibit BMP signaling is conserved.



Type I	Species	E6 Loop
Sax	*D. melanogaster*	FGKQGKP 386
Sax	*D. pseudoobscura*	FGTEGKP 390
Sax	*A. gambiae*	FGTEGKP 307
Sax	*T. castaneum*	FGTEGKP 275
Sax	*A. mellifera*	VGTQGKP 133
ALK-1/2	*H. sapiens*	FGTQGKP 330
Tkv	*D. melanogaster*	FGTPGKP 322

Figure 3.8. Differences in the E6 loop sequence of Sax from different Sax species. (Top) Cladogram depicting the evolutionary relatedness of 12 *Drosophila* species. (Bottom) Sequence alignment of the E6 loop of type I receptors from different species. The E6 loop of the *melanogaster* group (represented by *D. melanogaster*) is characterized by a KQ motif. The E6 loop of species from outside of the *melanogaster* group is characterized by a TE motif. The consensus motif is TQ (represented by *A. mellifera* and ALK-1/2 from *H. sapiens*). Underlined G represents the invariant glycine that when mutated in TβRI negatively affects activation of the receptor (Weis-Garcia and Massagué, 1996). It has also been implicated in type I receptor dimerization (Huse et al., 1999).

To begin to address this issue, we generated a $Sax^{K382T\ Q383E}$ double mutant to reconstitute the non-*melanogaster* group E6 loop and tested its effect on *gbb*-induced signaling in the *brkSE-lacZ* assay. Since the Sax^{K382T} mutant had no positive or negative effect on Gbb signaling (**Fig. 3.7**), we hypothesized that the addition of the Q383E mutation would reconstitute a receptor that either facilitates or inhibits BMP signaling. Alternatively, the Q383E mutation may not have any effect at all. In our cell culture assay, however, the $Sax^{K382T\ Q383E}$ double mutant behaved much like wild-type Sax and inhibited *gbb* signaling (**Fig. 3.7**). This result indicates that swapping the KQ motif for the TE motif does not change Sax's antagonistic behavior to a facilitating one. Furthermore, this result suggests that the TE motif found in the non-*melanogaster* group might act as compensatory mutations to preserve Sax's ability to inhibit BMP signaling in these species.

The GS loop and αGS2 of Sax can abolish Tkv signaling activity

The J-GSD region designated in the chimeric constructs actually consists of 4 distinct elements: the juxtamembrane domain, αGS1 helix, GS loop, and the αGS2 helix. To further refine the J-GSD region and identify the minimal inhibitory elements of Sax we constructed a TTST chimeric protein in which only the GS loop-αGS2 helix sequence of Tkv was swapped for this region in Sax (**Fig. 3.9A**). We targeted this region because it is important in regulating type I receptor kinase activity as evidenced by the fact that mutations that confer constitutive activity have been mapped to these sequences.

As expected, Sax inhibited Gbb signaling, whereas Tkv enhanced Gbb signaling in our *brk-lacZ* assay. However, the ability of TTST to enhance Gbb signaling was greatly reduced compared to Tkv; in fact, TTST neither enhanced nor inhibited Gbb signaling. This result suggests that Sax's GS loop and αGS2 helix is sufficient to abolish Tkv's ability to enhance Gbb signaling.

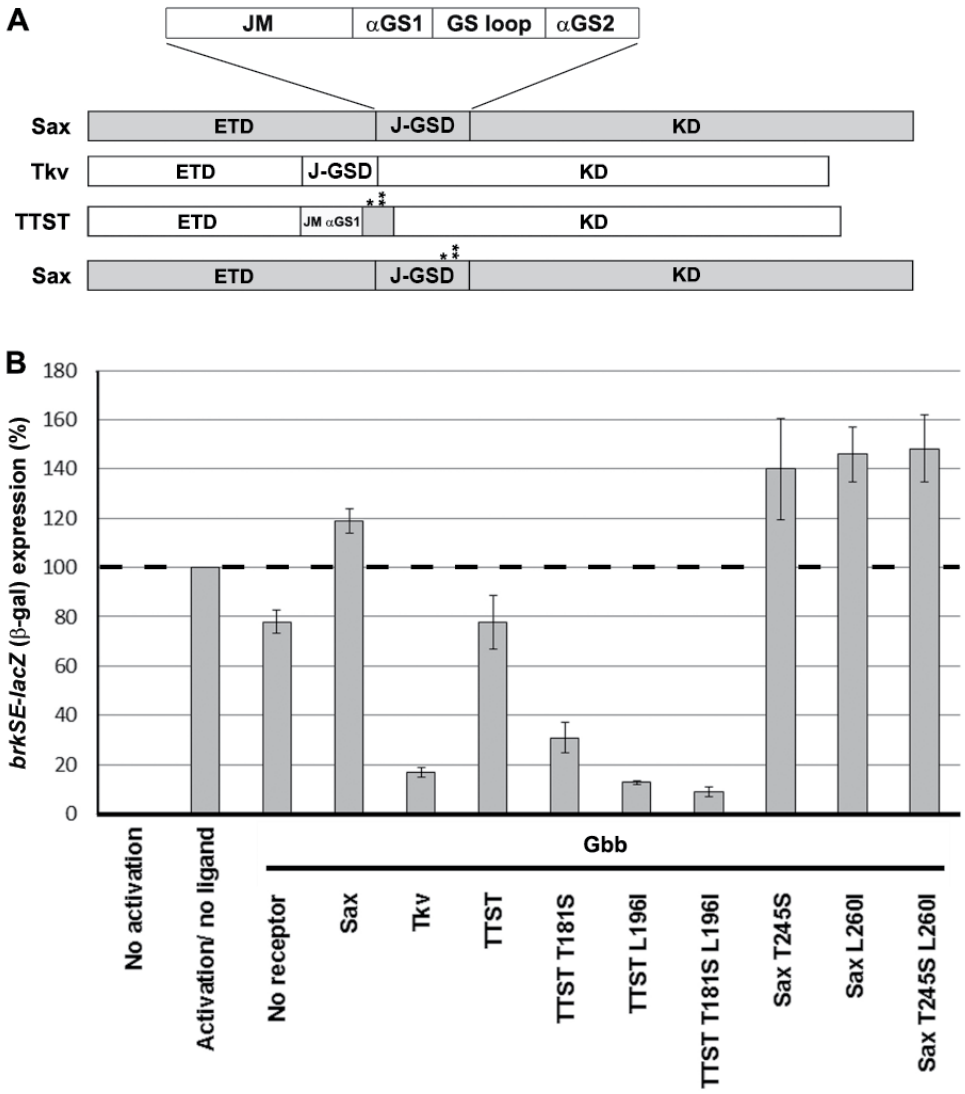


Figure 3.9. Effect of αGS2 domain mutations on the activity of Tkv. A. Diagram of Sax, Tkv, and TTST chimeric receptor. ETD, Extracellular-Transmembrane domain; J-GSD, juxtamembrane-GS domain; KD, kinase domain. The J-GSD is composed of 4 separate subdomains: JM, juxtamembrane, αGS1 helix, GS loop, and αGS2 helix. * location of residue swap in the GS loop. **, location of residue swap in αGS2. TTST is chimeric receptor in which the GS loop and the αGS2 helix of Tkv has been replaced with those of Sax . **B.** The effects of GS loop and αGS2 mutations on TTST and Sax. Single and double TTST mutants enhanced Gbb signaling, whereas single and double Sax mutants retained inhibitory behavior. Three independent experiments were performed. "No activation" and "Activation/no receptor" were used as negative and positive controls, respectively. Expression levels of β-galactosidase from "Activation / no receptor" were set as 100%. Error bars represent SD.

Interestingly, the GS loop and αGS2 helix of Tkv and Sax differ by only two amino acids. Serine 181 in the GS loop of Tkv corresponds to threonine 245 in Sax, and isoleucine 196 in the αGS2 helix of Tkv corresponds to leucine 260 in Sax. (Therefore TTST is essentially a Tkv^{S181T}). To determine which residue is responsible for Tkv's signaling activity we generated single and double mutant combinations of T181S and L196I found in the "S" region of the TTST chimeric receptor. We found that both $TTST^{T181S}$ and $TTST^{L196I}$ single mutant constructs were sufficient to enhance Gbb signaling. As expected, the $TTST^{T181S\ L196I}$ double mutant, which is the chimeric receptor reverted back to Tkv, also enhanced Gbb signaling. (**Fig. 3.9B**)

Next, we investigated the effect of swapping the corresponding residues in Sax given that mutating either one of these residues was sufficient to convert TTST behavior. We observed that both the Sax^{T245S} and Sax^{L260I} single mutants, as well as the Sax^{T245S} L260I (TSLI) double mutant inhibited Gbb signaling (**Fig. 3.9B**). These results indicate that neither of these two residues in Tkv, alone or in combination is sufficient to confer signaling activity onto Sax. These results are consistent with the proposal that an "inhibitory" element exists in the Sax KD in addition to the one in the Sax J-GSD. Therefore, to fully convert Sax into a type I receptor capable of enhancing Gbb signals we propose that both "inhibitory" elements must be exchanged for their signaling counterparts found in Tkv.

Since neither single nor double combinations of the T245S and L260I mutations could confer Tkv-like signaling activity onto Sax we turned our attention back to the E6 loop. We decided to test the effect of the K382T mutation on Sax $^{T245S\ L260I}$ double mutant signaling activity, since the K382T mutation was sufficient to convert STS into a Tkv-like receptor (**Fig. 3.7**). In contrast to STS^{K382T}, which enhanced Gbb signaling, the $Sax^{T245S\ L260I\ K382T}$ (TSLI K382T) triple mutant left Gbb signaling unaffected when compared to cells transfected with *gbb* alone (**Fig. 3.10**). Although $Sax^{T245S\ L260I\ K382T}$ did not exhibit

Tkv-like signaling, the addition of the K328T mutation abolished the ability of $Sax^{T245S\ L260I}$ to inhibit Gbb-signaling. These results indicate that the T245S L260I K382T triple mutant combination is not sufficient to confer Tkv-like signaling activity onto Sax. Although these results do not rule out the requirement of T245S (GS loop) or L260I (αGS2 helix) in the signaling activity of STS^{K382T}, it does indicate that other residues in the juxtamembrane domain or αGS1 helix are required.

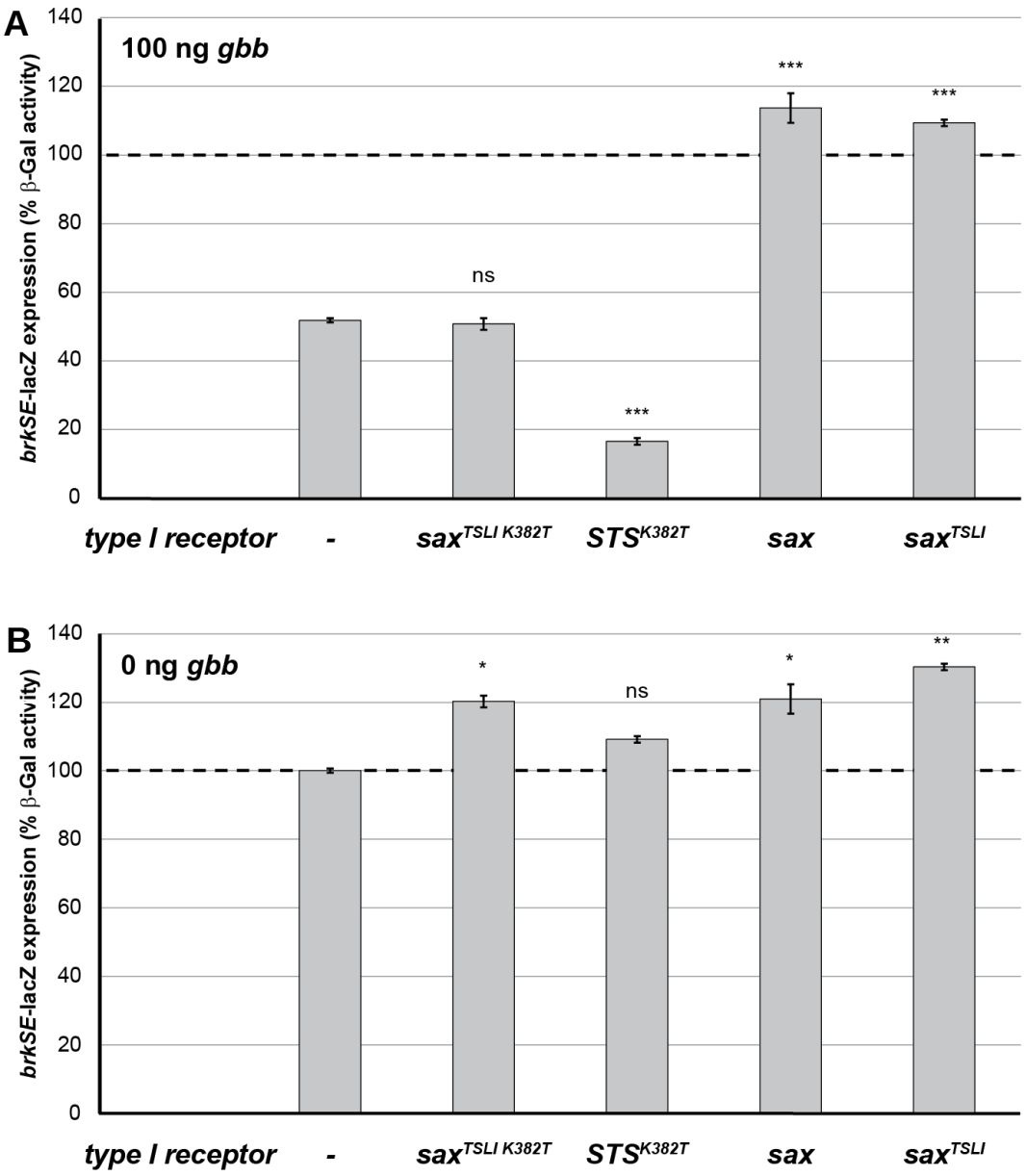


Figure 3.10. **Effect of the K382T mutation on signaling ability of Sax^{TSLI}. A.** Sax$^{TSLI\ K328T}$ triple mutant does not enhance Gbb signaling in S2 cells transfected with 100 ng *gbb*. **B.** Sax^{TSLIK382T}, Sax, Sax^{TSLI} inhibit background BMP signaling in the absence of transfected *gbb*. STS^{K382T} does not affect background BMP signaling. β-galactosidase activity represents *brkSE-lacZ* reporter expression. Background BMP signaling measured in S2 cells transfected with only the *brkSE-lacZ* reporter was set to 100% and is indicated by the dashed line. Data plotted are the mean (± SEM) of experiment performed in triplicate. Multiple pairwise comparisons were performed in R using the Tukey HSD test to determine statistical significance (* p≤0.05, ** p≤0.01, *** p≤0.001). TSLI = T245S L260I double mutation.

DISCUSSION

During wing development Sax exhibits an ability to both facilitate and antagonize BMP signaling(Bangi and Wharton, 2006b). Sax's inhibitory behavior appears to be a function of its inability to phosphorylate Mad within the context of a Sax:Sax receptor complex (Chapter 2). This dual behavior has yet to be observed in any of other BMP type I receptors. In this study, we reasoned that the elements that determine Sax's behavior must reside within differences between Sax and Tkv, the other *Drosophila* type I receptor that only promotes BMP signaling. To test this we generated chimeric receptors by swapping different domains of Tkv and Sax to identify regions of either protein that were important for signaling or inhibiting activity. We exploited a BMP-responsive *lacZ* reporter assay in S2 cells, to investigate the effect of each chimeric receptor on BMP signaling. Only the antagonistic behavior, and not the facilitating behavior, of wild-type Sax has been observed using this particular assay in S2 cells.

Two determinants dictate type I receptor behavior

Our results indicate that the inhibitory behavior of Sax and the signaling activity of Tkv reside in their respective intracellular domains. Furthermore, inputs from both the J-GSD and KD impact the behavior of the type I receptor. For full inhibitory or facilitating function, each receptor's KD must be paired with its respective J-GSD (juxtamembrane and GS domains). However, the TTS chimeric receptor appears to be the exception, since the pairing of the Tkv J-GSD with Sax KD in this context inhibited Gbb-signaling to the same extent that wild-type Sax did. This may reflect the ability of the TTS receptor to inhibit endogenous Dpp-signaling in S2 cells by virtue of Tkv-Dpp high affinity interactions. These observations suggest that the J-GSD and KDs of Tkv and

Sax are incompatible with each other in terms of each receptor's specific promotive or antagonistic function.

Further refinement of the KD suggested that the E6 loop impacts the nature of type I receptor activity. Substituting lysine 382 from the Sax E6 loop for the corresponding threonine in Tkv (K382T) in the STS backbone yielded a chimeric receptor that exhibited Tkv-like signaling (Fig. 3.6, compare STS^{K328T} to STT). This result suggests that the single-site K328T mutation renders the Sax KD compatible with the Tkv J-GSD to constitute chimeric receptor that can signal. Furthermore, the inability of the K382T mutation, by itself, to confer Tkv levels of signaling onto Sax (Fig. 3.6, compare $Sax^{K382T\ to}$ STT) is consistent with the notion that receptor behavior is impacted by two inputs. However, the K382T mutation did partially abrogate Sax's ability to inhibit Gbb signaling. Whether the converse mutation (T318K) in the Tkv KD would confer full inhibitory activity to the TST chimera remains to be addressed (Fig. 3.3B). However, experiments performed in S2 cells indicate that a Tkv $^{Q199D\ T318K}$ compound mutant receptor (Q199D confers constitutive activity) is still constitutively active, suggesting that The T318 residue may not play a role in Tkv signaling activity (Haerry, 2010).

Our attempts to identify sequences in the J-GSD yielded less conclusive results. The TTST chimera, which contains the GS loop and αGS2 helix of Sax, can be converted into a receptor capable of signaling like Tkv by mutations in either the GS loop (T181S) or the αGS2 helix (I196L) (**Fig. 3.9**). Both of these mutations convert the amino acids found in Sax to the corresponding residues in Tkv. Thus, either of these Sax-to-Tkv reverting mutations was sufficient to restore signaling "compatibility"between the J-GSD and KD in TTST. It should be noted that these two residues are the only differences between the TTST and Tkv amino acid sequences.

However, the same double amino acid substitutions (T245S L260I) in Sax were not sufficient to alter the inhibitory behavior of Sax (**Fig. 3.9**). Moreover, the triple mutant combination T245S L260I K382T in Sax did not confer Tkv-like activity, indicating that these residues are not the determinants in the J-GSD that cooperate with K382T to determine a facilitating function for type I receptors. These results suggest that other sequences residing in either the juxtamembrane domain or the αGS1 helix need to be altered to complement the K382T mutation in Sax to convert it from an inhibitor to facilitator. Although, structural studies implicate the αGS1 helix in hydrophobic interactions that stabilize the autoinhibited conformation of type I receptors (Chaikuad et al., 2012; Huse et al., 1999; Huse et al., 2001), the juxtamembrane domain of type I receptors is largely uncharacterized. Thus, chimeras in which the JM and αGS1 are swapped between Sax and Tkv will need to be generated to determine how these subdomains affect type I receptor behavior.

Given that the K382T mutation in concert with the Tkv J-GSD dictates a promotive function for type I receptors we considered how these results impact our current model for type I receptor behavior. As previously mentioned, the E6 loop has been implicated in type I dimerization and transphosphorylation of the GS domain by the type II receptor. We propose that the E6 loop and element(s) in the J-GSD of Sax forces Sax:Sax complexes into a non-productive configuration that may interfere with phosphorylation of the GS domain. The GS domain contains multiple serine/threonines that are the targets of the type II receptor kinase and there are data that suggest multiple phosphorylations are required for full activation of type I receptor kinase activity (Huse et al., 2001). It is possible that underphosphorylation of the Sax GS domain in Sax:Sax complexes accounts for its signaling incompetence. Preliminary phospho mass spec trials suggests that the GS domain of Sax can be phosphorylated **(Table 6)**, however

quantitative methods such as SILAC will need to be employed in order to compare differences in the level of phosphorylation between Sax and Tkv.

In contrast to Sax, the E6 loop and J-GSD of Tkv would permit homodimerization into productive configurations that can be phosphorylated by the type II receptor. Two scenarios are possible with regard to Sax:Tkv complexes. One possibility is that heteromeric interactions of the E6 loop and J-GSD permits a configuration in which both receptors can be activated. The other possibility is that only one of the receptors, most likely Tkv, is activated and is responsible for signaling in the heteromeric complex.

Molecular models for how the Sax E6 loop affects signaling activity

The E6 loop is highly conserved region that has been implicated in both the activation of the type I receptor kinase and dimerization of type I receptors. For instance, a G322D mutation in the E6 loop of the TGF-β type I receptor (TβRI) prevents the type II receptor (TβRII) from transphosphorylating the TβRI^{G322D} GS domain (G 322D corresponds to the glycine residue boxed in **Fig. 1.15**). Thus, the G322D mutation renders the TβRI kinase inactivatable. Importantly, this mutation does not prevent the TβRI^{G322D} from interacting with TβRII since the receptors can still be copurified (Weis-Garcia and Massagué, 1996). This result indicates that the loss of transphosphorylation may be a result of a non-productive receptor configuration rather than the inability of TβRI^{G322D} to interact with TβRII. Structural studies of TβRI also suggest the G322 residue in the E6 loop participates in intermolecular hydrogen bonds between type I:type I receptor dimers (Huse et al., 1999). The authors of this study suggest that replacing G322 with an acidic residue like aspartic acid could disrupt these hydrogen bonds and change the conformation of the type I: type I dimer in such a way that the GS domain transphosphorylation is negatively impacted.

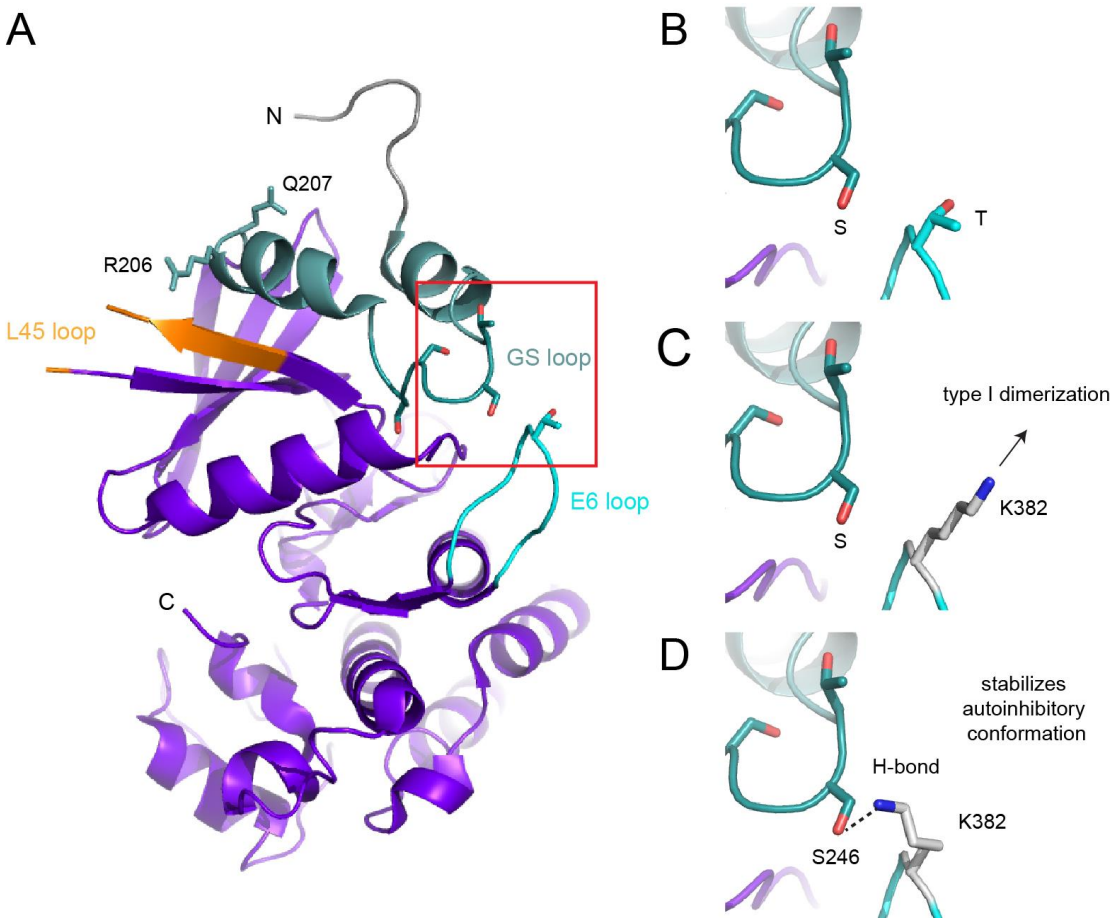


Figure 3.11. Structural models reveal how the Sax E6 loop may affect signaling activity. A. Crystal structure of the ALK2 intracellular domain with type I receptor subdomains labeled. Serine/threonine side chains of the GS domain and the conserved threonine in the E6 loop are shown with their hydroxyl groups in red. The area within the red box corresponds to close-up views in B-D. **B.** The second serine (S) residue in the GS loop is in close proximity to the conserved threonine (T) in the E6 loop. **C & D.** In Sax, the conserved threonine residue in the E6 loop is replaced by a lysine (K382) whose amine group is shown in blue. **C.** The long, polar side chain of K382 may affect Sax:Sax dimerization such that the GS domain is not phosphorylated. **D.** Alternatively, the side chain amine group of K382 may form a hydrogen bond with a GS domain serine (S246). This interaction may further stabilize the autoinhibitory conformation of Sax and prevent activation of its kinase.

Although the E6 loop is a highly conserved subdomain, lysine 382 in Sax represents a highly divergent amino acid change from the conserved threonine residue found in all of the other type I receptors (**Fig. 1.15)**. Given that the lysine 382 is situated one position downstream of the conserved glycine mentioned above, it is possible that the effects of lysine 382 on Sax signaling activity mimics the G322D mutation in TβRI. Interestingly, modeling the Sax K382 residue onto the ALK2 crystal structure provides two potential molecular mechanisms for how the E6 loop of Sax affects its signaling activity (**Fig. 3.11**). First, the long, polar side chain of lysine 382 could force Sax:Sax dimers into a configuration that prevents GS domain phosphorylation by disrupting hydrogen bonds between the Sax monomers, much like what has been proposed for the G322D mutation in TβRI (**Fig. 3.11C**). Alternatively, lysine 382 may form a hydrogen bond with a serine residue located in the nearby GS domain and thereby stabilize the autoinhibitory conformation of Sax. (**Fig. 3.11D**)

STT chimera can be used to probe how Tkv ICD influences Sax activity

Whether Sax in Sax:Tkv complexes can "signal" remains unresolved. One of the obstacles impeding our ability to address this question is how to faithfully and reliable induce formation of the Sax:Tkv complexes. Sax and Tkv have different affinities for the *Drosophila* BMPs and several studies suggest that Sax:Tkv complexes bind Gbb:Dpp heterodimers. One way to clear this hurdle is to purify Gbb:Dpp heterodimers. Another way is to exploit the STT chimeras generated in this study. With only the Sax extracellular domain to consider we can simplify our experimental design and use only Gbb, which should induce the formation of Sax:Sax, STT:STT, and Sax:STT complexes in equal ratios. We can then determine if the signaling activity of Sax can be activated by the presence of the Tkv-kinase domain by coexpressing Sax with a "kinase-dead" form of

STT. Using a "kinase-dead" STT would remove any signaling contribution of STT so that any signaling activity must originate from the Sax molecule.

Assuming that the presence of Tkv permits Sax to be activated, then the level of Gbb signaling in cells cotransfected with Sax and STT^{KD} should be intermediate to signaling levels observed in cells expressing only Sax (we expect Sax:Sax does not signal) and cells coexpressing Sax and wild-type STT (we expect Sax:STT does signal). Therefore, we predict that the relative activity levels would be: Sax:Sax < Sax:STTKD < Sax:STT. Thus far, we have induced in STT two different mutations that have been reported to inactivate the kinase activity of TβRI. However, neither mutation appears to fully knock out STT kinase activity **(Appendix 6.10)**. We are currently investigating other potential kinase-inactivating mutations.

Is the inhibitory behavior of Sax conserved?

The BMP signaling pathway is highly conserved from *C. elegans* to humans. In fact, many of the regulatory mechanisms and components that modulate BMP signaling that were initially identified in simple model organisms have also been found in vertebrates. In this study, we investigated the antagonistic behavior of Sax with an eye toward understanding the function of its vertebrate orthologs ALK1/2. Mutations in both ALK1/2 that dysregulate BMP signaling are associated with a number of human diseases, and determining if the inhibitory function of Sax is conserved in its orthologs could have profound impacts in our understanding of these diseases. For example, fibrodysplasia ossificans progressiva is a disease characterized by heterotopic ossification of muscle and soft tissue and is caused by gain-of-function mutations in ALK2. However, these gain-of-function mutations may also represent loss-of-function in terms of inhibitory activity. Therefore, effective therapeutic strategies may have to compensate for both to restore proper BMP signaling.

Although evidence for inhibitory functions of ALK1/2 have been observed in TGF-β superfamily signaling, it is not clear that inhibition results from an inability to phosphorylate R-Smads, as has been proposed for Sax. For instance, ectopic expression of ALK2 inhibited BMP6-induced signaling in COS cells (van Dinther et al., 2010), but no further characterization has been performed nor has this behavior been observed *in vivo*. ALK2 has also been proposed to inhibit Activin signaling by binding and preventing ligand from interacting with its type II receptor, thereby interfering with the formation of an activin signaling complex (Renlund et al., 2007). ALK1 can inhibit TβRI (ALK5)-mediated TGF-β signaling; however inhibition is a result of ALK1 mediated-signaling through Smad1/5.

In the course of my own studies, I have observed ALK2 facilitate BMP-7 signals but not Gbb, Dpp, or BMP4 **(Figure 4.5 C, D, & E)**. These results indicate that when provided the proper ligand to bind, ALK2 can activate BMP signaling. Morever, these results suggest that ALK2 may have ligand-specific dual behavior. A similar analysis of ALK1 remains to be performed.

The results from this study are consistent with a conserved antagonistic behavior of Sax within the *Drosophila* genus (**Fig. 3.7 & 3.8**). A cladogram constructed using the Sax sequence from all 12 *Drosophila* species largely agrees with the aforementioned phylogenetic tree, except for the branch points for *D. yakuba* and *D. willistoni*, and the placement of *D. ananassae* with the *obscura* group (**Fig. 3.12** compared to **Fig. 3.8**).

Sequence alignments revealed that the TE motif in the E6 loop of Sax is conserved between the six non-*melanogaster* species (Diptera), *Anopheles gambiae* (Diptera), and *Tribolium castaneum* (Coleoptera). The conservation of this motif combined with the cladogram suggests that the TE motif represents the ancestral Sax E6 loop sequence present in the last common ancestor between *Tribolium* and Diptera (**Fig. 3.12**). In contrast, the E6 loop of Sax in *Apis mellifera* (Hymenoptera) is characterized

by a TQ motif that is common to the type I receptors of vertebrates (**Fig. 3.12**). Based on the conservation of the TE motif it is possible that the inhibitory behavior of Sax is a feature that appeared only in Dipterans and Coleopterans. Moreover, the KQ motif found in the *melanogaster* group may represent a more recent evolutionary development that occurred after the split between the *melanogaster* and *obscura* groups and may be compensatory mutations to preserve the antagonistic behavior of Sax in the *melanogaster* group. Further investigation to identify inhibitory elements in (*D. melanogaster)* Sax's J-GSD coupled with sequence conservation analysis will help shed light on whether Sax's inhibitory behavior is conserved in all *Drosophila* species.

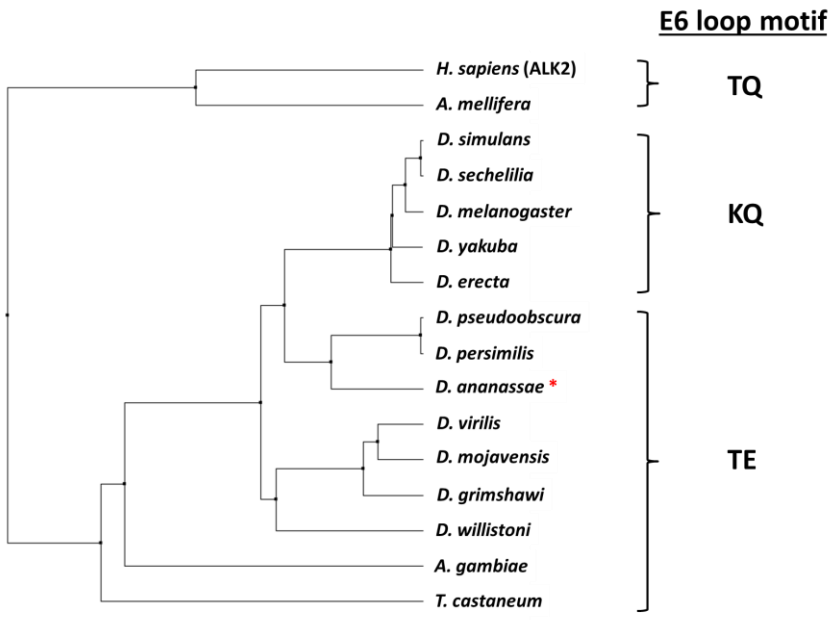


Figure 3.12. A phylogenetic tree of Sax/ALK2. Phylogenetic tree was constructed using Sax amino acid sequences from 12 *Drosophila* species, *A. mellifera*, *A. gambiae*, and *T. castaneum*, as well as ALK2 (*H. sapiens*). The different Sax proteins and ALK2 cluster by their E6 loop motif, with the exception of *D. ananassae* Sax *(*red *) which has a *melanogaster* E6 loop motif, but overall greater sequence similarity with *D. pseudoobscura* and *D. persmilis*.

Chapter 4

Hyperactive BMP signaling induced by ALK2^{R206H} requires type II receptor function in a *Drosophila* model for classic Fibrodysplasia Ossificans Progressiva

I performed all of the experiments presented in this chapter.

ABSTRACT

Fibrodysplasia Ossificans Progressiva (FOP) is an autosomal dominant disorder characterized by the episodic deposition of heterotopic bone in place of soft connective tissue. All mutations associated with FOP map to the BMP type I receptor, ALK2, with the vast majority of patients possessing a single amino acid change, ALK2^{R206H}. The mechanism(s) regulating the expressivity of hyperactive signaling associated with the ALK2^{R206H} receptor during the life of the patient is not well understood.

In the *Drosophila* system, the human ALK2^{R206H} receptor also induces hyperactive signaling. As observed in vertebrates, elevated signaling associated with ALK2^{R206H} in *Drosophila* is ligand-independent. We found that a key determinant for mutant ALK2^{R206H} receptor signaling is the presence of a type II receptor. Our results also demonstrate that wild-type ALK2 can antagonize, as well as promote, BMP signaling--a dual behavior previously exhibited by its *Drosophila* ortholog, Saxophone (Sax).

Due to the heterozygosity of ALK2^{R206H} in FOP, this dual behavior of ALK2 is of particular interest, since the interplay between the two disparate behaviors of wild-type ALK2 receptors could be shifted by the presence of hyperactive ALK2^{R206H} receptors. Our studies provide a compelling example for *Drosophila* as a model organism to study the molecular underpinnings of a complex human syndrome such as FOP.

INTRODUCTION

Fibrodysplasia Ossificans Progressiva (FOP) is a rare genetic disorder marked by the episodic deposition of heterotopic bone in place of muscle and connective tissues throughout the life of a patient. All individuals with FOP have been found to carry a point mutation in one copy of the gene encoding the bone morphogenetic protein (BMP) type I receptor, *ALK2/ACVR1* (Shore et al., 2006; Kaplan et al., 2009). FOP-associated mutations in ALK2/ACVR1 appear to produce hyperactive receptors, resulting in inappropriate BMP signaling (Billings et al., 2008; Fukuda et al., 2009; Kaplan et al., 2009; van Dinther et al., 2010).

Transforming Growth Factor- β (TGF-β)/BMP type I receptors are highly conserved, transmembrane serine/threonine kinases that are an integral part of the TGF-β/BMP signal transduction pathway, acting in a diverse array of cellular and developmental processes. The TGF-β/BMP type I receptors are characterized by a cysteine-rich, extracellular ligand-binding domain; a single-pass transmembrane domain; and a well-conserved, intracellular kinase domain (Massagué, 1998). The intracellular domain contains regulatory regions such as the L45 loop, which confers binding specificity for the intracellular transducer, the receptor-mediated Smad (R-Smad) (Feng and Derynck, 1997; Persson et al., 1998), and a glycine-serine rich GS domain required for activation of the type I receptor kinase (Wrana et al., 1994a; Franzén et al., 1995). Type I receptors mediate extracellular TGF-β/BMP signals as part of a receptor complex with the related type II receptor. Upon formation of the ligand-bound receptor complex, the type I receptor is activated by the constitutive kinase activity of the type II receptor through trans-phosphorylation. Once phosphorylated by the type II receptor, the GS domain forms a secondary binding site for R-Smads (Huse et al., 2001). The R-Smads are in turn phosphorylated by the activated type I receptors.

Phosphorlyated R-Smads complex with the Co-Smad, accumulate in the nucleus, and interact with other proteins to regulate transcription of specific target genes (Massagué, 1998; Wu and Hill, 2009).

Based largely on sequence homology and ligand specificity, two main groups emerge from the family of BMP type I receptors represented by the mammalian ALKs and *Drosophila* Tkv and Sax receptors: ALK3/ALK6/Tkv and ALK1/ALK2 (ACVR1)/Sax (Chen and Massagué, 1999; Newfeld et al., 1999). These two groups exhibit higher affinities for secreted BMP ligands from the two subfamilies, BMP2/4/Dpp and BMP5/6/7/Gbb, respectively. Although some functional redundancy exists amongst ligand and receptor families it is clear that the tissue context of various ligand/receptor combinations impacts signaling output. As such, mutations in type I receptors alone are associated with a number of unique diseases including, hereditary hemorrhagic telangiectasia type 2 (HHT2; ALK1/ACVRL1), juvenile polyposis syndrome (ALK3/BMPR1A), brachydactyly type A2 (ALK6/BMPR1B) and the focus of this study, fibrodysplasia ossificans progressiva (FOP; ALK2/ ACVR1) (Abdalla and Letarte, 2006; Bayrak-Toydemir et al., 2006; Wehner et al., 2006; Olivieri et al., 2007; Howe et al., 2001; Zhou et al., 2001; Kim et al., 2003; Lehmann et al., 2003; Lehmann et al., 2006).

FOP is characterized by progressive, heterotopic ossification that occurs through an endochondral process (Pignolo et al., 2005). Extraskeletal ossification is especially detrimental when it leads to immobilization of joints and restriction of organ function. Mortality associated with FOP often results from respiratory complications due to the fusion of rib bones that interfere with the function of muscles, connective tissue, and nerves in the intercostal space (Kaplan and Glaser, 2005). Interestingly, for the most part, clinical features are not apparent at birth other than great toe malformations, a characteristic that is invariant in classic FOP (Shore et al., 2006). The onset of FOP is generally delayed until early childhood, suggesting that the disease is developmental in

nature and may require other triggers. Ossification is episodic and tends to occur in association with trauma or inflammation, thus rendering surgery an ineffective treatment (Kaplan et al., 2008).

The vast majority of FOP patients harbor the classic mutation which is defined by a 617G>A mutation in one copy of the *ALK2* gene, resulting in a histidine substitution at arginine 206 (R206H) (Shore et al., 2006). The R206H mutation, which lies C-terminal to the GS domain in ALK2 (see **Fig. 4.2A**), leads to a high level of BMP signaling in a variety of systems (Billings et al., 2008; Shen et al., 2009; Song et al., 2010; van Dinther et al., 2010). Interestingly, this residue is located just N-terminal to a conserved Thr/Gln residue that, when mutated to Asp, confers constitutive activity to all members of the TGF-β and BMP family of type I receptors (Wieser et al., 1995; Attisano et al., 1996; Akiyama et al., 1997; Macías-Silva et al., 1998; Chen and Massagué, 1999). Both the classic FOP mutation and the Thr/Gln to Asp mutation emphasize the importance of the region near the GS domain.

While mutations responsible for FOP have been identified, the molecular details that result in the hyperactive behavior of the mutated ALK2 type I receptor are not yet fully understood. The episodic nature of FOP and the long latency or quiescent period prior to heterotopic bone formation in patients indicates that the hyperactivity of the mutant receptor must be unleashed. Studies to elucidate the mechanism of ALK2 receptor activation and its function within an organismal context will most certainly advance our understanding of FOP. Finally, identifying the molecular events that are responsible for FOP-induced hyperactive BMP signaling will reveal avenues for potential therapeutic approaches.

Drosophila has proven to be an outstanding model organism to study an increasing number of human diseases based on the high degree of molecular and functional conservation observed for genes known to be involved in both the signaling

pathways and regulatory mechanisms governing development and homeostasis (Veraksa et al., 2000; Reiter and Bier, 2002; O'Kane, 2003; Bier, 2005; Botas, 2007; Chintapalli et al., 2007; Pandey and Nichols, 2011). *Drosophila* signaling components are largely non-redundant which circumvents the potential difficulty in interpreting pathway manipulations made in vertebrate systems where two or more closely related proteins may exhibit functional redundancy. As such, the initial identification of the core TGF-β/BMP signaling components benefited from the genetically tractable *Drosophila* system (Sekelsky et al., 1995; Newfeld et al., 1996; Zhang et al., 1996; Botas, 2007). In addition, the high degree of functional conservation between the *Drosophila* and vertebrate BMP signaling pathway components is underscored by the interchangeability of their respective signaling components at each level of the pathway (Padgett et al., 1993; Sampath et al., 1993; Penton et al., 1994; Brummel et al., 1994; Newfeld et al., 1996; Fritsch et al., 2010).

Previous work from our lab has demonstrated that Sax, the *Drosophila* ALK2 orthologue, has a dual function that both promotes and antagonizes BMP signaling (Bangi and Wharton, 2006b). The ability of type I receptors from other organisms to antagonize BMP signaling has not been investigated, although recent studies suggest that ALK2 is able to inhibit Activin signaling in MA-10 cells and inhibit BMP6-induced signaling in COS cells (Renlund et al., 2007; van Dinther et al., 2010). These reports, coupled with the evolutionary relatedness of ALK2 and Sax, raises the possibility that ALK2, like Sax, may have the ability to inhibit BMP signaling.

Here we report on a series of studies that investigated the use of *Drosophila* as a model to assess the consequences of hyperactive receptor kinase activity associated with FOP and the molecular factors required therein. Our findings reveal that the *Drosophila* components are able to mediate hyperactive signaling by the mutant human receptor $ALK2^{R206H}$ ligand-independently hyperactive type I receptor both *in vivo* and in

Drosophila cell culture. Importantly, our findings have contributed to the mechanistic understanding of how defective FOP receptors signal by revealing that the type II receptor is a critical molecular determinant required for ALK2^{R206H} mutant receptor signaling. Additionally, we investigated the functional similarities between Sax and ALK2 and found that wild-type ALK2 is also able to block BMP signaling but it achieves this inhibition in a manner different from that employed by Sax. These results provide an important advance in our understanding of both the molecular events required for hyperactive signaling by a FOP mutant receptor and the wild-type behavior of the ALK2/ACVR1 receptor. Moreover, these studies provide a new tool for future investigations of the mechanistic attributes and the triggers responsible for activating FOP mutant receptors.

MATERIALS AND METHODS

Plasmid Constructs

Gateway cloning (Invitrogen) was used to clone all cDNAs into the following *Drosophila* Gateway Vectors: pTWF for GAL4-UAS driven expression in transgenic animals and the Actin5C vector pAWF (C-terminal 3xFLAG) for constitutive expression in cell culture. *ALK2 and ALK2^{R206H}* cDNAs were a generous gift from Eileen Shore. *punt* cDNA was a gift from Michael O'Connor. *pAW gbb*, *pAW dppHA* and *pAW hBMP4* were constructed by Takuya Akiyama.

Ligand-binding domain deletions: Ligand-binding domain deletion mutants were generated by Quikchange Site-directed Mutagenesis (Stratagene). For ALK2$^{\Delta LBD}$, sequences corresponding to Cys35 to Cys 99 were removed using the following primers: fwd 5'- CAA CCC CAA ACT CTA CAT GAA CAG GAA CAT CAC GGC C-3' and rev 5'- GGC CGT GAT GTT CCT GTT CAT GTA GAG TTT GGG GTT G-3'. For Sax, sequences corresponding to Cys67 to Cys148 were removed using the following primers: fwd 5'- CGC ATC CCA GAT ACA AAA ATG AGG GAG ACT TTC C-3' and rev 5'- GGA AAG TCT CCC TCA TTT TTG TAT CTG GGA TGC G-3'.

GS domain mutants: Two sets of GS domain mutations were generated in ALK2^{R206H} (Quikchange Site-Directed Mutagenesis). ALK2 $^{GS1-\ R206H}$: all three serines were mutated to alanine (TSGSGSG > TAGAGAG) using the following primers: (ALK2 S190,192,194A fwd) 5'- CAG ATT TAT TGG ATC ATT CGT GTA CAG CAG GAG CTG GCG CTG GTC TTC CTT TTC TGG TAC -3' and (ALK2 S190,192,194A rev) 5'- GTA CCA GAA AAG GAA GAC CAG CGC CAG CTC CTG CTG TAC ACG AAT GAT CCA ATA AAT CTG-3'.

ALK2 $^{GS2-\ R206H}$: a threonine and all three serines were mutated to alanine (TSGSGSG > AAGAGAG) using the following primers: (ALK2 T189A S190,192,194A fwd) 5'-CAG ATT TAT TGG ATC ATT CGT GTG CAG CAG GAG CTG GCG CTG GTC TTC CTT TTC TGG TAC-3' and (ALK2 T189A S190,192,194A rev) 5'-GTA CCA GAA AAG GAA GAC CAG CGC CAG CTC CTG CTG CAC ACG AAT GAT CCA ATA AAT CTG-3'.

Drosophila melanogaster strains and crosses

All fly strains were cultured using standard sucrose, yeast extract, agar food at 25°C. All fly strains are described in Flybase and obtained from Bloomington Stock Center, except where noted: *UAS-gbb9.1* (Khalsa et al., 1998), *A9-GAL4*, *UAS- tkv^{QD}* (Haerry et al., 1998), *UAS-wit-HA31* (Michael O'Connor). *UAS-putRNAi* (from NIG-FLY, NIG 7904 R-2*D*). *UAS-sax-3xFLAG(1-1M-A), UAS-ALK2-3xFLAG(8-1-9M-1a),* and *UAS-ALK2^{R206H}-3xFlag(3-4F1-a)* were germline transformants derived from constructs described above.

Receptor and *gbb* overexpression

Receptors and *gbb* were overexpressed using the UAS-GAL4 system (Brand and Perrimon, 1993). *A9-GAL4* and *ap-GAL4* drivers express primarily in the dorsal compartment of the wing imaginal disc.

in vivo Gbb signaling assay

A previously described *in vivo* assay (Bangi and Wharton, 2006b) was used to test for the ability of BMP type I receptors to affect Gbb signaling. Adult wings from the

following genotypes were mounted (DPX, EM Sciences) and scored: *w A9-GAL4/yw; +/+; UAS-gbb9.1/+* were compared to *w A9-GAL4/yw; UAS-sax(1-1M-A)/+; UAS-gbb9.1/+* and *w A9-GAL4/yw; UAS-ALK2(8-1-9M-1a)/+; UAS-gbb9.1/+*.

Immunohistochemistry

Everted third instar larvae were dissected and fixed in 4% paraformaldehyde/PBS (v/v) for 20 minutes at room temperature followed by 5 washes in PBST (0.3% Triton X-100). Fixed tissues were then incubated overnight in blocking solution (10% NGS in PBST) at 4^{o}C. After blocking, the cuticles were incubated in primary antibody diluted in blocking solution at the following dilutions: 1:1000 anti-FLAG M2 (Sigma, F3165), 1:1000 anti-HA 3F10 (Roche) and 1:1000 PS3 (Epitomics). Tissues were then washed 5 times with PBST and incubated in secondary antibody in blocking solution at the following dilutions: 1:1000 GAM Alexa Fluor 633, 1:1000 GARt Alexa Fluor405 (in WitHA experiments), 1:1000 GARb Alexa Fluor568. Following 5 washes in PBST, wing discs were removed and mounted in 80% glycerol/0.5% N-propyl gallate. Confocal images were collected using a Zeiss LSM510 Meta confocal laser scanning microscope.

***Drosophila* Schneider 2 (S2) cell maintenance and Transfections**

S2 cells were cultured in Shields and Sang M3 Insect Medium (Sigma S8398) pH 6.5 containing 10% Insect Medium Supplement (Sigma I7267) and 2% Fetal Bovine Serum (F3018). Transient transfections were carried out using Effectene Transfection Reagent (Qiagen 301427).

Quantitative Cell-based BMP Signaling Assay

An adapted protocol based on a previously described assay was used to measure BMP signaling activity (Bangi and Wharton, 2006b; Müller et al., 2003; Twombly et al., 2009). This assay makes use of a reporter construct expressing *lacZ* under the control of a *Su(H)* transcriptional activation response element as well as a *brk* transcriptional silencer element (*Su(H)/brkS-lacZ*). Co-transfection of the reporter construct with plasmids encoding Su(H) and an activated form of Notch (N*) lead to *lacZ* transcription while the activation of BMP signaling leads to a repression of *lacZ* expression by virtue of the BMP-responsive *brk* silencer element. BMP signaling can thus be measured as a loss of β-galactosidase activity.

$2.8x10^6$ cells were co-transfected with *Su(H), N*, Su(H)/brkS-lacZ*, and *luciferase* plasmids, all under the control of the actin 5C promoter. Constructs and their concentrations used in this assay are indicated in the figure legends. Cells were harvested and lysed 3 days post-transfection and β-galactosidase activity of cleared lysate was measured using the dual luciferase assay system (Dual-Light, Applied Biosystems) and normalized to luciferase activity which served as a transfection control for each sample. The normalized value obtained from the cleared lysate of cells co-transfected with only *Su(H), N*, Su(H)/brkS-lacZ* and luciferase was set to 100%. Statistical significance was determined using two-tailed T-Test with significance value of 0.05. Epitope tagged and untagged versions of the type I receptors investigated in this study were compared for signaling activity and showed no significant difference (data not shown).

Co-immunoprecipitation

$8x10^6$ S2 cells were transiently transfected with 300ng of pAWF type I receptor constructs and 700ng of either pAW *dppHA* or pAW *gbb*; cells were incubated at 25^oC for 4 days for protein production. Cells were solubilized in 1% Triton X-100 at 4^oC for 1

hour. Cleared lysate was incubated with 1ug anti-FLAG M2 (Sigma F3165) bound to 20uL of Dynabeads Protein G Dynabead (Invitrogen 100-04D) per sample at 4° for 1 hour. An aliquot of cleared lysate was saved as soluble input for western blot analysis. The beads were then washed once with one volume of Wash Buffer 1 (20mM Tris-HCl pH 7.4, 150mM NaCl, 0.2% Triton X-100), twice with one volume of Wash Buffer 2 (20mM Tris-HCl pH 7.4, 150mM NaCl), and boiled for 5 minutes in 50uL 2xSDS buffer. IP and soluble input fractions were run on 12% SDS-PAGE gels and analyzed by western blot using standard protocols. Anti-HA 3F10 (Roche) was used at 0.1ng/uL. Anti-Flag M2 (Sigma) was used at 4ng/uL, mouse anti-Gbb (gift from Guillermo Marquez) was used at a 1:1000 dilution. Secondary antibodies GAM IgG-HRP light-chain specific (Jackson) and GARat HRP (Jackson, preabsorbed) were used at a 1:10,000 dilution.

Image analysis

Intensity profiles of pMad distribution were measured by the Fiji Image Processing Package (http://fiji.sc/wiki/index.php/Fiji). The profiles shown are the average intensity plots measured in the dorsal and ventral compartments of five wing discs and aligned by the posterior and anterior peaks of pMad distribution in the ventral compartment.

RESULTS

FOP mutant receptor ALK2^{R206H} stimulates increased BMP signaling in *Drosophila*

In order to test the ability of the human ALK2^{R206H} classic FOP mutant receptor to signal in *Drosophila,* we generated transgenic lines that allowed us to control the expression of ALK2^{R206H} in a tissue-specific manner (Brand and Perrimon, 1993) and assayed for the ability of ALK2^{R206H} to induce BMP signaling in the developing wing. It is known that BMP signaling plays critical roles growth, patterning and differentiation in the wing imaginal disc during wing development (Rogulja and Irvine, 2005; Bangi and Wharton, 2006a; O'Connor et al., 2006; Affolter and Basler, 2007; Blair, 2007; Rogulja et al., 2008; Oh and Irvine, 2011; Schwank et al., 2011; Wartlick et al., 2011a; Wartlick et al., 2011b). In the primordial cells of the wing proper, a gradient of BMP signaling activity is generated through the action of two *Drosophila* BMP ligands, Dpp and Gbb, two type I receptors, Sax and Tkv, and the type II receptor, Punt. The resulting phospho-Mad (pMad) gradient reflects the output of BMP signaling and is critical for regulating its transcriptional targets.

The expression of *ALK2^{R206H}* was directed primarily to the dorsal compartment of the wing imaginal disc by crossing *ap-GAL4* or *A9-GAL4* lines to a *UAS-ALK2^{R206H}* transgenic line. Adults were obtained, albeit unable to fully emerge from the pupal case. The wings from these individuals were misshapen and marked by ectopic vein tissue (**Fig. 4.1B**; data not shown). In larvae of the same genotype, we found a higher level of pMad throughout the dorsal compartment compared to endogenous levels of pMad observed in the ventral compartment (**Fig. 4.1C',D**). The presence of ectopic pMad indicates that ALK2^{R206H} is able to stimulate BMP signaling in *Drosophila* imaginal disc tissues, presumably through the direct phosphorylation of the *Drosophila*

Smad1/5/8 orthologue, Mad (**Fig. 4.1D**). As expected from the known role of BMP signaling in tissue growth (Capdevila and Guerrero, 1994; Haerry et al., 1998; Rogulja and Irvine, 2005; Affolter and Basler, 2007), we observed an increase in the size of the dorsal compartment of *ap-GAL4>ALK2^{R206H}* wing discs (**Fig. 4.1D**) as well as the downwardly curved wings evident in adults resulting from enlargement of the dorsal surface.

As in our *in vivo* studies, we found that ALK2^{R206H} can induce an increase in BMP signaling in a quantitative cell-based BMP signaling assay (**Fig. 4.1F**). This cell-based assay makes use of a *lacZ* reporter construct under the control of the *brinker* silencer element (*brkS*) which is known to quantitatively repress transcription in response to Mad-mediated signaling (Müller et al., 2003; Bangi and Wharton, 2006b; Twombly et al., 2009). S2 cells transfected with a plasmid construct encoding the *Drosophila* ligand Gbb exhibited a reduction in β-gal activity, reflecting the repression of *lacZ* transcription in response to an increase in BMP signaling (**Fig. 4.1F**). Cells transfected with a construct encoding the FOP mutant receptor ALK2^{R206H} showed very high levels of BMP signaling (**Fig. 4.1F**).

Extracellular ligand binding domain is not required for hyperactivity of ALK2^{R206H}

The wild-type ALK2 receptor has been shown to promote Müllerian-inhibiting substance (MIS)-dependent signaling in mammalian systems (Clarke et al., 2001; Visser et al., 2001) and to bind the vertebrate ligands, Activin and BMP7 (Attisano et al., 1993; ten Dijke et al., 1994). In zebrafish embryos and mammalian cells, the mutant ALK2^{R206H} receptor has been reported to signal independently of BMP ligands (Billings et al., 2008; Fukuda et al., 2009; Shen et al., 2009) however, the possibility that the

hyperactive nature of ALK2^{R206H} depended on interactions with other ligands was not fully excluded in these studies.

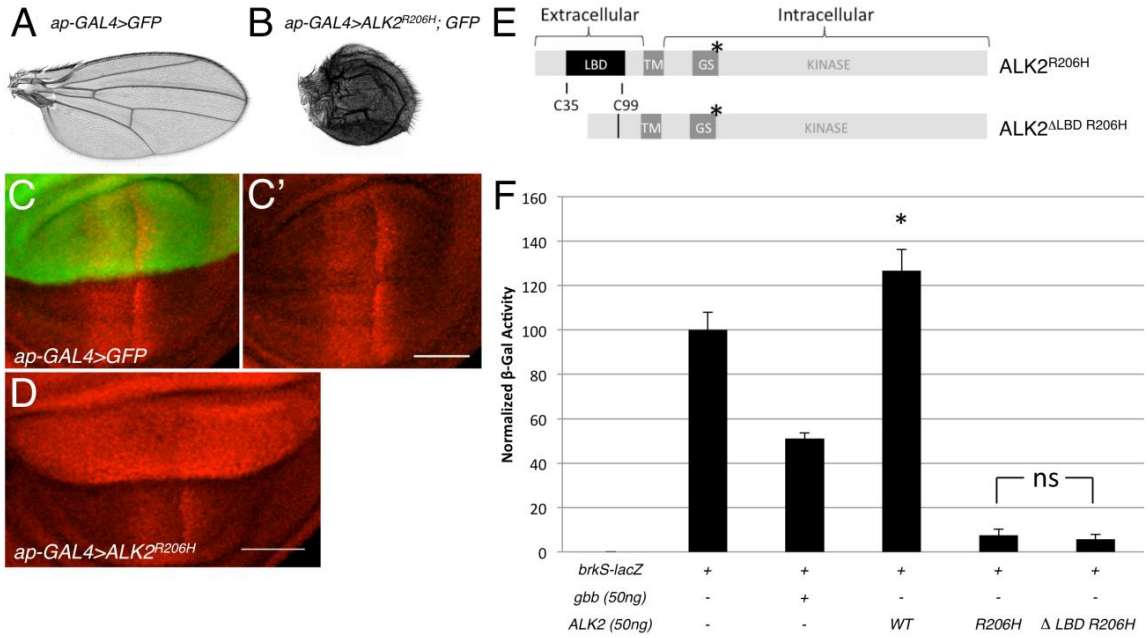


Figure 4.1. In the *Drosophila* system the ALK2^{R206H} FOP mutant receptor stimulates BMP signaling even in the absence of its ligand binding domain. A. Wild-type wing from control *ap-GAL4, UAS-GFP/+* adult. **B.** Wing from *ap-GAL4, UAS-GFP/UAS-ALK2^{R206H}* adult. **C-D**. Confocal images of pMad distribution (red) in the wing pouch of third instar larval wing discs. Scale bar= 50μm. **C,C'.** A representative *ap-GAL4, UAS-GFP/+* control wing disc. The dorsal expression domain of *ap-GAL4* is marked by GFP expression (green). The ventral compartment lacks expression of GFP. **D.** A representative *ap-GAL4,UAS-GFP/UAS-ALK2^{R206H} wing disc*. **E.** Diagram of full length ALK2^{R206H} and ligand-binding domain deletion mutant ALK2$^{\Delta LBD\ R206H}$ drawn to scale. Amino acids from Cys35 to Cys99 were removed by site-directed mutagenesis. An asterisk (*) indicates position of the R206H mutation. **F.** Quantitative *brkS-lacZ* assay measuring BMP signaling activity of ALK2^{R206H} and ALK2$^{\Delta LBD\ R206H}$ in S2 cell culture. Data represent mean +/- standard deviation (n=4). ns indicates not significant at P<0.05 when comparing *brkS-lacZ +ALK2^{R206H}* versus *brkS-lacZ+ALK2$^{\square LBD\ R206H}$*(p=0.38). An asterisk (*) indicates significance at P<0.05 when compared to *brkS-lacZ* transfection alone (P=0.005). We interpret this difference to reflect the ability of ALK2 to inhibit endogenous BMP signaling in S2 cells. LBD = ligand binding domain. TM= transmembrane domain. GS = glycine-serine rich domain/box.

In *Drosophila,* orthologues are evident for both BMP ligand subfamilies (BMP2/4 = Dpp and BMP5/6/7 = Gbb) as well as for the TGF-β/Activin subfamily (Daw, Actβ, Myo, Mav) (Kutty et al., 1998; Lo and Frasch, 1999; Nguyen et al., 2000; Parker et al., 2004; Parker et al., 2006; Moustakas and Heldin, 2009). Given ALK2's promiscuity in binding ligands from different subfamilies, we generated an ALK2^{R206H} receptor that lacked the cysteine-rich (C38-C99), ligand-binding domain (LBD) to definitively test for the ability of ALK2^{R206H} to signal independently of any ligand in the *Drosophila* system. Cells transfected with the ALK2$^{\Delta LBD\text{-}R206H}$ construct were able to induce BMP signaling at comparable levels to that achieved by the full-length ALK2^{R206H} receptor (**Fig. 4.1F**), indicating that the signaling activity of the FOP mutant receptor is not only ligand-independent but that it is able to function in the promotion of BMP signaling despite lacking of a large portion of its extracellular domain (ECD) (**Fig. 4.1E**).

Hyperactivity of ALK2^{R206H} requires type II receptor function

To transduce BMP signals, a type I receptor must be activated through phosphorylation of its GS domain by the type II receptor. Given the proximity of the R206H FOP mutation to the GS domain, we questioned whether the hyperactive nature of the ALK2^{R206H} receptor depended on a phosphorylated GS domain. The importance of trans-phosphorylation of the ALK2^{R206H} GS domain by the type II receptor kinase was tested by mutating the GS domain Ser/Thr residues to Ala and assaying the mutated constructs for signaling activity (**Fig. 4.2A,B**). Both ALK2$^{GS1\text{-}R206H}$ (three Ser mutated to Ala) and ALK2$^{GS2\text{-}R206H}$ (three Ser and the Thr mutated to Ala) resulted in abrogation of signaling as indicated by the failure of *brkS-lacZ* expression to be repressed (**Fig. 4.2B**). These results indicate that the Ser or Thr residues are critical for signaling, suggesting that their phosphorylation is required for the hyperactive signaling of the ALK2^{R206H} receptor.

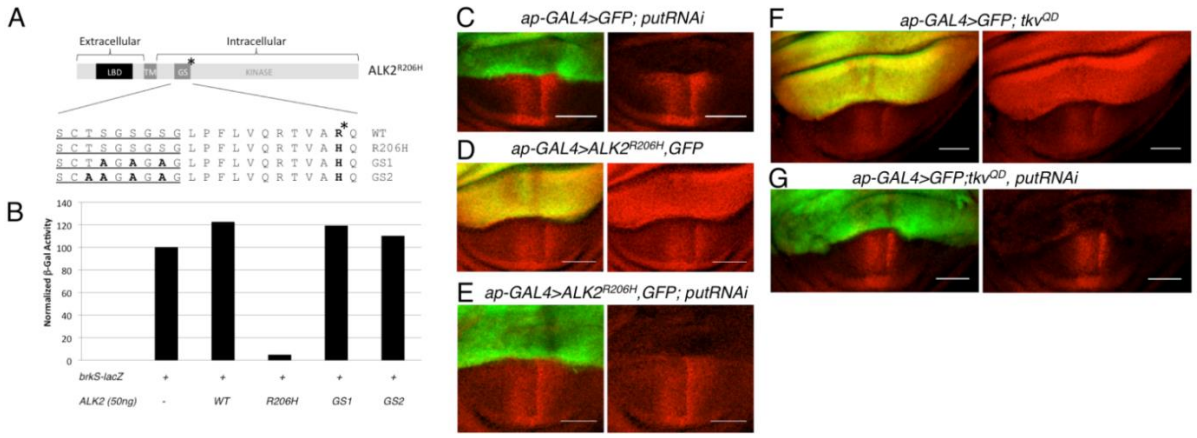


Figure 4.2. Hyperactive signaling induced by ALK2^{R206H} requires BMP type II receptor function. A. (top) Diagram of the full length ALK2^{R206H} receptor drawn to scale. LBD = ligand binding domain. TM= transmembrane domain. GS = glycine-serine rich domain/box. An asterisk (*) indicates position of R206H mutation. (Below) Amino acid alignment of GS domains (from ALK2, ALK2^{R206H}, ALK2$^{GS1\text{-}R206H}$ (GS1)and ALK2$^{GS2\text{-}R206H}$ (GS2). Glycine-serine rich sequence containing serine and threonine targets of type II receptor phosphorylation is underlined. **B.** *brkS-lacZ* signaling assay indicates ALK2$^{GS1\text{-}R206H}$ (GS1) and ALK2$^{GS2\text{-}R206H}$ (GS2) lack BMP signaling activity. Data plotted are mean of two experiments performed in duplicate. **C-G.** Stimulation of BMP signaling by ALK2^{R206H} and Tkv^{QD} in the wing disc requires the type II receptor Punt. Confocal images of pMad distribution (red) in wing pouch of third larval instar wing discs, *ap-GAL4* expression domain marked by *UAS-GFP* (green). **C:** Expression of *put* RNAi in dorsal compartment leads to dramatic reduction in pMad. *ap-GAL4, UAS-GFP/+; UAS-put RNAi/+* **D.** Expression of *ALK2^{R206H}* in dorsal compartment leads to an increase in pMad (red) levels, *ap-GAL4, UAS-GFP/UAS-ALK2^{R206H}*. **E.** Co-expression of *put* RNAi eliminates pMad increase associated with *ALK2^{R206H}* as well as endogenous pMad, *ap-GAL4,UAS-GFP /UAS-ALK2^{R206H}; UAS-putRNAi/+*. **F.** High levels of pMad are associated with expression of *tkv^{QD}* in dorsal wing compartment, *ap-GAL4, UAS-GFP/+; UAS-tkv^{QD}/+*. **G.** Co-expression of *put* RNAi eliminates BMP signaling induced by *tkv^{QD}* as indicated by the loss of pMad, *ap-GAL4, UAS-GFP/+; UAS-tkv^{QD}/UAS-put RNAi*. Scale bar = 50μm.

To specifically test the importance of type II receptor function in enabling ALK2^{R206H} hyperactivity, we made use of a *UAS-putRNAi* construct to knock down the expression of the *Drosophila* type II receptor, Punt, *in vivo*. Directed expression of *put* RNAi to the dorsal compartment of the wing imaginal disc using *ap-GAL4* results in a

dramatic loss of pMad (**Fig. 4.2C**) consistent with the requirement for *put* in BMP signaling (Letsou et al., 1995; Ruberte et al., 1995). The previously observed elevated levels of pMad induced by expression of *ALK2^{R206H}* in the dorsal wing compartment (**Fig. 4.2D**) were largely suppressed when *put* RNAi was co-expressed (**Fig. 4.2E**), demonstrating that the activity of ALK2^{R206H} is dependent on the presence of type II receptor function *in vivo*.

The QD activating mutation in BMP type I receptors is dependent on type II receptor function

Previous reports have shown that mutation of the conserved Thr/Gln residue neighboring R206 (in ALK2) to Asp results in constitutive signaling in all members of the TGF-β/BMP family of type I receptors (Wieser et al., 1995; Attisano et al., 1996; Akiyama et al., 1997; Macías-Silva et al., 1998; Chen and Massagué, 1999)**.** The constitutive activity associated with TβR1^{T204D} has been described to be independent of TGF-β type II receptor (TβRII) activity (Wieser et al., 1995). However, as shown above, we found that the presence of a type II receptor is absolutely required for the signaling hyperactivity associated with ALK2^{R206H}. These somewhat conflicting observations led us to ask whether activating mutations in other type I receptors have a requirement for type II receptors. Indeed, we found that, unlike TβR1^{T204D}, constitutive signaling produced by the *Drosophila* BMP type I receptor, Tkv, carrying the equivalent mutation (Gln to Asp; Tkv^{QD}) is in fact type II receptor-dependent. The high levels of pMad induced by Tkv^{QD} are suppressed by knocking down Punt with *put* RNAi (**Fig. 4.2F,G**). These results suggest an inherent difference in how TβRI and the BMP type I receptors, Tkv and Alk2, respond to an activating mutation. However, it is not yet clear whether these results reflect a fundamental difference in the mechanism by which TGF-β and BMP type I receptors can be activated, as it still remains a possiblity in the previous study that TβRI

could have been acted upon by BMP or Activin type II receptors (Wieser et al., 1995; Chen et al., 1997).

Drosophila Type II receptor Wit can activate ALK2^{R206H}

We next tested if the type II requirement for ALK2^{R206H} hyperactivity was dependent on a specific type II receptor. Given that knocking down endogenous Punt completely suppressed the elevated pMad levels in wing discs induced by ALK2^{R206H}, we tested for the ability of the other *Drosophila* type II receptor, Wit, to restore hyperactive signaling in this experimental context. Indeed, we found that the expression of *wit-HA* with *ALK2^{R206H}* and *put* RNAi led to elevated pMad, indicating that the ability of ALK2^{R206H} to signal is not limited to a specific type II receptor (**Fig. 4.3**). The ability of Wit to restore signaling by ALK2^{R206H} in the absence of Punt is consistent with our finding that ALK2^{R206H} signaling requires the presence of a type II receptor.

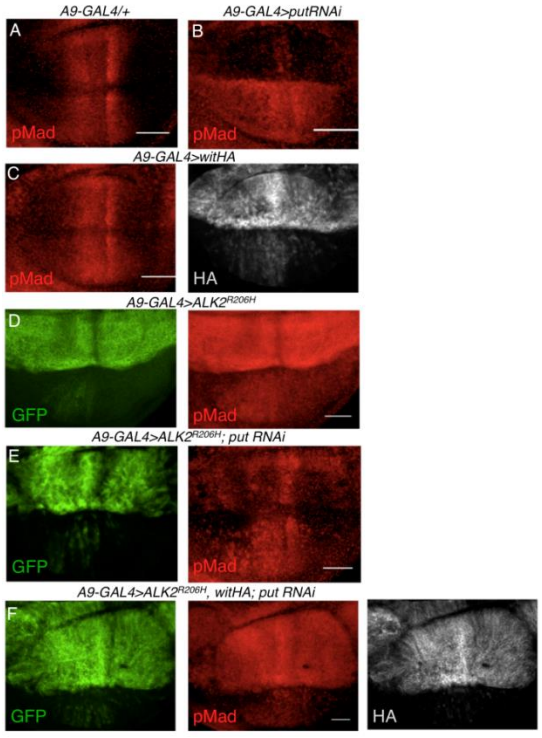


Figure 4.3. Wit enables ALK2^{R206H} hyperactive signaling. The pMad distribution in A9GAL4/+; UAS-witHA wing discs is slightly increased compared with A9-GAL4/+ control wing discs, consistent with its role in mediating bone morphogenetic protein (BMP) signals. A9-GAL4/+;UAS-putRNAi /+ exhibits a nearly complete loss of pMad in the dorsal compartment of the wing disc similar to that observed in ap-GAL4, UAS-GFP/+; UAS-putRNAi wing discs. A9-GAL4/+; UAS-ALK2R206H/+ leads to a dramatic increase in pMad intensity in the dorsal compartment that is significantly down-regulated in A9-GAL4/+; UAS-ALK2R206H/+; UAS-putRNAi/+. The dramatic increase in pMad distribution and intensity is restored in A9-GAL4/+; UAS-ALK2R206H/+; UAS-putRNAi/ UAS-witHA. pMad (red), ALK2R206H (green), Wit (white).

ALK2 can inhibit BMP signaling

Clearly the FOP mutant receptor, ALK2^{R206H}, exhibits high levels of signaling in *Drosophila* when expressed *in vivo* as well as in cell culture (**Fig. 4.1, 4. 2**). We have previously shown that the *Drosophila* ALK2 orthologue, Sax, exhibits a dual function in its transduction of BMP signals (Bangi and Wharton, 2006b). Given that the wing phenotypes associated with ectopic expression of Gbb or Dpp can be suppressed by overexpression of Sax and enhanced by the loss of endogenous *sax*, we proposed that Sax could antagonize BMP signaling by binding ligand into signaling incompetent receptor complexes. Consistent with this hypothesis, we found that Sax was able to block Gbb-induced signaling in a quantitative manner (Bangi and Wharton, 2006b). Despite this inhibitory behavior, it is clear that the complete loss of endogenous Sax results in a reduction in BMP signaling, indicating that Sax must play a role in promoting signaling output. However, Sax is unable to promote signaling on its own since a complete loss of the other type I receptor, Tkv, eliminates all BMP signaling despite the presence of endogenous Sax. The ability of Sax alone to inhibit signaling is borne out by the fact that the overexpression of Tkv enhances rather than inhibits signaling induced by either Gbb or Dpp. Given this unusual behavior of Sax and its evolutionary relatedness to ALK2, , we considered the possibility that ALK2 mutations associated with FOP may in fact actually mask a normally occurring dual function of ALK2, such that the inhibitory function is lost and the mutation manifests itself as one that hyperactivates ALK2.

To test for the ability of wild-type ALK2 to inhibit BMP signaling, we first compared the effect of overexpressing wild-type ALK2 to that of Sax under conditions known to reveal the inhibitory function of Sax *in vivo* (**Fig. 4.4A,B**). In both cases, we observed a loss or thinning of longitudinal vein 5 (L5), a phenotype associated with a loss of *gbb* function, as well as a reduction in the overall size of the wing, an indication of reduced BMP signaling (Wharton et al., 1999). In general, ectopic expression of *ALK2*

produced phenotypes indicative of a more severe reduction in BMP signaling than those achieved by overexpression of *sax* including a greater reduction in wing size and the additional loss of L4 (**Fig. 4.4A, B**). An actual reduction in BMP signaling was indeed observed as the dramatic loss of pMad observed within the dorsal compartment of the wing pouch when ALK2 is overexpressed compared to the overexpression of Sax (**Fig. 4.4C-D'**). Thus, like overexpression of Sax, the overexpression of ALK2 leads to an effective reduction in BMP signaling.

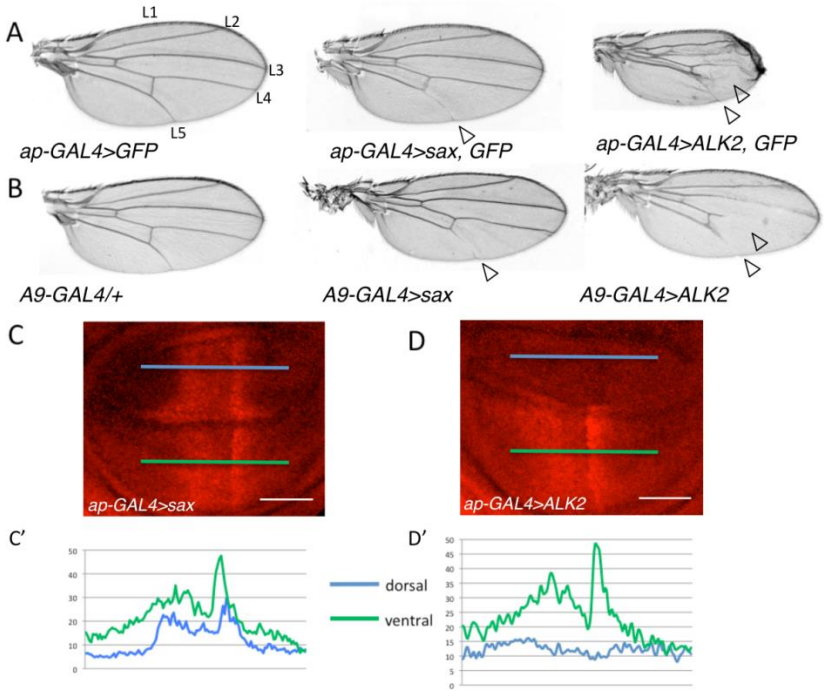


Figure 4.4. ALK2 can inhibit endogenous BMP signaling. A-B. Expression of *ALK2*, like *sax*, leads to loss of vein tissue (open arrowheads). **A:** Adult wings from *ap-GAL4, UAS-GFP/+* (left), *ap-GAL4, UAS-GFP/UAS-sax* (middle), and *ap-GAL4, UAS-GFP/UAS-ALK2* (right). **B.** Adult wings from *A9-GAL4/+* (left), *A9-GAL4/+; UAS-sax/+* (middle) and *A9-GAL4/+; UAS-ALK2/+* (right). **C-D'.** ALK2 reduces pMad levels. **C,D.** Representative confocal images of pMad distribution (red) in wing pouch of third instar larval wing discs (C) *ap-GAL4, UAS-GFP/ UAS-sax,* (D) *ap-GAL4, UAS-GFP/UAS-ALK2*. Scale bar = 50μm **C'.** Average pMad intensity profiles of the dorsal (blue line) and ventral (green line) compartments of *ap-GAL4, UAS-GFP/UAS-sax* wing discs (n=5). **D'.** Average pMad intensity profiles of the dorsal (blue line) and ventral (green line) compartments of *ap-GAL4, UAS-GFP/UAS-ALK2* wing discs (n=5).

We and others have observed that Sax binds Gbb more effectively than Dpp and, as such, we found that Sax can suppress the wing phenotypes produced by overexpression of Gbb better than those produced by the overexpression of Dpp (Haerry et al., 1998; Haerry, 2010). ALK2 has been shown to bind BMP7 but not BMP4 (ten Dijke et al., 1994; Macías-Silva et al., 1998; Greenwald et al., 2003) and given the evolutionary relatedness of Gbb and Dpp to BMP7 and BMP4, respectively (Sampath et al., 1993; Fritsch et al., 2010), we hypothesized that ALK2 would be able to effectively inhibit Gbb-induced BMP signaling. As observed previously, *A9-GAL4>UAS-gbb* resulted in an array of wing phenotypes marked by ectopic vein material, indicative of an increase in BMP signaling. The distribution of wing phenotypes is shifted toward less severe phenotypic classes when *sax* is coexpressed with *gbb* (Bangi and Wharton, 2006b) (**Fig. 4.5A**). In a second set of experiments we made use of this phenotypic assay to test for the ability of *ALK2* to antagonize signaling by Gbb. We found that not only did co-expression of *ALK2* and *gbb* suppress wing defects associated with ectopic Gbb signaling, but that all *A9-GAL4>UAS-ALK2*; *UAS-gbb* wings exhibited phenotypes consistent with a decrease in endogenous BMP signaling, such as a reduction in wing size and a loss of longitudinal vein material (class 6) (**Fig. 4.5A**). An examination of pMad distribution in the wing disc confirmed this conclusion as not only was the ectopic pMad induced by *gbb* overexpression eliminated, but alsothat pMad associated with endogenous BMP signaling was dramatically reduced (**Fig. 4.5B**).

The ability of ALK2 to inhibit BMP signaling was also tested in the quantitative, cell-based BMP signaling assay. Cotransfection of either *sax* or *ALK2* with *gbb* resulted in a suppression of Gbb-induced signaling, indicating that both receptors can inhibit signaling (**Fig. 4.5C**). Interestingly, we found that signaling induced by transfection of either *dpp* or human *BMP4* was also inhibited by ALK2 (**Fig. 4.5D**) whereas signaling

induced by transfection of mouse *BMP7* was enhanced by ALK2, indicating that the ability of ALK2 to inhibit or promote BMP signaling is ligand-specific (**Fig. 4.5D,E**).

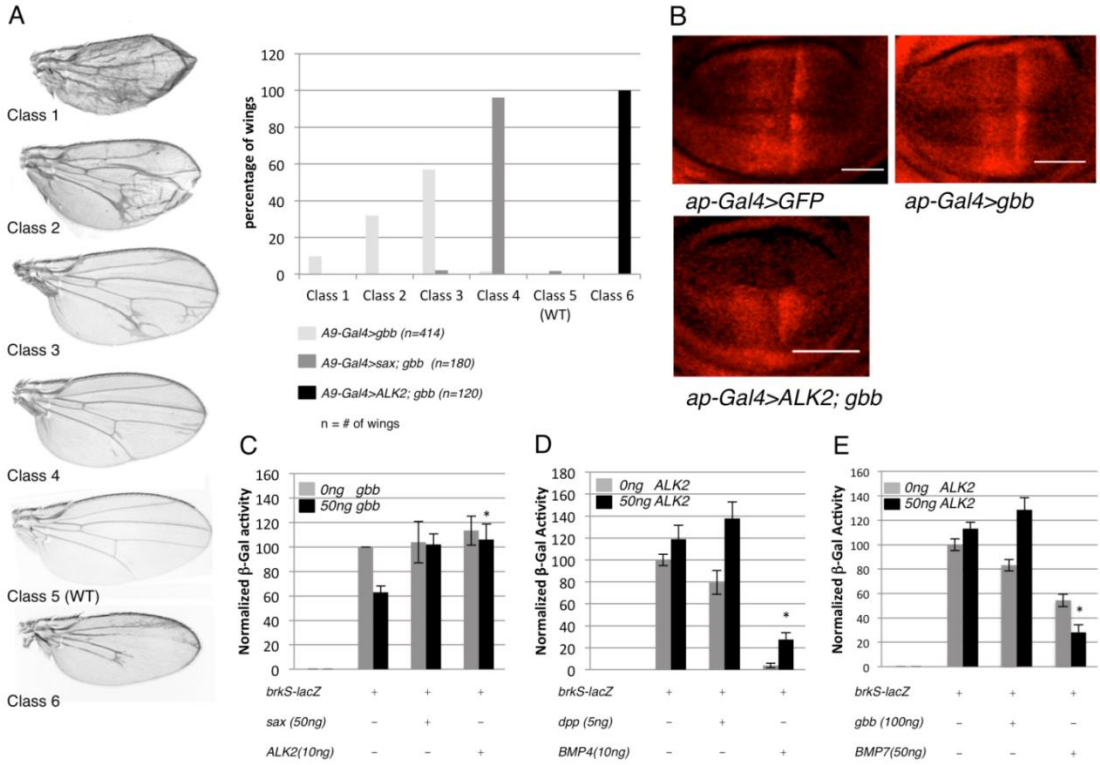


Figure 4.5. ALK2 can inhibit exogenous, ligand-induced BMP signaling in a ligand-specific manner. A. (left) Class 1 to Class 4: phenotypic distribution of adult wings from *A9-GAL4/+;UAS-gbb9.1/+*; Class 5: Wild-type; Class 6: phenotype of *A9-GAL4/+; UAS-ALK2/+; UAS-gbb9.1/+* adult wings. (right) The shift in the *gbb* over-expression phenotype associated with co-expression of either *sax* or *Alk2* suggests Gbb-induced signaling is antagonized. **B.** ALK2 can inhibit the increase in pMad (red) associated with Gbb expression in the dorsal compartment. (top left) *ap-GAL4, UAS-GFP/+* (top right) *ap-GAL4, UAS-GFP/+;UASgbb9.1/+* (bottom left) *ap-GAL4, UAS-GFP/UAS ALK2; UASgbb9.1/+*. **C.** ALK2 can antagonize BMP signaling induced by Gbb as measured by the *brkS-lacZ* reporter assay in S2 cell culture. Data represent mean +/- standard deviation (n=4). An asterisk (*) indicates significance at P<0.05 when compared to *brkS-lacZ + 50 ng gbb* (P=0.006). **D.** ALK2 can antagonize BMP signaling induced by Dpp and human BMP4 (hBMP4) as measured by the *brkS-lacZ* reporter assay in S2 cell culture. Data represent mean +/- standard deviation (n=6). An asterisk (*) indicates significance at P<0.05 when compared with *brkS-lacZ + 10ng BMP4* (P=0.0006). **E.** ALK2 enhances BMP signaling induced by mouse BMP7 (mBMP7) in the *brkS-lacZ* S2 cell culture assay. Data represent mean +/- standard deviation (n=3). An asterisk (*) indicates significance at P<0.05 when compared with *brkS-lacZ + 50ng BMP7* (P=0.005).

Ligand-binding is not required for ALK2-mediated inhibition

The ability of ALK2 to inhibit signaling induced by BMP4, a ligand it does not bind, raised the possibility that ALK2 may only inhibit signaling induced by BMP ligands that do not interact with ALK2. We investigated this possibility by testing the ability of ALK2 to bind Gbb and Dpp by co-immunoprecipitation. We were not able to detect an interaction between ALK2 with either Gbb or Dpp (**Fig. 4.6A**, lane 4 & **Fig. 4.6B**, lane 13, respectively) while the expected association between Gbb and Sax (**Fig. 4.6A**, lane 2) was apparent, as was a strong interaction between Dpp and Tkv (**Fig. 4.6B**, lane 11) with no to little interaction between Dpp and Sax (lane 12).

It is possible that the affinity of ALK2 for the *Drosophila* BMP ligands is below the detectable limit of co-immunoprecipitations. Therefore, to more rigorously test for the importance of ALK2-ligand interactions, we deleted the cysteine-rich region (C38-C99) ligand-binding domain of ALK2 ($ALK2^{\Delta LBD}$) (**Fig. 4.6C**) and tested for the ability of this mutant receptor to block signaling. Interestingly, we found that $ALK2^{\square LBD}$ was able to effectively block Gbb induced signaling in S2 cells (**Fig. 4.6D**), indicating that the ability of ALK2 to block BMP signaling is independent of a direct interaction with ligand and, for that matter, independent of a large portion of its extracellular domain. Thus, ALK2 must inhibit BMP signaling by a mechanism other than ligand sequestration.

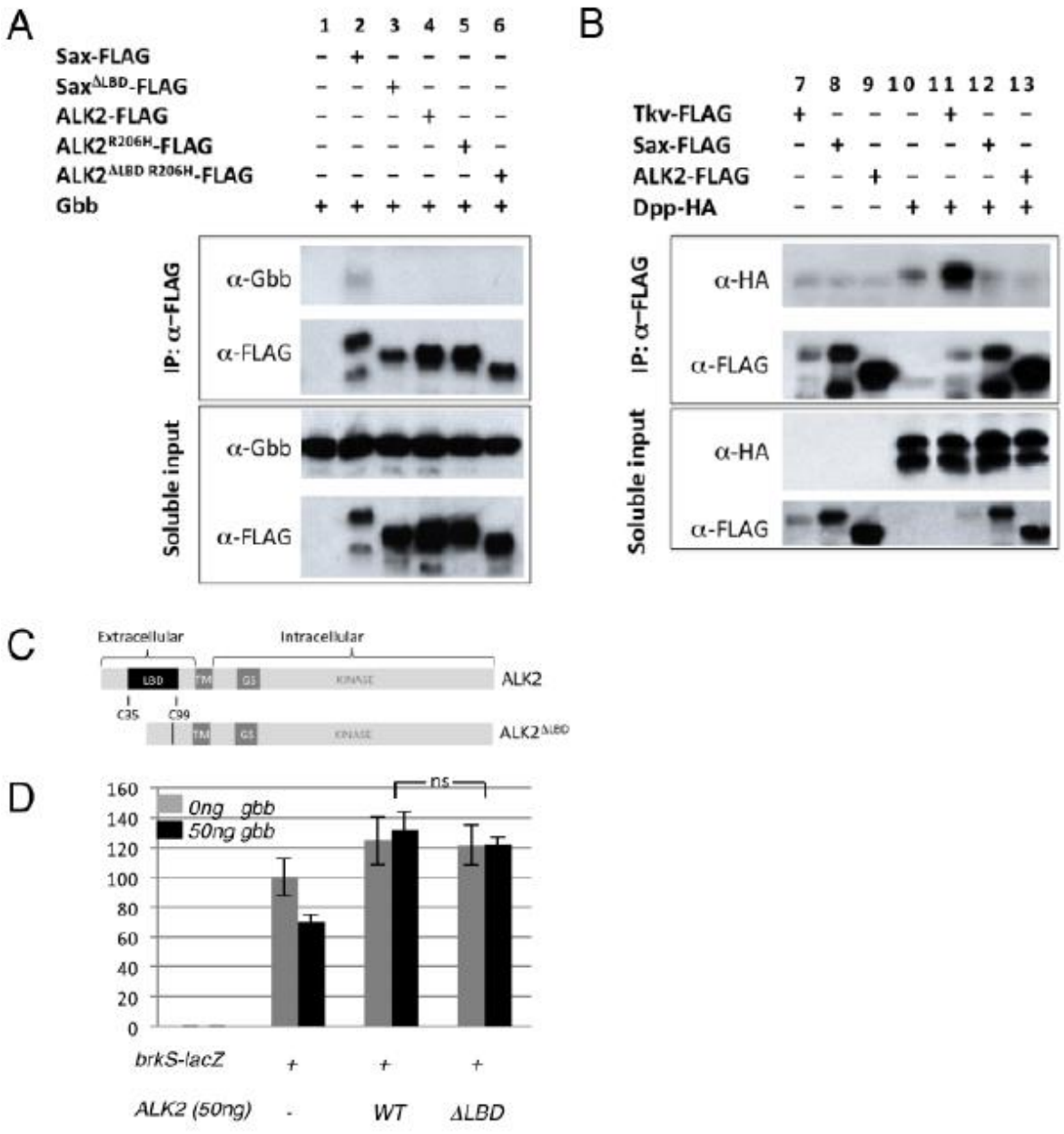


Figure 4.6. ALK2 does not bind the *Drosophila* bone morphogenetic proteins (BMPs), Dpp or Gbb. A,B. Gbb co-immunoprecipitates with its high-affinity receptor Sax but not ALK2. Dpp-HA co-immunoprecipitates with its high-affinity receptor Tkv but not ALK2. **C.** Diagram of the full-length ALK2 (LBD, ligand binding domain; TM, transmembrane domain; GS, glycine-serine rich domain/box) and ligand-binding domain deletion mutant ALK2$^{\Delta LBD}$ with amino acids from Cys35 to Cys99 removed by site-directed mutagenesis. **D.** ALK2$^{\Delta LBD}$ can inhibit Gbb-induced signaling in S2 cells. Data represent mean +/- standard deviation (n = 3). ns indicates not significant at $P < 0.05$ when comparing *brkS-lacZ* + ALK2^{WT} + 50 ng gbb vs. *brkS-lacZ* + ALK2$^{\Delta LBD}$ + 50 ng gbb ($P > 0.3$).

DISCUSSION

The BMP signaling pathway exhibits a high degree of conservation across metazoans (Newfeld et al., 1999). Consistent with this, we found that when the mutant form of the human ALK2 type I receptor ($ALK2^{R206H}$), which is associated with the vast majority of fibrodysplasia ossificans progressiva (FOP) cases, (Shore et al., 2006; Billings et al., 2008) is expressed in *Drosophila,* it mimics the misregulation of BMP signaling previously described in vertebrate systems (Billings et al., 2008; Fukuda et al., 2009; Shen et al., 2009; Song et al., 2010; van Dinther et al., 2010). Our results provide clear evidence that *Drosophila* BMP signaling components are compatible with the human ALK2 type I receptor, such that the classic FOP mutation $ALK2^{R206H}$ manifests as hyperactive BMP signaling in *Drosophila* tissues as well. This finding bodes well for the use of the *Drosophila* system as a future tool to elucidate the molecular details responsible for misregulated BMP signaling associated with FOP despite the obvious differences in the ultimate consequence of this hyperactive signaling in *Drosophila* compared to heterotopic bone formation in mammals.

Hyperactive BMP signaling requires type II receptor function

Consistent with experiments conducted in mammalian cells, we found that in *Drosophila* $ALK2^{R206H}$ is able to induce high levels of phosphorylated Mad in the absence of ligand binding. Importantly, we found that the signaling hyperactivity of $ALK2^{R206H}$ requires the function of a type II receptor kinase, which is responsible for activating type I receptors at their GS domain (**Fig. 4.7A**). Accordingly, the ability of $ALK2^{R206H}$ to induce high levels of pMad is abrogated when the activation domain (GS domain) of $ALK2^{R206H}$ receptor is mutated. While it remains possible in the various *Drosophila* assay systems we have tested that the endogenous *Drosophila* type I receptors Sax or Tkv are,

instead, responsible for Mad phosphorylation in response to ALK2^{R206H} expression, this seems unlikely considering that the level of these receptors should be far lower than that of overexpressed ALK2^{R206H}. Additionally, the requirement for an intact GS domain in ALK2^{R206H} to induce high levels of pMad makes this possibility less likely.

The dependency of hyperactive signaling by ALK2^{R206H} on the type II receptor had not previously been appreciated in studies of FOP, and the fact that ALK2^{R206H} can signal independently of ligand but requires type II receptor function indicates that ALK2^{R206H} must be able to interact with type II receptors independently of ligand. While interactions between type I and type II receptors are largely thought to be induced by complex formation with secreted ligands (Groppe et al., 2008; Nickel et al., 2009), ligand-independent interactions have been reported (Haerry, 2010). Furthermore, BMP type I and type II receptors can interact independently of ligand to generate preformed complexes that then bind ligand to initiate signal transduction (Nohe et al., 2002; Hassel et al., 2003; Ehrlich et al., 2011; Marom et al., 2011). Taken together, we envision a model in which the classic FOP mutation exposes the serine/threonine residues in the GS domain to phosphorylation by the type II receptor, thus circumventing the requirement for ligand to activate signaling (**Fig. 4.7A**).

Interestingly, our results show that the constitutively active BMP type I receptor Tkv^{QD} also shows a dependency for type II receptor function (**Fig. 4.2G**). This result contrasts what was previously observed for the constitutively active TβR1^{T204D}, which signals in the absence of the TGF-β type II receptor, TβR-II (Wieser et al., 1995; Chen et al., 1997). Although it has not yet been definitively shown that TβR1^{T204D} signals independently of BMP or Activin type II receptors, these apparently conflicting data could reflect a fundamental difference in the requirement for type II receptors between BMP and TGF-β signaling. Other key distinctions between TGF-β and BMP receptor signaling have been previously noted. For example, structural studies of BMP receptor

complexes have shown that the extracellular domains of the BMP type I and II receptors do not contact one another, whereas in TGF-β receptor complexes an N-terminal extension in the extracellular domain of TGF-β type II receptors directly interacts with the TGF-β type I receptor (Kirsch et al., 2000; Allendorph et al., 2006; Groppe et al., 2008). In addition, the minimal receptor complex that is sufficient for BMP versus TGF-β signaling appears to differ such that a heterotrimeric (type I:type II:type II) BMP receptor complex is can transduce BMP signals (Isaacs et al., 2010), whereas autonomously functioning TβRI:TβRII (type I:type II) heterodimers have been shown to transduce TGF-β signals (Huang et al., 2011).

In addition to divergent type II receptor requirements, mutations that confer hyperactivity or constitutive activity to TGF-β/BMP type I receptors differ in their respective effect on binding of the intracellular inhibitor FKBP12 to the type I receptor. FKBP12 has been proposed to prevent "leaky" ligand-independent signaling by masking the GS domain in the absence of ligand binding. (Chen et al., 1997; Huse et al., 1999; Huse et al., 2001; Wang and Donahoe, 2004). In a number of experiments, it has been shown that the R206H mutation reduces binding of FKBP12, making this an attractive molecular explanation for the hyperactivity displayed by $ALK2^{R206H}$ (Groppe et al., 2007; Shen et al., 2009; Song et al., 2010; Groppe et al., 2011). In contrast, the constitutively active Q207D mutation in ALK2 does not disrupt binding of FKBP12, whereas the equivalent mutation in TβR1(T204D) does (Chen et al., 1997). In the case of the *Drosophila* FKBP12 orthologue FKBP2, our preliminary studies indicate that loss of FKBP2 function *in vivo* did not produce phenotypes consistent with a substantial increase in BMP signaling (**Appendix 6.12,** S. Ballard, data not shown). Taken together, there does not appear to be a clear correlation between a loss or disruption of FKBP12 binding and the hyperactivity of mutant type I receptors. While we do not yet understand the mechanisms underlying the differential association of FKBP12 with

ALK2^{R026H} versus ALK2^{Q207D}, such differences raise the possibility that *in vivo,* the constitutively active ALK2^{Q207D} receptor behaves differently from the ALK2^{R206H} FOP mutant receptor.

Exploiting the type II receptor requirement for therapeutic strategies

The finding that ALK2^{R206H} hyperactive signaling depends on type II receptor function provides a new angle in the search for FOP therapeutics. Current strategies for drug development have focused on identifying small molecule inhibitors of type I receptor kinase activity (Yu et al., 2008a; Yu et al., 2008b; Hao et al., 2010). One such inhibitor, dorsomorphin, has been shown to effectively inhibit ALK2^{R206H} kinase activity (Yu et al., 2008a; Fukuda et al., 2009; Shen et al., 2009; van Dinther et al., 2010) but, unfortunately, dorsomorphin also non-specifically inhibits the kinase activity of other BMP type I receptors and appears to exhibit "off-target" effects on VEGF signaling (Yu et al., 2008b; Hao et al., 2010).

In addition to future efforts to improve the selectivity of dorsomorphin analogs (Hao et al., 2010), alternative approaches that disrupt FOP-induced signaling are needed. An exciting new prospect for drug development could exploit our recently identified type II receptor requirement for ALK2^{R206H} hyperactivity by focusing on the identification of molecules or factors that specifically block the interaction between ALK2^{R206H} and type II receptors in FOP cells. One intriguing possibility would be to take a biologics-based approach. For instance, de novo inhibitory BMP ligands could be designed that bind to the ALK2 ectodomain with high affinity while exhibiting no affinity for type II receptors. This inhibitory ligand would therefore act as a steric block against ALK2^{R206H} interactions with type II receptors. Interestingly, mutations in the type II receptor binding epitope ("knuckle, **Fig. 1.7 A**) of the BMP ligand have been used in a previous study to disrupt binding (Isaacs et al., 2010). Furthermore, an inhibitory BMP

ligand, BMP3, has been identified, however BMP3 appears to mediate its inhibition through interactions with the type II receptor ACVR2B (Gamer et al., 2005; Kokabu et al., 2012)

Wild-type ALK2 receptor can inhibit BMP signaling

In addition to our studies of ALK2^{R206H} in the *Drosophila* system, we also analyzed the ability of wild-type ALK2 to mediate signaling. Since FOP is a dominant autosomal disease and all mutations isolated thus far are protein-coding point mutations, the FOP mutant receptors must always be expressed in the presence of wild-type ALK2 receptor. In order to understand the mechanistic underpinnings of FOP it is therefore critical that we have a full understanding of wild-type ALK2 receptor function, in addition to elucidating the consequences of the R206H mutation. Thus, we investigated the possibility that ALK2 can both promote and antagonize signaling, a behavior exhibited by the *Drosophila* ALK2 orthologue, Sax (Bangi and Wharton, 2006b). Our results revealed that wild-type ALK2 receptor is, indeed, able to inhibit BMP signaling *in vivo* as well as in *Drosophila* cell culture. Interestingly, we found that the mechanism by which ALK2 accomplishes signaling inhibition likely differs from that employed by Sax. Whereas Sax likely inhibits signaling by incorporating its high-affinity ligand Gbb into inactive complexes, our results indicate that ALK2 appears to inhibit signaling induced by ligands that ALK2 itself does not actually bind (i.e. Gbb, Dpp, and BMP4). Furthermore, ALK2 has been observed to inhibit signaling induced by BMP6 (van Dinther et al., 2010), a ligand that ALK2 has not been reported to bind. Based on these observations, we propose that ALK2 inhibits signaling by interacting with a type II receptor, such as Punt, and prevents binding of ligands, such as Dpp, Gbb, BMP4, or BMP6, to the ALK2/Punt complex (**Fig. 4.7B, left**). A similar mechanism has been proposed to explain the negative effect of ALK2/ACVR1 on signaling induced by Activin,

which acts through a different set of core signaling components (Renlund et al., 2007). Conversely, in the case of BMP7, a ligand that has been shown to bind ALK2 (ten Dijke et al., 1994; Greenwald et al., 2003), the presence of ALK2 in complex with the type II receptor would facilitate BMP7 binding and enhance BMP7-induced signaling (**Fig. 4.7A**).

In our model, we propose that ALK2 acts as a modifier of receptor complex activity by dictating which BMP ligand can or cannot bind the complex of ALK2 and type II receptor. Through this function of imparting ligand specificity, ALK2 ultimately determines whether the receptor complex that a type II receptor has participated in will be active or inactive, depending on which BMP ligand is present (**Fig. 4.7B, left**). Moreover, in the absence of BMP7 and under conditions where ALK2 becomes enriched over other type I receptors at the cell membrane, BMP signaling is suppressed as ALK2 begins to titrate type II receptors (**Fig. 4.7B**, right). Therefore, the ability of ALK2 to regulate signaling based on BMP ligand type may have a profound impact on ligand-specific responses and warrants further investigation to determine if this dual behavior of ALK2 is observed in tissues normally expressing ALK2.

On a separate note, the inability of ALK2 to bind Gbb was unexpected based on the demonstrated ability of ALK2 to bind BMP7 and the evolutionary relatedness of Gbb to the BMP5/6/7 subgroup. While the conserved domains of BMP5, BMP6 and BMP7 can reportedly rescue *gbb* mutant phenotypes (Fritsch et al., 2010), our results suggest that it is unlikely that Gbb can fully substitute for BMP7 function in vertebrates, specifically for BMP7-induced signaling mediated by ALK2.

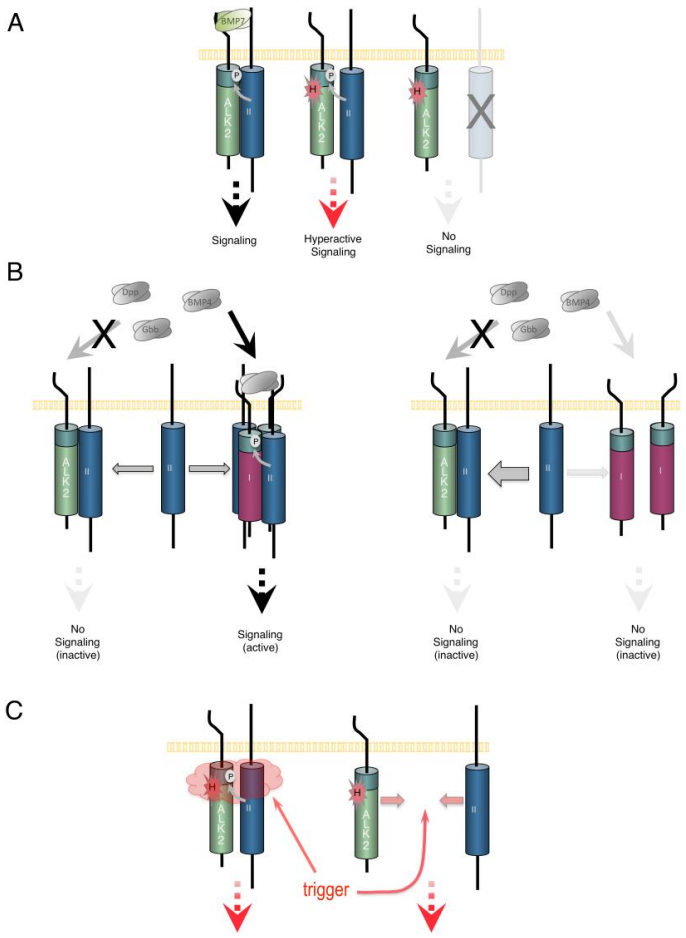


Figure 4.7. Models for ALK2^{R206H} hyperactivity and ALK2 inhibition of BMP signaling. A. (left) When bound by BMP7, the GS domain (green domain adjacent to membrane) of ALK2 is phosphorylated (P in white circle) by a type II receptor (blue receptor labeled "II") leading to BMP signal transduction. (Middle) The classic R206H FOP mutation in ALK2 (H in red starburst) circumvents the ligand requirement for signaling by increasing the accessibility of ALK2's GS domain to the type II receptor resulting in hyperactive signaling. (right) In the absence of a functional type II receptor, ALK2^{R206H} is not activated and unable to signal. **B.** (left) By its virtue of imparting BMP7-binding specificity to the receptor complex, ALK2 is unable to mediate signaling by BMP4, Gbb, or Dpp. These ligands, however, can signal through other type I receptors (purple receptor labeled "I"). (right) In the absence of BMP7 and under conditions when the type I receptor population at the cell surface is enriched for ALK2 (as is the case during experimental overexpression of ALK2), BMP signaling in general is suppressed as a result of titrating type II receptors away from productive signaling complexes into inactive complexes with ALK2. **C.** Various events (trigger) that may act to allow hyperactive signaling of ALK2^{R206H} could further increase GS domain accessibility by disrupting the interaction of a putative inhibitor, or could facilitate the interaction of ALK2^{R206H} with available type II receptors.

Impact of *Drosophila* models for the study of FOP

Perhaps the least well understood aspect of FOP, and most difficult for patients, is the sporadic and progressive nature of the disease. One of the primary difficulties still facing the FOP field is reconciling the molecular events of hyperactive signaling induced by the FOP receptor in animal models with the clinical features that manifest in patients. The sporadic nature of the disease contrasts with the hyperactivity that the mutant receptor displays in experimental assays, suggesting that under endogenous conditions the activity of the mutant receptor must be regulated or muted until some event triggers a flare-up.

To date, all FOP patients are heterozygous for mutations in ALK2 regardless of whether they harbor the classic R206H or an atypical mutation. It is possible that one copy of $ALK2^{WT}$ can compete with $ALK2^{FOP}$ receptors for type II availability, thereby keeping the final output of BMP signaling below a threshold required for bone formation. Thus, in an endogenous context the relative ratio between FOP type I receptors, wild-type type I receptors, and type II receptors could be the determining factor in whether or not activation of the pathway reaches a threshold necessary for bone deposition. As such it is possible that when at physiological levels the FOP mutant receptor activity might be inhibited by a different factor in *trans* and it is only when the mutant receptor is overexpressed under experimental conditions that it escapes this negative regulation. Thus, in the future it will be important to study the behavior of the FOP mutant receptors when expressed at physiologically relevant levels, achievable through homologous recombination. Making use of the *Drosophila* model system to express both mutant and wild-type receptors at endogenous levels will enable *in vivo* mutagenic screens to identify factors that suppress or enhance the effects of the $ALK2^{R206H}$ activity and, in turn, provide us with new targets for therapy and treatment. Furthermore, given the correlation between ossification and trauma, it has been

suggested that inflammation associated with injury may in some way trigger heterotopic ossification. Such a triggering event could act to increase the accessibility of the GS domain to the kinase activity of the type II receptor by disrupting the binding of a putative inhibitor or by influencing the ability of the ALK2^{R206H} receptor to interact with available type II receptors (**Fig. 4.7C**). Although the precise mechanism(s) by which such putative modulators may influence the behavior of ALK2^{R206H} remains unknown, the *Drosophila* system is a particularly attractive model organism in which to undertake such studies given the high conservation of pathways governing cellular physiology.

In closing, our work has demonstrated the value of using a *Drosophila* genetic system to study the molecular foundation of altered BMP signaling characteristic of individuals with FOP. Our experiments reveal a requirement for type II receptor function in the hyperactivity displayed by the ALK2^{R206H} mutant receptor, a fact previously unappreciated. Although the majority of FOP patients are characterized by the classic R206H mutation, a small but growing list of variant mutations in other domains of the receptor are being identified (Billings et al., 2008; Furuya et al., 2008; Bocciardi et al., 2009; Petrie et al., 2009; Ohte et al., 2011). Whether the activity of these variant mutant receptors also requires type II receptor function will be the subject of future studies. Lastly, we have also observed the ability of wild-type ALK2 to inhibit BMP signaling in a ligand-specific manner. How these findings contribute to the sporadic nature of FOP, as well as impact our broad understanding of other diseases associated with the misreglation of type I receptor activity, warrants further investigation. We intend to exploit the comprehensive genetic tools in the *Drosophila* system to screen for potential modifiers of FOP mutant receptor activity as a means to bridge this gap.

CHAPTER 5

Conclusion and future directions

The *Drosophila* BMP type I receptor Saxophone has been shown to have both a facilitating and an antagonistic behavior during wing development. Its role in mediating BMP signals in the wing disc is evidenced by genetic loss-of-function studies. Mitotic clones lacking *sax* function exhibit reduced levels of pMad and a decrease in the expression of the BMP target gene *sal* (Bangi and Wharton, 2006b; Singer et al., 1997). However, Sax can also inhibit BMP signaling induced by the BMP ligand Gbb. Overexpression of *gbb* in the developing wing results in wing phenotypes consistent with activation of BMP signaling (Bangi and Wharton, 2006b; Haerry et al., 1998; Khalsa et al., 1998). Coexpression of *sax* with *gbb* suppressed these phenotypes, indicating that Sax was acting in opposition to Gbb (Bangi and Wharton, 2006b). Furthermore, these phenotypes were exacerbated when *gbb* was over expressed in a sax^{4} null heterozygous background, strongly suggesting that inhibiting Gbb-induced signaling is an endogenous behavior of Sax (Bangi and Wharton, 2006b).

The dual behavior of Sax can be explained by a model in which Sax's function is determined by its type I receptor partner in the signaling complex (**Fig. 1.3**). Sax can mediate signals as part of a complex with Tkv, but Sax cannot transduce signals when in a complex with another molecule of Sax. This model does not, however, address whether Sax phosphorylates Mad in a Sax:Tkv complex or how Sax is prevented from signaling in a Sax:Sax complex. One possible explanation for Sax's dual behavior is that Sax is a type I receptor that lacks kinase activity. This scenario would satisfy the condition that Sax:Sax complexes do not transduce BMP signals. To comply with the condition that Sax mediates signals in a Sax:Tkv complex, a "dead-kinase" Sax could serve as a silent co-receptor that helps Tkv bind BMP ligand. In this case, Tkv would be the only active kinase in the complex.

Regulation of Sax kinase activity

Our results indicate that Sax is not a dead-kinase and that its activity can be revealed by mutations in the GS domain. In addition, Sax kinase activity can be stimulated by either type II receptor. Furthermore, cooperative signaling between the type II receptors and Sax could be enhanced by the GS domain mutations. Taken together, these results highlight the importance of the GS domain in maintaining Sax in an inactive state.

The analysis of Sax-Tkv chimeric receptors revealed that sequence differences in the E6 loop and the region encompassing the juxtamembrane and GS domain (J-GSD) accounts for the antagonistic behavior of Sax. Importantly, we found that chimeric receptors required the pairing of the Tkv J-GSD with the Tkv E6 loop to display full Tkv-like signaling activity. Similarly, full inhibitory behavior required paring of the Sax J-GSD with another element in the Sax kinase domain. Whether or not that element is the E6 loop remains to be tested, as it was not examined in this study. Moreover, further refinement of the J-GSD will be required to identify the specific differences that account for the different behaviors of Sax and Tkv.

Both the GS domain and the E6 loop are involved in the activation of the type I receptor kinase. As mentioned before, the GS domain is phosphorylated by the type II receptor which, in turn, activates the type I receptor kinase. How the E6 loop participates in the activation process is unknown, but mutations in the E6 loop have been shown to negatively impact phosphorylation of ALK5 (TβRI) by the TGF-β type II receptor. Intriguingly, the E6 loop mutation did not abolish the interaction of TβRI with the type II receptor, indicating that the effect on transphosphorylation must occur downstream of type I-type II interaction. Structural studies of the TβRI receptor, however, implicate the E6 loop in type I receptor dimerization. Therefore it is possible that the changes in the E6 loop can affect the configuration of the type I receptor dimer

such that transphosphorylation of the GS domain is hindered. These results further underscore the importance of the GS domain in Sax regulation.

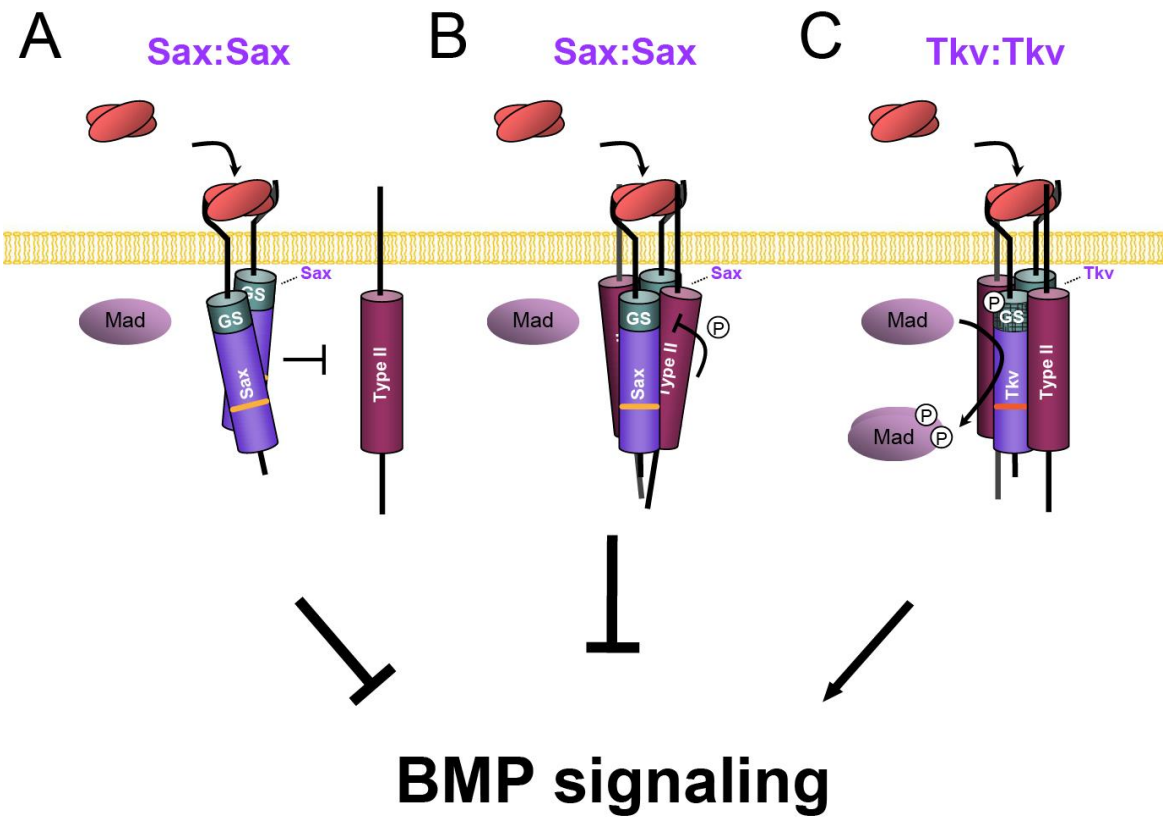


Figure 5.1. Model – Sax:Sax dimer negatively regulates Sax kinase activation. The GS domain and E6 loop (orange line) were identified as critical determinants of the antagonistic behavior of Sax. Given their roles in kinase activation and type I receptor dimerization we propose two models to explain how Sax kinase activity remains inactive in Sax:Sax complexes. **A.** Sax:Sax dimers adopt configurations that prevent interactions with type II receptors **B.** Sax:Sax dimers interact with type II receptors in such a way that the Sax GS domain is inaccessible to the type II receptor kinase and therefore cannot be phosphorylated. **C.** In contrast, Tkv:Tkv dimers can interact with type II receptors productively.

Another intriguing possibility to consider stems from recent data that suggests the minimal BMP signaling complex consists of a BMP dimer bound to two type II receptors and one type I receptor (**Fig. 5.2A**) (Isaacs et al., 2010). In this study, a BMP2-BMP6 heterodimer was produced and purified in which the binding epitope for the type I receptor in the BMP6 monomer was abolished by mutagenesis while leaving the remaining binding interfaces (type I on BMP6, type I and II on BMP2) intact (Isaacs et al., 2010). This mutant heterodimer was still capable of activating BMP signaling as measured by a Smad1-dependent *luc* reporter assay in C2C12 cells. The removal of additional receptor binding sites on either BMP2 or BMP6 rendered a heterodimer incapable of activating the Smad1-dependent *luc* reporter, suggesting that a minimum of two type II receptors and one type I receptor was required for signaling. Similar experiments involving TGF-β ligands in which type I and type II receptor binding sites were mutated suggest that autonomous TβRI:TβRII pairs can mediate TGF-β signaling (Huang et al., 2011). Therefore, the antagonistic behavior of Sax could also be explained by a model in which Sax:Sax dimers can only interact with one type II receptor thereby forming a complex that is not sufficient for signaling (**Fig. 5.2B**).

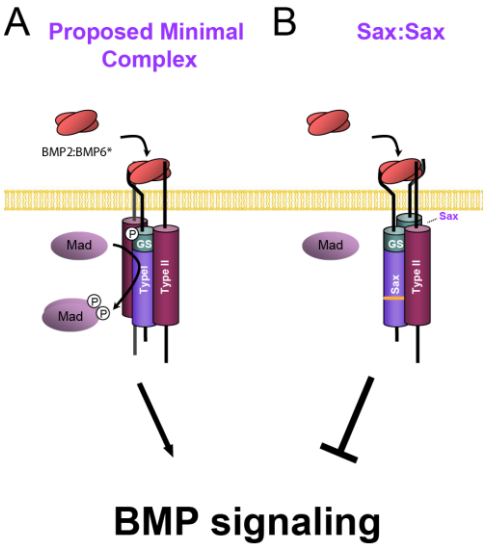


Figure 5.2. Model – Sax:Sax dimer forms an incomplete receptor signaling complex. A. It has been proposed that the the minimal signaling complex consists of one type I receptor and two type II receptors (Isaacs et al., 2010). **B.** A Sax:Sax dimer may only be able to interact with one type II receptor to form an incomplete receptor complex that is incompetent for signaling. This model is consistent with the observation that increasing type II receptor concentration can stimulate Sax kinase activity (**Fig. 2.10 & 2.11**) presumably by forcing the formation of complete receptor complexes.

Alternatives mechanism underlying the antagonistic function of Sax

Incomplete phosphorylation of Mad

Although our data strongly suggests that the inhibitory function of Sax is a result of additional regulation mediated through the Sax GS domain that keeps its kinase inactive, we cannot rule out the potential for other mechanisms that underlie the antagonistic behavior of Sax. For instance, it is possible that the Sax kinase in Sax:Sax complexes is activated, but the Sax kinase domain is only competent to phosphorylate one of the two serines in the C-terminus of Mad. Since Smad-mediated BMP signaling requires phosphorylation of both serines, Sax could inhibit BMP signaling by titrating BMP ligands into complexes that incompletely phosphorylate Mad. In Sax:Tkv complexes, however, Sax would facilitate BMP signaling by cooperating with Tkv to phosphorylate Mad. It is conceivable that Sax-mediated phosphorylation of one site and Tkv-mediated phosphorylation the other site would increase the rate of Mad phosphorylation. Single and double mutations of the C-terminal serines in Mad have been generated to test this hypothesis (**Appendix 6.13 & 6.14**).

Sax kinase activity is regulated by a factor in trans

Another possibility is that a specific interaction with a factor in *trans* keeps the Sax kinase inactive. An obvious candidate would be FKBP2, the *Drosophila* homolog of FKBP1A, which has been proposed to inhibit type I receptors of the TGF-β superfamily (Wang et al., 1996). FKBP1A binds to the GS domain of type I receptors and is thought to stabilize the autoinhibitory conformation of the type I receptor and also shield the GS domain from type II receptor transphosphorylation (Chen et al., 1997; Huse et al., 2001; Wang and Donahoe, 2004). Consequently, ligand binding triggers the dissociation of

FKBP1A to allow activation of the type I receptor (Chen et al., 1997; Huse et al., 1999; Wang and Donahoe, 2004). Furthermore, GS domain mutations have been predicted to disrupt FKBP1A binding (Groppe et al., 2007). Our preliminary experiments indicate that FKBP2 can inhibit BMP signaling and interact with Sax and Sax^{K262H} (**Fig. 6.12""**), and thus warrants further analysis. It is possible that FKBP2 remains bound to wild-type Sax even in response to Gbb ligand thereby keeping its kinase inactive. In contrast, we expect that binding of Gbb would cause the dissociation of FKBP1A from Sax^{K262H} given that Sax^{K262H} can transduce Gbb signals. It will important to test how ligand binding affects the interaction of FKBP2 with Sax versus Sax^{K262H}.

Posttranslational regulation of type I receptor activity

Type I receptor activity is subject to post-translational modifications. As previously mentioned, transphosphorylation of the type I receptor GS domain is required for kinase activation. Therefore, the signaling activity of type I receptors may be attenuated by phosphatases acting on the GS domain. In *Drosophila,* it has been proposed that the phosphatase PP1c is brought to the type I receptor by SARA (Smad anchor for receptor activation), a protein normally involved in recruiting R-Smads to the type I receptor (Bennett and Alphey, 2002). In *Xenopus* the phosphatase Dullard can regulate the phosphorylation state of ALK3 in addition to its role in promoting proteosomal degradation of BMP type II receptors (Satow et al., 2006). Whether these phosphatases target the GS domain or other regions of the type I receptor remains unknown.

Type I receptors are also targeted for proteosomal degradation by ubiquitination. In *Xenopus* embryos, the E3 ubiquitin ligase Smad ubiquitin regulatory factor 1 (Smurf1) ubiquitinates BMP type I receptors (Murakami et al., 2003). This interaction between Smurf1 and BMP type I receptors is mediated by Smad6, an inhibitory Smad that also

negatively regulates BMP signaling by competing for type I receptor binding and preventing R-Smad phosphorylation. In the germline stem cell niche of the *Drosophila* ovary, Smurf is targeted to the *Drosophila* type I receptor Tkv as part of a complex with Fused (Fu), a serine/threonine kinase that regulates Hedgehog signaling (Xia et al., 2010). Little is known, however, regarding the posttranslational modification of Sax. It is possible that the inability of Sax to transduce BMP signals may reflect either rapid turnover of Sax:Sax complexes or dephosphorylation of the Sax GS domain.

Sax as a regulator of BMP signaling in the larval wing disc

In *Drosophila*, both secreted factors and cell surface proteins modulate BMP signaling extracellularly (**Fig. 1.2**). These regulators generally act upon BMP ligands and can either prevent or facilitate their binding to BMP receptors. Furthermore, extracellular modulators can also serve to mediate the transport of BMP ligands across a field of cells as well as regulate the availability of BMP ligands to participate in signaling. For example, during embryogenesis the extracellular modulators Sog and Tsg bind to BMP ligands to inhibit signaling in the ventral half of the embryo while simultaneously facilitating their transport to the dorsal midline (reviewed in O'Connor et al., 2006). Here, proteolytic cleavage of Sog and Tsg mediated by Tolloid releases BMP ligands, which in turn induces signaling at the dorsal midline. The secreted factors CV and CV-2 along with Sog function similarly during pupal wing development to help form the crossveins (**Fig. 1.2 & 1.9A**; reviewed in Blair, 2007 & O'Connor et al., 2006; Serpe et al., 2008).

During larval wing development, however, these secreted, extracellular factors do not appear to modulate BMP signaling. Instead, cell surface proteins such as Dally, Dally-like, Ltl, and Pent cooperate to modulate ligand distribution as well as facilitate ligand binding to the BMP receptors (reviewed in Raftery and Umulis, 2012). Given its

dual behavior, Sax may also contribute to the regulation of ligand distribution and availability. For instance, Sax:Sax complexes may function as a ligand sink that sequesters BMPs away from signaling competent complexes. In this way, Sax could help maintain proper BMP signaling activity in the larval wing disc by providing a buffering mechanism that deals with fluctuations or increases in BMP ligand concentration. Thus, the gradient of BMP signaling activity may reflect not only inputs from signaling competent complexes, but also the balance of regulatory inputs including the antagonistic function of Sax (**Fig. 1.2, 1.10B, & 5.1B**)

Inhibitory function of Sax outside of wing development

Thus far, the inhibitory behavior of Sax has only been observed during wing development. The identification of Sax GS mutants that transduce rather than inhibit BMP signals may, however, provide a useful tool to probe for the requirement of Sax's antagonistic function in other tissues. I have generated rescue constructs derived from genomic sequences of the *sax* locus in which the *sax* gene has been replaced with *lacZ*, sax^{WT}, or sax^{K262H} cDNA (**Appendix 6.3**). Using the genomic sequences of sax should place *cDNA* expression under endogenous *sax* regulation. In preliminary experiments, I observed that the sax^{WT}rescue construct dramatically rescues embryonic lethality associated with sax^{2} mutations, whereas the sax^{K262H} rescue construct exhibits only weak rescue. Although these results do not necessarily indicate that the antagonistic behavior of Sax is specifically required, it could indicate that the balance between the antagonistic and facilitating roles of Sax are important since Sax^{K262H}, a receptor that can only transduce signals, cannot function in place of wild-type Sax in during embryonic development.

Effect of Tkv and Gbb on Sax kinase activity

The question remains however, whether Sax within a Sax:Tkv complex can transduce BMP signals by phosphorylating Mad. Is the Sax kinase activated in the presence of Tkv? Unfortunately, we have not been able to adequately address whether Sax signals in a Sax:Tkv complex. The complication in answering this question extends from the lack of knowledge regarding the formation of Sax:Tkv complexes. Given the respective affinities of Sax and Tkv for Gbb and Scw versus Dpp, the most obvious scenario would be that Gbb:Dpp or Scw:Dpp heterodimers mediate the formation of Sax:Tkv complexes. Furthermore, in cell culture experiments both Gbb:Dpp and Scw:Dpp heterodimers require the presence of both Sax and Tkv to induce Mad phosphorylation, consistent with the ligand heterodimers signaling through Sax:Tkv complexes(Shimmi et al., 2005a; Shimmi et al., 2005b). However, direct evidence of a heteromeric receptor complex bound to a BMP heterodimer has not been observed in *Drosophila* like it has in zebrafish where ALK2:ALk8 complexes are bound by BMP2b:BMP7 heterodimers (Little and Mullins, 2009).

We can, however, exploit the STT chimeras generated in this study to address whether the Tkv intracellular domain affects Sax kinase activity. Since Sax and the STT chimera share the Sax extracellular domain, Gbb can be used to mediate Sax:STT interactions. This experiment (as outlined in the Chapter 3 discussion and **Appendix 6.10**) would require using kinase dead versions of STT (STT^{KD}) to remove any signaling contribution from the STT receptor. Therefore, any Gbb-induced signaling observed in cells expressing Sax and STT^{KD} would have to be mediated by the Sax receptor.

Extending our model, it is possible that the particular configuration of Sax:Tkv complexes either allows type II receptors to bind productively or grants the type II receptor access to the Sax GS domain. Future studies should employ coimmunopreciptation to determine whether Sax interacts with type II receptors and if

the presence of Tkv can affect this interaction. Additionally, a quantitative mass spectrometry approach could be taken to determine if the extent GS domain phosphorylation differs between Sax and Tkv.

In the course of this study, we also observed that Gbb was unable to enhance the cooperative signaling observed between type II receptors and Sax (**Fig. 2.11**). This result was unexpected given that Gbb is the preferred ligand for Sax (**Fig. 4.6 A**). Furthermore, we observed that Wit enhanced Gbb signaling in our *brkSE-lacZ* assays, implying that Gbb can bind and signal through Wit (**Fig. 2.11**). These data raises questions as to whether Gbb can interact with or mediate the formation of Sax:Wit complexes despite being able to interact with either receptor independently. To address whether Gbb can interact with Sax and Wit simultaneously we can sequentially immunopurify Sax and Wit to enrich for Sax:Wit complexes and probe for the coimmunoprecipitation of Gbb.

BMP type II receptor activity in fibrodysplasia ossificans progressiva (FOP): function or scaffold?

The importance of the type II receptor in signal transduction was also underscored by our *Drosophila* model for FOP. Our data indicate that hyperactive signaling from the ALK2^{R206H} receptor requires type II receptor activity to phosphorylate its GS domain. Furthermore, given that ALK2^{R206H} activity is ligand-independent, these results indicate that ALK2^{R206H} can interact with type II receptors in the absence of ligand binding. This observation is consistent with data that point to the existence of preformed receptor complexes at the cell surface (Gilboa et al., 2000; Marom et al., 2011; Nohe et al., 2002).

A recent study in a mouse model for FOP has also reported the requirement of a type II receptor in ALK2^{R206H} signaling (Bagarova et al., 2013). However, their results

indicate that the type II receptor activity was dispensable for ALK2^{R206H} signaling and suggests that ALK2^{R206H} requires the presence of type II receptors possibly for scaffolding purposes to form a ligand-independent active signaling complex.

These results, in contrast to ours, may represent a difference in what "constitutively active" BMP type I receptors require to transduce signals in mice versus *Drosophila*. Alternatively, it may reflect the difference in how we assayed for the requirement of type II receptor activity. In the study by Bagarova et al., 2013, a kinase-dead type II receptor was sufficient to cooperate with ALK2^{R206H} and activate BMP signaling. In our study, we mutated the ALK2^{R206H} GS domain serines, which are the targets of type II receptor transphosphorylation, and observed that constitutive signaling activity was abolished and inferred that type II receptor activity was required. It is possible that the GS domain serine mutations affected ALK2^{R206H} signaling downstream of type II receptor transphosphorylation. Given the observation that the phosphorylated GS domain in TβRI also acts as a secondary binding site for Smad2 (Huse et al., 2001), the GS domain serine mutations may have disrupted Mad (Smad) binding.

To investigate the discrepancy between our results and those reported by Bagarova et al., we can test the requirement for type II receptor activity more rigorously by assaying the ability of a kinase-dead type II receptor to restore ALK2^{R206H} signaling activity in the absence of endogenous type II receptors. This can be performed in the wing disc where it is possible to remove type II receptor activity by *RNAi* knockdown while simultaneously coexpressing ALK2^{R206H} and a kinase-dead variant of Punt. Furthemore, the interaction between Mad and ALK2$^{R206H\ GS1}$ and ALK2$^{R206H\ GS2}$ should be tested by coimmunoprecipitation to determine if mutating the serines in the GS domain disrupts Mad binding.

The suggestion that ALK2^{R206H} requires the type II receptor for scaffolding purposes brings up an intriguing question: Is there a minimal signaling complex

required for "constitutively active" type I receptors? As mentioned previously, recent data suggests that a minimal signaling complex for the BMP pathway consists of two type II receptors and one type I receptor. Is this minimal ratio the same for constitutively active receptors or can signaling be initiated by just a heterodimer consisting of a constitutively active type I receptor paired with a type II receptor (**Fig. 5.3A**)? Additionally, can constitutively active type I receptors participate in a fully mature, quadripartite complex (**Fig. 5.3B**)? In which case, could the signaling activity of ALK2^{R206H} potentially be influenced by the presence and identity of the other type I receptor? For instance, as a result of the R206H mutation the GS domain of ALK2^{R206H} may be rendered a target of type I phosphorylation.

It is not immediately clear why ALK2^{R206H} would need a type II receptor for scaffolding purposes. As proposed by Bagarova et al., interaction with the type II receptor might be required for proper positioning of the Smad substrate for phosphorylation (Bagarova et al., 2013). It is also possible that the type II receptor acts as a scaffold for a factor that may be required for full activation of the pathway functioning downstream of ALK2^{R206H} (**Fig. 5.3C**). This is bolstered by the fact that the C-terminal tail of BMP type II receptors may represent a hot-spot for mediating interactions with other proteins such as the tyrosine kinase c-Src, LIM kinase 1, and Trb3 (Chan et al., 2007; Foletta et al., 2003; Hassel et al., 2004; Lee-Hoeflich et al., 2004; Wong et al., 2005). Alternatively, interaction with the type II receptor may affect ALK2^{R206H}'s membrane localization and endocytosis, both of which can influence BMP signaling (Hartung et al., 2006; Jiang et al., 2011)

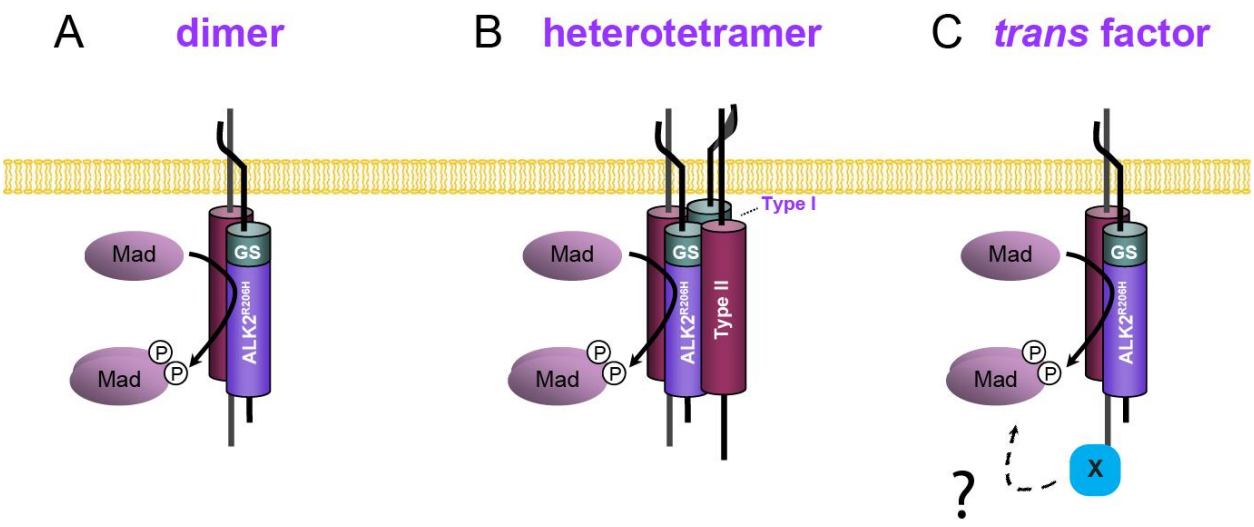


Figure 5.3. Model – The nature of type II receptor requirement for ALK2^{R206H} signaling activity. ALK2^{R206H} signaling activity requires the type II receptor but the nature of this requirement is unclear. The work presented in this thesis supports a model in which type II receptor activity is required, whereas the work from (Bagarova et al., 2013) also points to a requirement for the type II receptor but argues that type II receptor kinase activity is dispensable. However, the requirement for a type II receptor does imply that ALK2^{R206H} requires complex formation to signal. It is unknown whether a dimer consisting of ALK2^{R206H} and a type II receptor is sufficient for signaling (**A.**) or if a multimeric complex is required (**B.**). Assuming a multimeric complex is required, could the other type I receptor in the complex affect the kinase activity of ALK2^{R206H}? Furthermore, BMP type II receptors are known to interact with a host of other factors that can modulate BMP signaling as well as other pathways. It is also possible that the type II receptor mediates the interaction between ALK2^{R206H} and an unidentified factor in *trans* that is required for signaling (**C.**).

Evolutionary conservation of a dual functioning type I receptor

While this dual behavior of Sax plays a critical role in Drosophila development, the question still remains whether this behavior of Sax an evolutionary quirk specific to *Drosophila melanogaster*. Given the high degree of sequence conservation among the *Drosophila* species that make up the *melanogaster* group, it is very likely that Sax functions as a dual behavior type I receptor in this group. It is also possible that the dual behavior of Sax is conserved in the non-*melanogaster* species as well. The E6 loop in the

six species that make up this group is characterized by a TE amino acid motif, whereas the *melanogaster* E6 loop is characterized by a KQ motif (**Fig. 3.8**). This difference in E6 loop sequence raised the possibility that the activities of Sax could be different between these two groups of *Drosophila* species. However, when we generated a Sax $^{K382T\ Q393E}$ double mutant to recapitulate the E6 loop of the non-*melanogaster* group, we demonstrated that this form of Sax also inhibited Gbb-induced signaling (**Fig. 3.7**). This result indicates that the non-*melanogaster* group E6 loop was not sufficient to confer facilitating function only to Sax. Therefore, it remains possible that the inhibitory activity of Sax is retained in all *Drosophila* species.

Although the Sax ortholog ALK1 has not yet been characterized, several pieces of data suggest that the other Sax ortholog, ALK2, behaves as a dual function BMP type I receptor. In COS cells, ALK2 was able to inhibit signaling induced by BMP6 (van Dinther et al., 2010), a ligand that has been shown to strongly bind ALK2 (Ebisawa et al., 1999). Furthermore, ALK2 was also shown to inhibit activin signaling by preventing activin from binding its type II receptor, ACVR2A (ActRIIA) (Renlund et al., 2007). In our cell culture assays, ALK2 inhibited Gbb-, Dpp-, and BMP4-induced BMP signaling (**Fig. 4.5 C & D**), most likely due to a mechanism downstream of ligand-binding since ALK2 did not bind Gbb or Dpp (**Fig. 4.6 A & B**). In contrast, ALK2 transduced signals initiated by BMP7 (**Fig. 4.5 E**), a ligand known to bind ALK2 (ten Dijke et al., 1994).

These results suggest that the dual behavior of Sax may not be an evolutionary "one-off" after all. However, further analysis is required to determine whether ALK2 exhibits dual behaviors *in vivo* and how it impacts developmental processes. Sax's dual behavior has been proposed to serve as a buffering mechanism in maintain the robustness of the pMad gradient in the wing disc. Could this buffering mechanism mediated by dual functioning type I receptors be a more generalized mechanism? Furthermore, how does the potential loss of an antagonistic function in ALK2^{R206H} affect

the disease progression of fibrodysplasia ossificans progressiva? These outstanding questions underscore the importance of further characterizing ALK2 to determine whether it, like its *Drosophila* counterpart Sax, is a dual functioning BMP type I receptor.

Chapter 6

Appendices

I performed all of the experiments presented in this chapter with the exception of Fig. 6.5 A-C, which were performed by Erdem Bangi, Ph.D.

PREFACE

The results presented in this chapter represent experiments performed to address issues that arose during the experiments described in the preceding chapters and includes preliminary data. Results are described along with the rationale for each experiment, materials and methods used, and any conclusions that could be drawn.

APPENDICES

Appendix 6.1 - Sax antagonizes whereas Sax^{K262H} enhances Gbb-induced BMP signaling in the posterior wing, respectively.

Rationale

The ability of Sax to antagonize and Sax^{K262H} to enhance Gbb-induced BMP signaling has been in observed during wing development and in S2 cells. In both cases, however, we could not ignore the potential impact of the other BMP ligands, namely Dpp. In the studies conducted in wing discs, the *ap-GAL4* and *A9-GAL4* drivers were used to direct expression of UAS transgenes in the dorsal compartment of the wing disc overlapping the *dpp* expression domain, which runs along the anterior side of the A/P boundary **(this study; E. Bangi 2006)** . Similarly, we know that S2 cells express *dpp* **(T. Akiyama, unpublished RT-PCR data; MODENCODE)**. Therefore, we could not exclude the possibility that endogenous Dpp was interacting with exogenous Gbb—*perhaps* through the formation of heterodimers—thereby altering the impact of *sax* or *sax^{K262H}* coexpression. To analyze the effect of Sax and Sax^{K262H} on Gbb-induced signaling independent of Dpp, we exploited the *hh-GAL4* driver, whose expression domain is in the posterior compartment where *dpp* is not expressed.

Materials and Methods

Stocks used in this experiment were: *w; UAS-GFP; hh-GAL4/T(2;3). yw;;UAS-gbb9.1. (y)w;UAS-sax-3xFLAG. (y)w; UAS-sax-3xFLAG; UAS-gbb9.1. (y)w; UAS-sax^{K262H}-3xFLAG; UAS-gbb9.1.* All crosses were done using *w; UAS-GFP; hh-GAL4/T(2;3)* virgins. Flies were raised at 25^{o}C. Adult wings were cut, washed in Ethanol, Acetone, Xylenes and mounted in DPX mountant. Wing discs were dissected from late

3rd instar larvae and immunohistochemistry was performed as previously described. Anti-PS3 (Epitomics) was used at 1:1000 dilution. Images were collected by confocal and pMad intensity was measured across the width of the wing pouch at the midpoint of the dorsal compartment. Average pMad intensity profiles were generaged from 5 wing discs for each genotype. The A/P border as defined by *hh-GAL>GFP* expression was used to align all the intensity profiles.

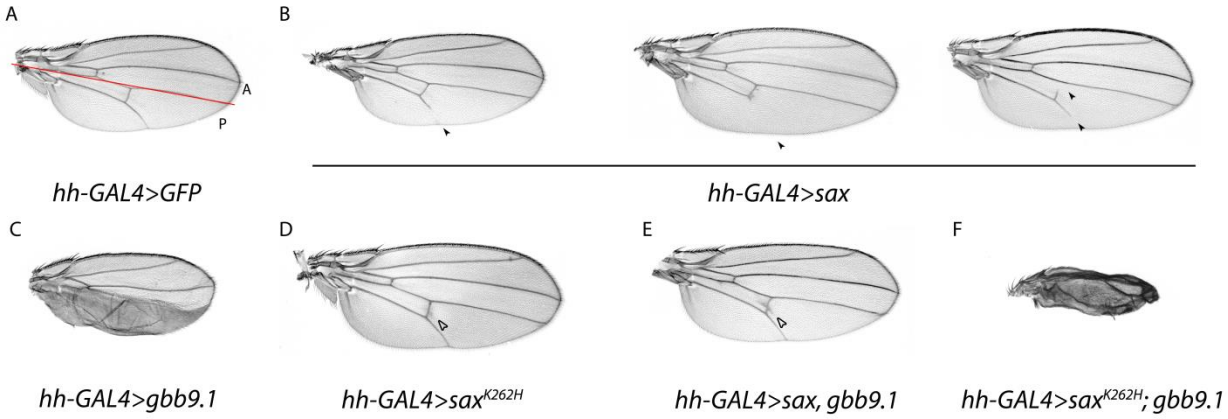


Figure 6.1. *sax* rescues, whereas *sax^{K262H}* enhances *hh-GAL4>gbb9.1* adult wing phenotypes. **A.** *hh-GAL4>GFP* control wing. Red line indicates the anterior (A)-posterior (P) boundary. *hh-GAL4* drives UAS transgene in the posterior compartment only. **B.** *hh-GAL4>sax* wings exhibiting a loss of vein tissue for longitudinal vein 5 (L5) or the posterior crossvein (PCV). **C.** *hh-GAL4>gbb9.1* wings exhibit moderate vein tissue patterning defects in the posterior compartment. **D & E.** *hh-GAL4>sax^{K262H}*and *hh-GAL4>sax; gbb9.1* adult wings are mostly wild-type except for PCV "spurs" (open arrowheads). **F.** Severe patterning defect is apparent in the entire wing of *hh-GAL4>sax^{K262H}; gbb9.1* adults.

Results and Conclusions

Overexpression of *sax* in the posterior compartment resulted in the partial loss of longitundal vein 5 (L5) or the partial loss of the posterior crossvein (PCV) (**Fig 6.1B**). Overexpression of *sax^{K262H}*, on the other hand, resulted in a mostly wild-type wing, albeit with extra PCV tissue referred to as "spurs" (**Figure 6.1 D**). Wings overexpressing

gbb9.1 displayed moderate patterning defects in the posterior compartment of the wing. This *gbb9.1* phenotype was rescued when *sax* was coexpressed, but enhanced with the coexpression of sax^{K262H} (**Fig. A6.1 E&F**)

Directly visualizing pMad in wing discs of the corresponding genotypes also yielded results that suggest *sax* inhibits *gbb*-induced signaling, whereas sax^{K262H} enhances *gbb*-induced signaling (**Fig. 6.2, top**). Posterior expression of *gbb9.1* (compare red line vs dark blue line) increased pMad in both the anterior (grey shaded area) and posterior (white shaded area) compartments. Coexpression of *sax* resulted in a decrease of *gbb*-induced BMP signaling only in the posterior compartment, whereas it slightly increased the width of the pMad gradient in the anterior compartment (light blue). In contrast, posterior coexpression of sax^{K262H} dramatically enhanced *gbb*-induced phosphorylation of Mad in both the anterior and posterior compartments (orange line).

Posterior overexpression of *sax* alone, however, increased pMad in both the anterior and posterior compartments(green line vs dark blue line in **Fig 6.2, bottom**). This is contrary to the reduced pMad levels observed when *sax* is overexpressed throughout the dorsal compartment **(Fig. 2.2 C)**. In fact, the level of pMad that is induced by *sax* is greater than that induced by the sax^{K262H} mutant (compare green line to purple line in **Fig. 6.2**).

These results indicate that *sax* inhibits BMP signaling when coexpressed with *gbb* in the posterior compartment. This is consistent with our model that exogenously produced Gbb is not sufficient to activate wild-type Sax kinase activity. On the other hand, sax^{K262H} enhances *gbb*-induced signaling—further supporting our model that mutations in the GS domain of *sax* render is "activatable" by Gbb ligand. The inability of posteriorly expressed *sax* to inhibit exogenous *gbb* signaling in the anterior compartment suggests that exogenous Gbb, which is a secreted and diffusible ligand, is escaping *sax*-mediated sequestration and is moving into anterior compartment where it

can activate BMP signaling. This scenario may arise if the level of exogenous Gbb produced is in excess of the amount of Sax receptor expressed.

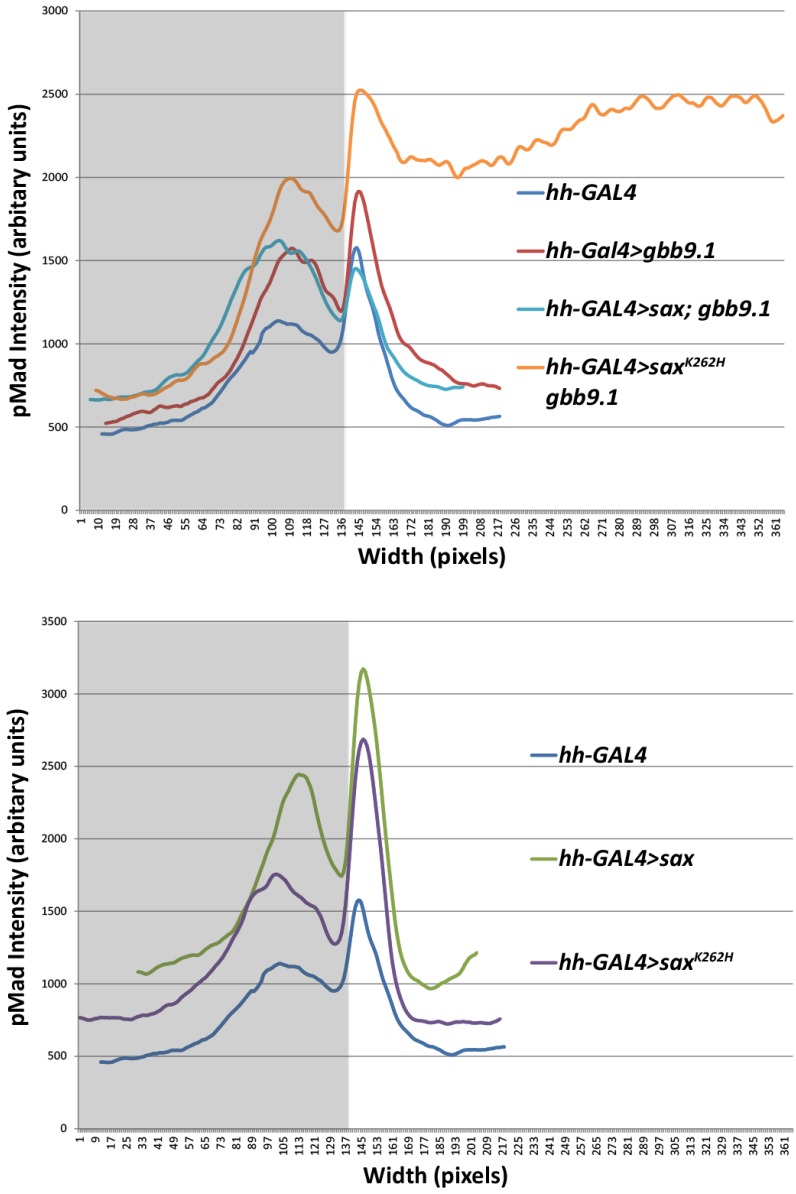


Fig. 6.1' *sax* antagonizes whereas *sax*^{K262H} enhances *hh-GAL4>gbb9.1* induced pMad in third instar larval wing discs. **Top panel.** pMad intensity profiles of *hh-GAL4, hh-GAL4>gbb9.1, hh-GAL>sax; gbb9.1,* and *hh-GAL4> sax*^{K262H}*; gbb9.1* wing discs. **Bottom panel.** pMad intensity profiles of *hh-GAL4*, *hh-GAL4>sax*, and *hh-GAL4>sax*^{K262H} wing discs. Average intensity profiles were generated from 5 wing discs for each genotype. Gray-shaded area indicates the the anterior compartment. White-shaded area indicates the posterior compartment, which is the *hh-GAL4* expression domain.

The curious observation that overexpression of *sax* alone in the posterior compartment increases pMad levels to a greater extent than sax^{K262H} overexpression remains a bit of conundrum. Whereas the "activatable" Sax^{K262H} receptor might be transducing BMP signals by binding to endogenously expressed BMP ligand, much of our previous data point to the wild-type Sax receptor inhibiting signaling by acting as ligand-sink. It should be noted that the overexpression of *sax* results in a dramatic decrease in the width of the wing disc. It is possible that decreasing the size of the disc effectively increases the ratio of BMP ligand available for signaling. This would be especially true for *dpp*, which is expressed in a narrow stripe along the anterior edge of the A/P boundary and would presumably be unaffected by changes in the width of the disc. The impact of overexpressing *sax* in the posterior compartment on *dpp* expression can be monitored by the various *dpp-lacZ* transgenic fly lines that have been generated in our lab (M. Aldrich)

Appendix 6.2 - Kinase activity is required for Sax^{K262H} to enhance Gbb-induced BMP signaling

Rationale

Our results indicated that *sax^{K262H}* was capable of enhancing *gbb*-induced BMP signaling both *in vivo* and in S2 cells. This suggested that the K262H mutation renders the kinase activity of Sax "activatable." To determine whether the ability of *sax^{K262H}* to transduce BMP signals is dependent on its kinase assay, I induced the A289D mutation associated with the loss-of-function *sax^{5}* allele to abolish the kinase activity in the resulting *sax$^{K262H\ A289D}$* double mutant. The effect of *sax$^{K262H\ A289D}$* on *gbb*-induced signaling was measured using the previously described *brkSE-lacZ* signaling assay in S2 cells.

Materials and Methods

Transfection of S2 cells and *brkSE-lacZ* signaling assays were performed as previously described. The following constructs were used in this analysis *pAW gbb 1-1-2* (pKW 299), *pAWF sax (pKW 270), pAWF sax^{K262H}*(pKW 231), *pAWF sax^{A289D},* and *pAWF sax$^{K262H\ A289D}$*. To generate the *pAWF sax$^{K262H\ A289D}$* I performed QuikChange site-directed mutagenesis (Stratagene) using the following primers: to induce the A289D mutation in *pDONR221 sax^{K262H} (T)*. The *sax$^{K262H\ A289D}$* cDNA was shuttled from *pDONR221 sax$^{K262H\ A289D}$* into the *pAWF* Gateway vector by LR recombinase (Invitrogen).

Results and Conclusions

Transfection of *gbb* activated BMP signaling as indicated by the repression of *brkSE-lacZ*. Consistent with our previous observations, *gbb*-induced repression of the *brkSE-lacZ* reporter was antagonized by *sax* and sax^{A289D}, whereas it was enhanced by sax^{K262H}. On the other hand, the $sax^{K262H\ A289D}$ double mutant inhibited *gbb* signaling. This antagonistic effect was indistinguishable from sax^{A289D} indicating that the effect of the A289D mutation is dominant over the effect of the K262H mutation.

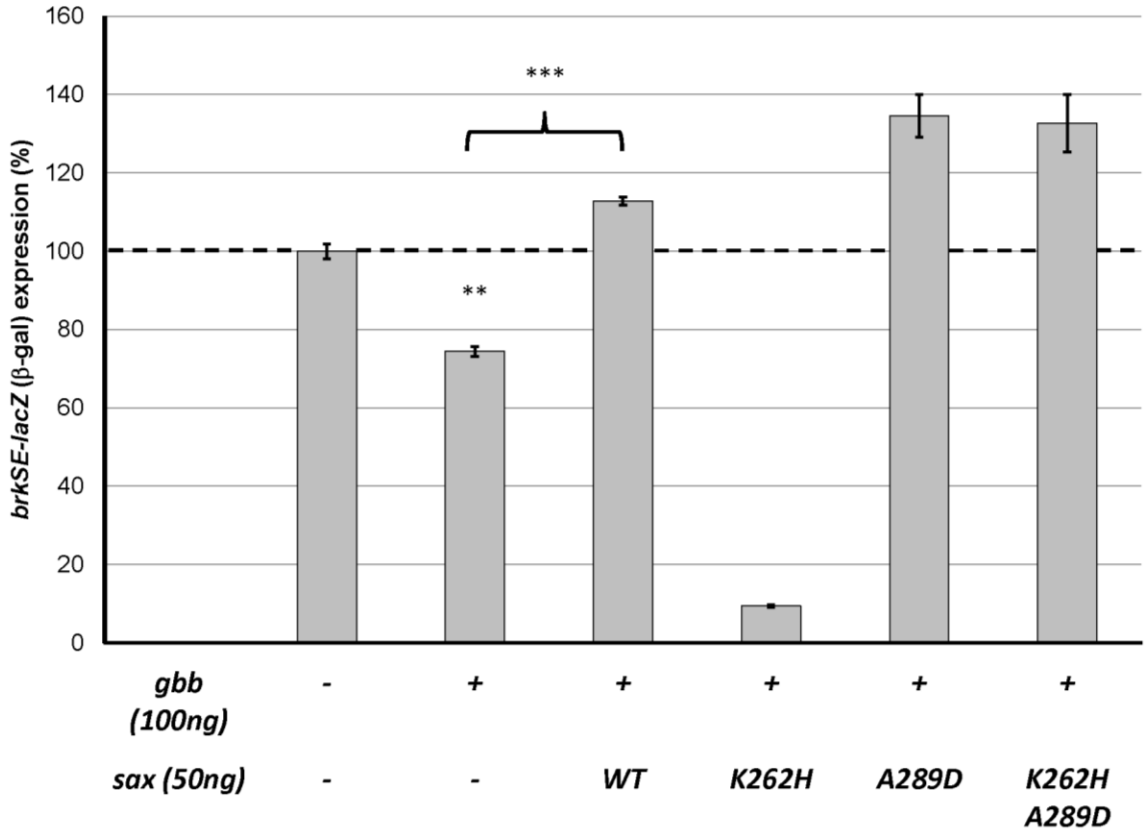


Figure 6.2. A289D mutation abolishes Sax^{K262H} signaling activity. The $sax^{K262H\ A289D}$ double mutant antagonizes *gbb*-induced repression of the BMP responsive *brkSE-lacZ* reporter as measured by β-Gal activity. Data plotted are the mean (± SEM) of an experiment performed in triplicate. Multiple pairwise comparisons were performed in R using the Tukey HSD test to determine statistical significance (* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$). Dashed indicates background β-gal activity set to 100% in cells transfected with only *brkSE-lacZ*.

Given the location of the A289D mutation in the Sax kinase domain and that it corresponds to the severe loss-of-function *sax*^{5} allele, we interpret the inability of *sax*$^{K262H\ A289D}$ to enhance *gbb* signaling to indicate that the A289D mutation abolishes the double mutant's kinase activity. These results are consistent with our model that the ability of *sax*^{K262H} to enhance *gbb*-signaling is dependent an "activatable" kinase activity that is not present in wild-type *sax*.

Appendix 6.3 - *sax*^{K262H} exhibits minor rescue of *sax*^{2}-associated embryonic maternal effect lethality

Rationale

BMP signaling is required for specifying dorsal cell fates during dorsal/ventral patterning of the embryo. Consistent with this requirement, pMad distribution accumulates and becomes restricted in a stripe along the dorsal midline during early embryogenesis. The original *sa*x alleles, *sax*^{1} and *sax*^{2}, were originally identified in a maternal effect lethality (MEL) screen, in which offspring of mothers who were homozygous for *sax*^{1} or *sax*^{2}, or transheterozygous *sax*^{1}/*sax*^{2} displayed significant embryonic lethality (Schupbach and Wieschaus, 1989). Cuticle preparations of these embryos revealed a severely ventralized phenotype--a hallmark of BMP signaling defect (Brummel et al., 1994; Nellen et al., 1994; Schupbach and Wieschaus, 1989). Furthermore, when *sax* function is specifically removed from the maternal germline through the generation of loss-of-function clones, the derived embryos lose the dorsal stripe of pMad. This underscores the importance of maternally loaded sax during embryonic development (Dorfman and Shilo, 2001). Taken together these results implicate *sax* as a facilitator of BMP signaling during early embryogenesis.

Thus far, the inhibitory behavior of Sax has only been observed during wing patterning. The *sax*^{K262H} mutant, however, offers a valuable tool in potentially identifying other developmental processes that require Sax's antagonistic behavior since Sax^{K262H} no longer displays the ability to inhibit Gbb signals. Given that the early embryo is sensitive to loss of *sax* function, we asked whether the *sax*^{K262H} mutant allele could rescue *sax* MEL. If *sax*^{K262H} cannot, then it would be the first suggestion that the inhibitory function of Sax may be required in the early embryo.

Materials and Methods

Previous work demonstrated that an 8.9 kb SalI fragment of the *sax* locus was sufficient to rescue MEL lethality associated with the sax^{1} and sax^{2} alleles (Nellen et al., 1994). To test whether K262H could rescue MEL we generated rescue constructs in which we inserted *sax*, sax^{K262H}, or *lacZ* cDNA in place of the *sax* gene within the context of the 8.9 kb SalI fragment (renamed 6.2 to reflect the size of the fragment when the *sax* gene was removed). In order to control for position effect, we generated transgenic lines using the phiC31 system to integrate the rescue constructs into the same site in the genome. The rescue constructs were then recombined onto a chromosome carrying the MEL-associate sax^{2} allele (genotype: sax^{2} *cn bw*).

To test each rescue construct's ability to rescue MEL, we mated *yw* males with sax^{2} homozygous females and females carrying one or two copies of each construct in a sax^{2} homozygous background. Embryos were collected on apple juice and the number of hatched embryos versus embryos that died before hatching was scored.

Results and Conclusions

As expected, embryos derived from sax^{2}/sax^{2} mothers exhibited severe MEL with only about 14% of embryos hatching (Fig MELb). The addition of one or two copies of the *6.2:lacZ* construct in the maternal genotype enhanced sax^{2}-associated MEL as only 0.42% and 1.9% of the embryos hatched, respectively (Fig MELb). The sax^{2} *6.2:lacZ* recombinant chromosome was subsequently used as a negative control in this analysis.

Next, we characterized a sax^{2} *6.2:sax-3xFLAG* recombinant chromosome designated 1aL. The 1aL chromosome dramatically rescued sax^{2}-associated MEL in dose-dependent fashion (Fig MELb). One copy of the 1aL chromosome increased embryonic viability to 33%, where as two copies increased viability to 65% (compared to 0.42% and 1.9% in sax^{2} *6.2:lacZ* negative controls). In contrast two independent sax^{2} *6.2:*sax^{K262H}-

3xFLAG recombinant chromosomes, 1R and 2M, exhibited minor rescue of MEL. The 1R chromosome was more effective at rescuing MEL. One copy of the 1R increased embryonic viability to 18%, however two copies of 1R did not improve this rescue. One and two copies of the 2M chromosome, on the other hand, increase embryonic viability by 1.9% and 9.5%, respectively. The difference in the ability to rescue MEL between the 1M and 2R lines may reflect differences in recombination sites.

It should be noted that while some embryos derived from *sax²*, *sax² 6.2:lacZ,* and *sax² 6.2:sax^{K262H}* mothers survived through embryogenesis, these progeny were mostly inviable (only a handful of adults were ever recovered). In contrast, many of the progeny derived from mothers carrying one or two *sax² 6.2:sax-3xFLAG* -1aL chromosomes survived to adulthood. This suggests that *sax* function early on in embryogenesis impacts the animal later on in development. Further analysis will be required to determine the lethal stages for the progeny of *sax²*, *sax² 6.2:lacZ,* and *sax² 6.2:sax^{K262H}* mothers. It will also be important to determine how embryonic morphology and pMad is affected in progeny derived from the different maternal genotypes. For instance, we might expect that embryos derived from *sax² 6.2:sax^{K262H}* would exhibit dorsalized phenotypes and an increase in pMad levels consistent with upregulation of BMP signaling.

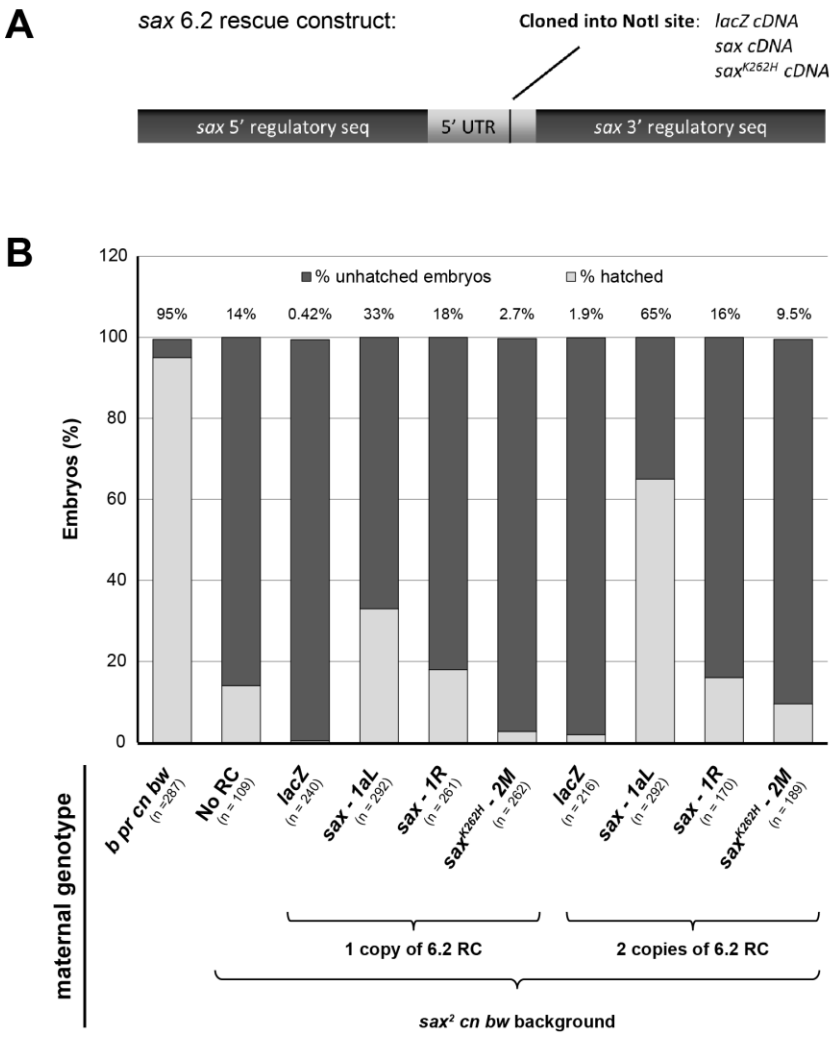


Figure 6.3 Effect of different *sax* rescue constructs on *sax²*-associated maternal affect lethality (MEL). *sax^{K262H}* exhibits minor rescue of *sax²*-associated (MEL). **A.** Diagram of the *sax* 6.2 rescue construct derived from an 8.9 kb EcoRI fragment from genomic clone BACR04B09 (Berkeley Drosophila Genome Project). This fragment includes a region of the *sax* locus that is sufficient for rescuing *sax¹* and *sax²* MEL. The *sax* sequence encompassing the ATG and stop codon was replaced with a NotI restriction site via site-direct mutagenesis. *lacZ, sax, and sax^{K262H}* cDNAs were then cloned into the NotI site. The rescue constructs were then inserted into the *Drosophila* genome via PhiC31 integrase-mediated transgenesis **REF**. Chromosomes carrying the rescue constructs were subsequently recombined with a chromosome carrying the *sax²* allele. **B.** Percentage of hatched embryos derived from different maternal genotypes carrying 6.2 rescue constructs. Embryos derived from the indicated maternal genotypes were collected and the number of embryos that hatched versus the number that died before hatching were counted. RC, rescue construct.

Appendix 6.4 - Mass spectrometry identifies phosphopeptides orresponding to the GS domain of Sax and potentially a novel phosphorylation site.

Rationale

The GS domain consists of several glycine-serine repeats that is highly-conserved between the type I receptors of the TGF-β/BMP signaling pathways (**Fig. 1.13**). Phosphorylation of the serines in the GS domain by the type II receptor is critical for activation of the type I receptor kinase. In this study, we consistently that *gbb*-induced BMP signaling was antagonized by *sax*, which suggests, perhaps, that the *sax* kinase is inactivatable. One possibility is that the GS domain of *sax* is not phosphorylated in response to *gbb* binding and therefore its kinase activity is kept latent. To confirm that the GS domain of *sax* is phosphorylated, we employed mass spectrometry to identify Sax phosphopeptides.

Materials and Methods

Stable lines of S2 cells overexpressing *sax, sax^{K262H},* or *gbb* were generated by cotransfecting 1.8 μg of *pAWF-sax*, *pAWF-sax^{K262H},* or *pAW-gbb-1xHA* respectively with 0.2 μg of the pCoPURO puromycin-resistance plasmid followed by puromycin selection. Stable cell lines were split (1:4) in M3 Media (pH 6.5)(Sigma) supplemented with 10% Insect Media Supplement (Sigma), 2% HI-FBS (Sigma), and 1:400 PenStrep (Hyclone) and cultured for seven days to allow for sufficient expression. On day 7, the stable cell lines were pelleted in a clinical centrifuge at setting 4 for 5'. Gbb-HA conditioned media was collected and used to resuspend the pellets of Sax-3xFLAG and Sax^{K262H}-3xFLAG expressing S2 cells. Ligand treatment was carried out for 3h, rotating at RT.

The Gbb-HA treated cells were then solubilized by Triton X-100 added directly to the media at a final concentration of 0.1%. M2 FLAG antibody (Sigma) bound to

Dynabeads Protein G (Invitrogen) was used to immunopurify Sax-3xFLAG and Sax^{K262H}-3xFLAG from solubilized cell extracts overnight at 4°C. The eluted samples were run on 12% SDS-PAGE gel and stained with GelCode Blue Safe Protein Stain. Bands corresponding in size to of Sax-3xFLAG and Sax^{K262H}-3xFLAG were cut out and subjected to In-gel tryptic digests (Pierce Thermofisher according to protocol). Digested peptides were analyzed by mass spec at Brown's Proteomics facility.

QuikChange II site-directed mutagenesis was used to generate the sax^{SR} and tkv^{RS} mutants. Primers used for sax^{S287R} mutagenesis:

Sax S287R fwd

5'-GGTCACTGGCATGGCGAACGCATCGCCGTGAAGATATTC-3'

GC-Sax S287R fwd

5'-GAATATCTTCACGGCGATGCGTTCGCCATGCCAGTGACC-3'

Primers used for tkv^{R223S} mutagenesis:

Tkv R223S fwd

5'-GCCAAATGGCGCGATGAGAG GTGGCCGTCAAGACC TTC-3'

GC-Tkv R223S fwd

5'-GAAGGTCTTGACGGCCACGCTCTCATCGCGCCATTTGGC-3'

sax^{S287R} *and* tkv^{R223S} cDNAS were shuttled from pDONR221 into pAWF Gateway vector using LR recombinase (Invitrogen). 100 ng of *gbb* and 50 ng of the indicated receptors were transfected into S2 cells and assayed using the *brkSE-lacZ* signaling assay.

Results and Conclusions

Mass spectrometry identified three phosphopeptides for Sax and four phosphopeptides for Sax^{K262H}. However, only one phosphopeptide for Sax (peptide# 2) was high scoring (MOWSE > 20, Expect < 0.05) while there were two high scoring phosphopeptides for Sax^{K262H} (peptide# 4 & 7). All but one phosphopeptide for either

protein corresponded to the GS domain—with one low scoring Sax phosphopeptide corresponding to the β3 domain in the kinase domain. Phosphorylation of this serine in the β3 domain has previously never been observed.

	Phosphopeptides	Domain	MOWSE	Expect
	Sax			
1	R.EYLQH**S**VTSGSGSGLPLLVQR.T	GS	2.18	20
2	R.EYLQHSVTSG**S**GSGLPLLVQR.T	GS	31.84	0.022
3	R.GHWHGE**S**IAVK.I	β3	8.46	3.4
	Sax^{K262H}			
4	R.EYLQHSVTSG**S**GSGLPLLVQR.T	GS	32.09	0.022
5	R.EYLQH**S**VTSGSGSGLPLLVQR.T	GS	13.67	1.4
6	R.EYLQHSVT**S**G**S**GSGLPLLVQR.T	GS	21.5	0.31
7	R.EYLQHSVT**S**GSGSGLPLLVQR.T	GS	45.87	0.00087

Table 6. Sax and SaxK262H phosphopeptides identified by mass spectrometry. Indicated are the domains of the Sax protein to which the phosphopeptides correspond along with MOWSE values and Expectation scores (Expect). Phosphoserines are indicated in red.

The identification of phosphopeptides corresponding to the GS domain suggests that Sax can be phosphorylated by the type II receptor and is consistent with the observation that both *punt* and *wit* can activate BMP signaling in conjunction with *sax* (**Fig. 2.10 & 2.11**) Previous work done in mammalian cell culture demonstrated a positive correlation between Smad binding to the type I receptor and subsequent Smad phosphorylation with the number of phosphoserines in the GS domain of the type I receptor (Huse). This suggests that optimal signaling requires a fully phosphorylated GS domain. It's possible that Sax's inability to enhance *gbb*-signaling may reflect a GS domain that is only partially phosphorylated, which keeps the Sax kinase in an inactive state. Furthermore, the experiments done in mammalian cell culture only addressed the importance of the number of phosphorylated serines to signaling. It remains unknown

whether each phosphoserine contributes equally to type I receptor signaling activity or if some phosphoserines are more important than others.

While our mass spec data fails to identify any Sax GS domain phosphopeptides that contained more than one phosphoserine, it should be noted that the phosphopeptides that were identified were mainly low scoring. It's possible that multiply-phosphorylated GS domain peptides were too rare to be observed. This could be improved by using TiO_2-based protocols to enrich for phosphoserine-containing peptides before submitting our samples for mass spectrometry.

Given that the corresponding amino acid (by sequence alignment) in Tkv is an arginine, we decided to investigate whether this serine—a potentially novel phosphorylation site—might underscore the apparent difference in signaling between Tkv and Sax. It's possible that phosphorylation of this serine could be an additional way to regulate Sax's kinase activity such that its phosphorylation state would reflect whether Sax's kinase is active. Therefore, we hypothesized that swapping the corresponding serine and arginine residues between the receptors would generate a Sax^{SR} protein that now enhances *gbb* signaling and a Tkv protein that would inhibit *gbb* signaling.

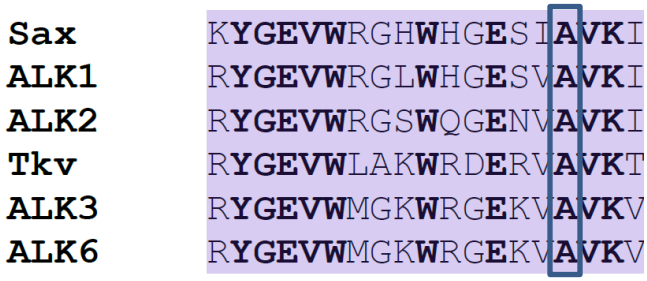


Figure 6.4. Alignment of β3 sequence of BMP type I receptors. The β3 sequences is highly conserved. Location of the phosphoserine in Sax identified by mass spec (**Table 6**) is boxed.

Results and Conclusions

brkSE-lacZ signaling assays in S2 cells, however, revealed that *tkv*^{RS} did not differ from wild-type *tkv* in its ability to enhance *gbb*-induced repression of the reporter. Neither did *sax*^{RS} differ from wild-type *sax* in its antagonistic behavior toward *gbb*-induced signaling. These results do not support this (phospho)serine as the underlying explanation for the antagonistic behavior of *sax*. We cannot, however, rule out the possibility that this (phospho) serine may be impacting *sax*'s function in other ways.

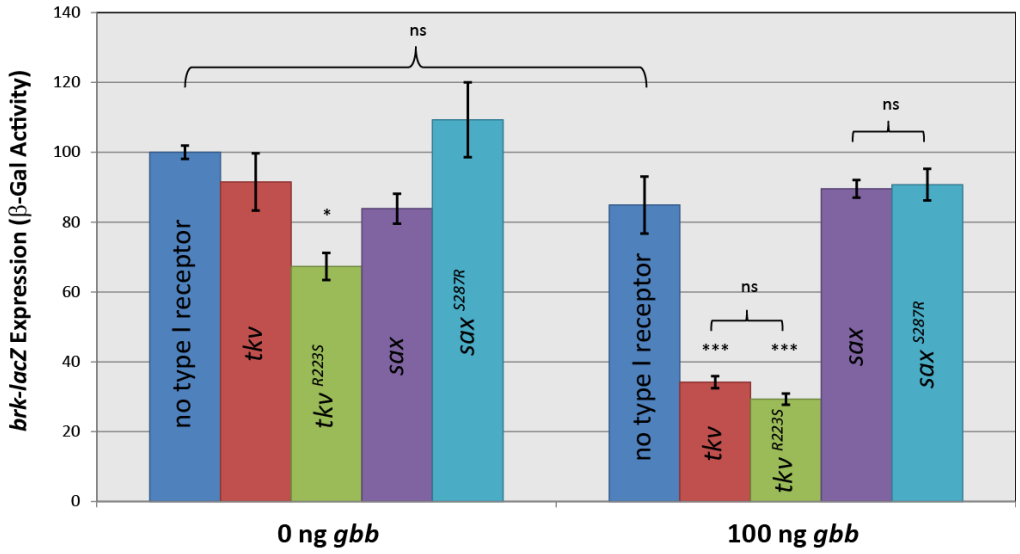


Figure 6.4'. Effect of swapping Sax and Tkv β3 residues corresponding to Sax^{S287} and Tkv^{R223S}. Sax^{S287R} inhibited Gbb-signaling and Tkv^{R223S} enhanced Gbb-signaling indicated that this residue is not responsible for the inhibitory behavior of Sax BMP-mediated repression of the *brkSE-lacZ* reporter was measured from β-galactosidase activity. Background BMP signaling measured in S2 cells transfected with the *brkSE-lacZ* alone was set to 100%. Data plotted are the mean (± SEM) of experiment performed in triplicate. Multiple pairwise comparisons were performed in R using the Tukey HSD test to determine statistical significance (* p≤0.05, ** p≤0.01, *** p≤0.001).

Appendix 6.5 - *sax*^{5} exhibits dominant-negative behavior.

These results were published in (Twombly et al., 2009). *The clonal analysis in Fig. 5 was performed by Erdem Bangi, Ph.D.*

Rationale

The difference in the ability of *sax*^{5} to enhance dpp lethality compared to *sax*^{4} or a *sax* deficiency (*Df(2R)H23*), prompted us to investigate in more detail the possibility that *sax*^{5} may exhibit a mild dominant-negative effect. We had previously examined the role of sax in wing patterning (Bangi and Wharton 2006b) and sought to compare the phenotype associated with a *sax*^{4} vs. a *sax*^{5} clone in the adult wing. While large posterior clones of *sax*^{4} show no wing patterning abnormalities, large clones of *sax*^{5} show a significant loss of longitudinal vein 4 (L4) and a narrowing of the L4/L5 intervein, a phenotype associated with a loss of dpp function (**Fig. 6.5**). This result is consistent with the enhancement of *dpp*^{d5}/*dpp*^{hr56} lethality by *sax*^{5} (Table 4 in Twombly et al., 2009), and supports the conclusion that *sax*^{5} is able to negatively impact *dpp* function.

Materials and Methods

Cell-based BMP signaling assay: A cell-based BMP signaling assay using S2 cells has been described previously (Muller et al. 2003; Bangi and Wharton 2006b) and depends on the endogenous expression of BMP signaling components. In this assay, a reporter construct expressing *lacZ* is controlled by a Su(H) transcriptional activation response element as well as a *brk* transcriptional silencer element (Su(H)/*brkSE-lacZ*). Transcription is activated by cotransfection of the reporter construct with plasmids encoding Su(H) and an activated form of Notch (N*). Activation of BMP signaling leads to repression of *lacZ* expression due to the presence of the brk silencer element, and thus, a reduction in β-galactosidase activity. BMP signaling levels are inversely

correlated with the level of β-galactosidase activity. Plasmids containing the coding sequences of tkv (pAcpA-*tkv1-FLAG*), sax (pAcpA-*sax-FLAG*), or *sax*^{5} (pAW-*sax*^{5}) were cotransfected with Su(H), N*, Su(H)/*brkSE-lacZ*, and luciferase plasmids, all under the control of the actin 5C promoter using Effectene Transfection (QIAGEN). b-Galactosidase values were measured using the dual luciferase assay system (Dual-Light, Applied Biosystems) and normalized to luciferase for each sample.

Results and Conclusions

We next made use of a cell-based BMP signaling assay to assess the ability of the *sax*^{5} mutation to affect BMP signaling. As described previously, lacZ expression in this assay is repressed by BMP signaling in a quantitative manner and thus, b-galactosidase activity is inversely correlated with the level of BMP signaling (Bangi and Wharton 2006b; Muller et al. 2003). As observed previously, Tkv exhibits some degree of signaling when wild-type tkv constructs are transfected into S2 cells alone (samples 2 and 3, **Fig. 6.5D**), while Sax does not (sample 4, **Fig. 6.5D**). We have found that S2 cells express gbb (T. Akiyama, unpublished results) and transfection with a wild-type sax construct appears to block endogenous Gbb signaling, likely as a result of ligand being bound by nonsignaling Sax–Sax complexes (Bangi and Wharton 2006b). When cotransfected with tkv, a wild-type sax construct results in the antagonism of signaling in a dose-dependent manner (samples 5 and 6, **Fig. 6.5D**). In agreement with our genetic analysis of *sax*^{5} mutants, cotransfection of tkv with a *sax*^{5} construct leads to a complete inhibition of Tkv-mediated BMP signaling (samples 8 and 9, **Fig. 6.5D**), indicating that the Sax^{5} protein can completely disrupt successful signaling.

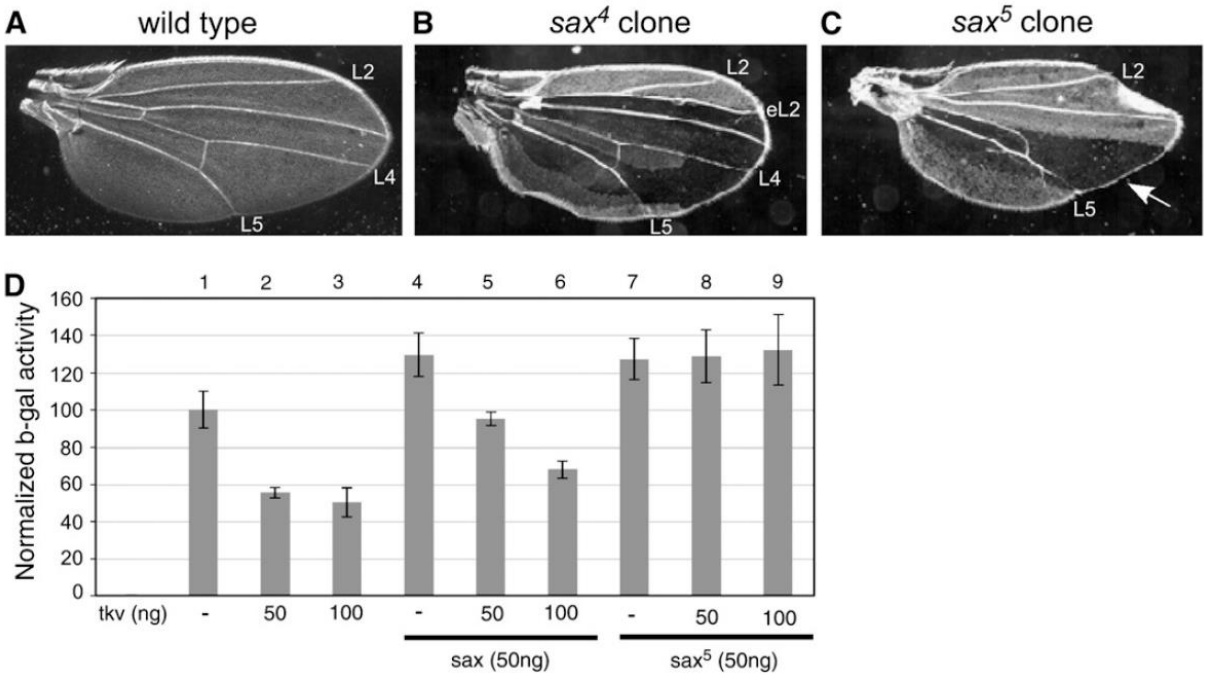


Figure 6.5.—*sax*^{5} produces more severe phenotypes than *sax*^{4}. A. Dark field image of a wild-type wing. Longitudinal veins 2 (L2), 4 (L4), and 5(L5) are indicated. (**B** and **C**) Wings resulting from *sax* mutant clones as described in Bangi and Wharton (2006b). Clones marked with *shv* appear dark in images. **B.** A *sax*^{4} clone encompassing the entire posterior compartment shows no patterning defects. Consistent with previous studies (Singer et al. 1997; Bangi and Wharton 2006b), a small *sax*^{4} clone in the anterior compartment leads to an ectopic L2 (eL2) vein. **C.** A *sax*^{5} clone in the posterior compartment results in the loss of L4 (arrow) and a narrowing of the L4/L5 intervein, a phenotype never seen in an equivalent *sax*^{4} clone. The more severe phenotype of *sax*^{5} suggests that the presence of a defective Sax receptor is more detrimental to BMP signaling during wing patterning than the complete loss of the Sax receptor. **D**. A cell-based BMP signaling assay indicates that the *sax*^{5} mutation is able to negatively affect BMP signaling mediated by Tkv. S2 cells were cotransfected with the *Su(H)/brkSE-lacZ* reporter construct, Su(H), and N* constructs to stimulate transcription (sample 1), and *tkv*, and/or *sax* and *sax*^{5} constructs under the control of the actin 5C promoter (samples 2–9). Values depicted are the fold activation of b-galactosidase over the basal activity of the reporter construct alone. All values represent the average of samples measured in triplicate and normalized for transfection efficiency.

Appendix 6.6 – Tkv^{KD} did not reveal Sax kinase activity

Rationale

Our current model of Sax dual behavior proposes that Sax can mediate signaling when in a complex with Tkv (**Fig. 1.3**). To determine whether the Sax kinase is active in a Sax:Tkv complex, a S2 cell-based Mad phosphorylation was employed. Furthermore, we made use of a a Tkv "kinase deficient " (Tkv^{KD}) mutant receptor to remove any signaling contribution from Tkv. We reasoned that any Gbb-induced phosphorylation of Mad detected in S2 cells cotransfected with Sax, Tkv^{KD}, and Mad would have to be mediated through Sax.

Materials and Methods

S2 cells (8 x 10^6 cells) were transfected with 700 ng pAC FLAG-Mad alone or in combination with 300ng of pAWH sax, pAWF Tkv, or pAWF Tkv^{KD} (kinase deficient mutation: Tkv^{G212E}). In cells where more than one receptor was transfected, 150 ng of each receptor was used. 5 days post transfection cells were treated with Gbb-HA conditioned media for 3 hours. Protein extracts were prepared and analyzed by western blot using the indicated antibodies. pAWH Tkv^{KD} was generated by site-directed mutagenesis using the pDONR221 tkv(T) contruct as template with the following the primers:

prKW146 tkv G212E fwd

5'-CAAAGGACGATATGAAGAGGTCTGGCTGG-3'

prKW 147tkv G212E rev

5'-CCAGCCAGACCTCTTCATATCGTCCTT TG-3'

The *tkv^{G212E}* cDNA was shuttled from the pDONR221 vector into pAWF using LR Clonase (Invitrogen).

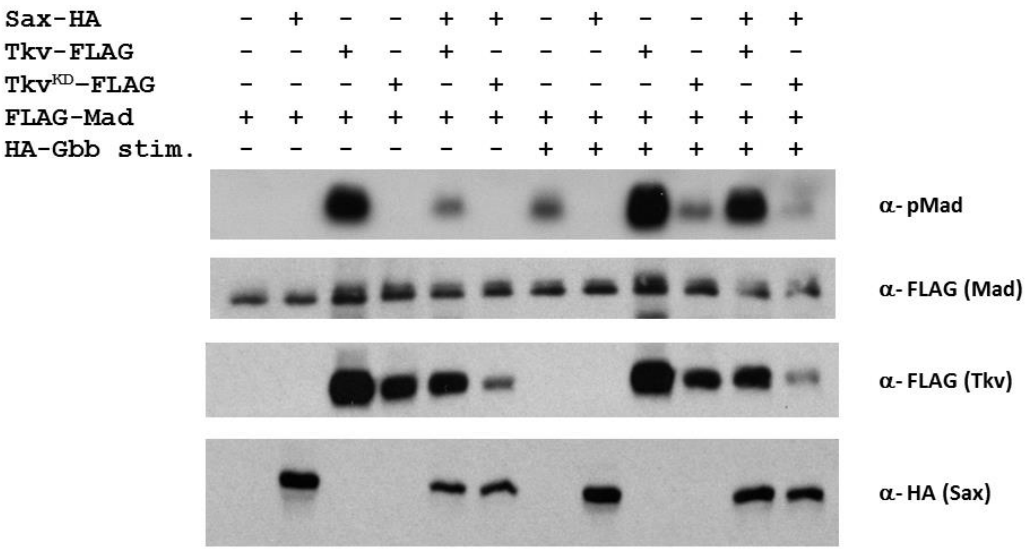


Figure 6.6. Tkv^{KD} did not reveal Sax kinase activity. Western blot of S2-cell Mad Phosphorylatoin assay. α-pMad signal is a measure of BMP signaling activity. α-FLAG blots indicate FLAG-Mad and Tkv-FLAG protein expression. A-HA blot indicates Sax-HA potein expression.

Results and Conclusion

In the absence of Gbb-HA treatment (lanes 1-6), only wild-type Tkv was sufficient to induce pMad (3). This Tkv-induced pMad was reduced when Tkv and Sax are coexpressed (5), consistent with an antagonistic behavior of Sax. No pMad was detected in cells coexpressing Sax and Tkv^{KD} together or alone (2, 4, & 6) indicating that neither receptor was sufficient to activate BMP signaling.

In the presence of exogenous Gbb-HA (lanes 7-12), Gbb-HA alone could induce pMad (7). This Gbb-induced Mad phosphorylation was enhanced by Tkv (9) consistent with Tkv transducing BMP signals. In contrast, cells expressing Sax completely inhibited Gbb-HA induced pMad consistent with Sax's antagonistic behavior (8). Furthermore, Tkv^{KD} had essentially no effect on Gbb-signaling (10). Similar to observations seen in the absence of Gbb-HA treatment, Sax negatively affected Tkv-enhanced Gbb signaling (11). Gbb-induced pMad was also repressed in cells coexpressing Tkv^{KD} and Sax, but not completely (12).

These data are difficult to interpret because Tkv^{KD} did not inhibit Gbb-HA induced signaling (10) like we would expect a kinase deficient receptor to do. Therefore, we could not conclude which receptor was responsible for phosphorylating Mad in cells coexpressing Sax and Tkv^{KD}. The experiment is also limited because we could not be sure that Sax:Tkv or Sax:Tkv^{KD} complexes were being formed. Given Gbb's apparent higher affinity for Sax than Tkv, it is very likely that Gbb preferentially mediates Sax:Sax complexes even in the presence of either Tkv or Tkv^{KD}. A different approach involving the use of a chimeric receptor in which the Sax extracellular domain is fused to the Tkv kinase domain could circumvent this obstacle and is outlined in **Appendix 6.10**.

Appendix 6.7 – *saxP* retains significant function:

These results were published in (Twombly et al., 2009).

Rationale

sax^{P} was previously reported to be a null allele, on the basis of the identification of a P-element insertion in the sax locus immediately following codon 36 of the sax PA open reading frame (Nellen et al. 1994). However, given that the *sax^{P}* allele exhibits traits in common with both *sax^{1}* and *sax^{2}* (missense mutations that likely produce aberrant proteins), we considered the possibility that the Pelement insertion responsible for *sax^{P}* may not completely disrupt normal sax transcription and may allow for the production of a wild-type protein product or one that is abnormal in some way.

Materials and Methods

For RT–PCR, RNA was isolated from yw1118 and yw; FRTG13 saxP homozygous third instar larvae (n = 10) using QIAGEN RNeasy and treated with Promega RQ1 DNase before cDNA synthesis. cDNA was synthesized using MLV RTase (Sigma) and oligo (dT)12-18 primer (Invitrogen). RT–PCR analysis was conducted using the following primers: (sax 9396 fwd) GCTGTGCCGGTGATTA CTG and (sax 11119 rev) GTCTTGTACTTGGATTAG; (P{lacW} 9661 fwd) GGATCTTC TTGAGATCC and (P{lacW}10648 fwd) GGATGTCTCTTGCC GACGGG; (8103) CGTTTCTGCTGTACAATAATGCCAG and (10228) GCCCATTAGCTATGGACAGGC.

Results and Conclusions

We performed RT–PCR on RNA from homozygous *sax*^{P} animals and detected the clear presence of an mRNA derived from sequences downstream of the proposed placW insertion site (**Fig. 6.6B**). The *sax* open reading frame (PA) (Brummel et al. 1994; Nellen et al. 1994) may be generated by one of two possible transcripts from the *sax* locus, the *sax-RA* transcript (see www.flybase.org).

A second transcript, *sax-RB*, can generate a second Sax protein (PB) that initiates at the methionine at codon 36 of the PA open reading frame. We considered the possibility that a transcript from the *sax*^{P} locus could encode an open reading frame similar to PB. We first determined the orientation of the placWelement responsible for *sax*^{P} by genomic Southerns (data not shown) and then to verify the site of the P-element (placW) insertion, we produced cDNA from *sax*^{P} homozygotes, generated PCR products, and sequenced the junction between the P39 end and the sax locus. In contrast to the previous report (Nellen et al. 1994), we found that the placW element is inserted just 59 to the GAC codon encoding aa 35 of the SaxPA open reading frame and thus, upstream from the ATG encoding methionine aa 36 (underlined in **Fig. 6.6A**). Thus, the SaxPB open reading frame remains intact in *sax*^{P} mRNA. Two additional ATG codons are upstream within the P39 end sequences and could encode methionine codons in frame with SaxPB, providing other possible alternative translational start sites (**Fig. 6.6 C**).

Our identification of lethal, null *sax* alleles and the genetic analyses indicating that *sax*^{P} retains some function, led us to conclude that the Sax-PB protein (or a Sax-PB protein with 2–12 additional amino acids) must have activity and importantly, must contribute in part to BMP signaling during development. *sax*^{P} homozygotes are viable and display only weak mutant phenotypes, indicating that most requirements for sax function throughout development can be met by the Sax-PB protein.

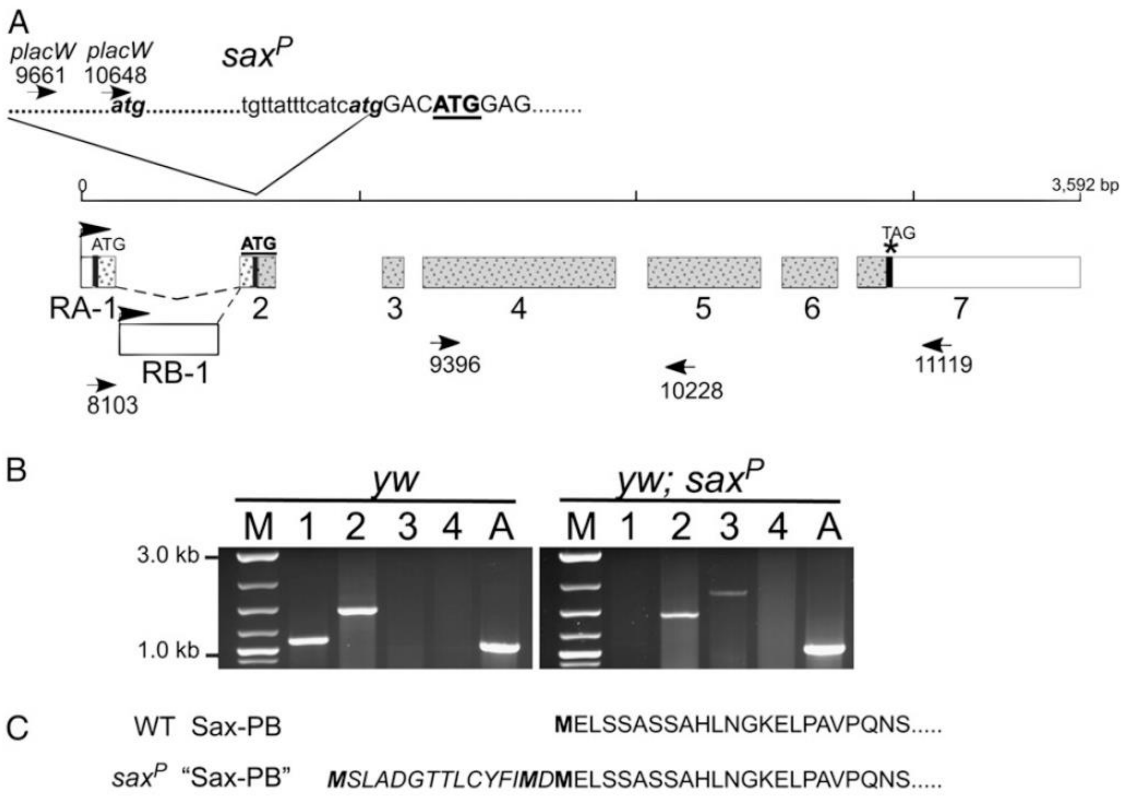


Figure 6.7. P-insertion site of *sax^P* does not disrupt transcription. A. Genomic structure of *sax* locus shown with exon (numbered) distribution for the two splice forms of sax mRNAs (RA and RB). The locations of two ATG initiation codons and the TAG termination codon (vertical thick solid lines) indicate the two overlapping open reading frames (speckles) giving rise to the putative protein products PA and PB (shaded speckles). Positions of PCR primers are indicated by arrowheads. The site and junctional sequence of the placW insertion giving rise to *sax^P* is shown at the top. The endogenous second ATG within the sax transcription unit is bold and underlined. **B.** RT–PCR products from different primer pairs generated from RNA isolated from control (yw) (left) and *sax^P* homozygous mutant flies (right). Lane 1, primers 8103 1 10228; lane 2, primers 9396 1 11119; lane 3, primers placW10648 1 11119; lane 4, primers placW9661 1 10228. Note the insertion of placW disrupts the wild-type transcription unit initiating at RA-1 (lane 1) but allows transcription to initiate within the placW element between primers placW9661 and placW10648 (presence of PCR product in lane 3 of *sax^P* animals and not in wild type, or in lane 4 of *yw* or *sax^P*). Transcription in both genotypes extends through the expected translational termination site (PCR product present in lane 2 of both genotypes). M, marker lane. A, actin control. **C.** The predicted amino acid sequence of SaxPB produced by open reading frame initiating at second endogenous ATG (bold and underlined in **A**) is shown in normal type with potential additional amino acids (italics) if translation initiated at an atg within the 3' Pelement of placW (shown in **A**).

Appendix 6.8 – Sax-PB isoform inhibits Gbb signaling

Rationale

Three isoforms of Sax have been identified (Sax-PA, -PB, and –PC)(Marygold et al., 2012), and in this study I have focused on the Sax-PA isoform. Given the observation that sax^{P} homozygous animals are viable (Nellen et al., 1994) and that the sax^{P} allele has the potential to produce a Sax-PB like isoform, we hypothesized that the facilitating and antagonistic functions of Sax are divided between the isoforms. We decided to assay Sax-PB's effect on Gbb-induced signaling in the *brkSE-lacZ* assay.

Materials and Methods

Transfection of S2 cells and *brkSE-lacZ* signaling assays were performed as previously described. 50 ng of each receptor cDNA and 50 ng of *gbb* cDNA were transfected as indicated. The following constructs were used in this analysis *pAW gbb 1-1-2* (pKW 299), *pAWF sax (pKW 270), pAWF* sax^{K262H}(pKW 231), and *pAWF sax-RB*. To generate *pAWF sax-RB,* I amplified the region corresponding to the PB coding region that is encompassed by *sax-RA* cDNA using the following primers:

Sax-PB attB1 (prKW16)

5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGGGAGGACATACCGGACTTAGACATGG-3'

sax 3' attB2 tag

5'-ggggaccactttgtacaagaaagctgggtgAACGCAGACCTCGTCGAAGTCC-3'

The amplified *sax-RB* cDNA was recombined into pDONR221 via BP Clonase (Invitrogen) and then shuttled into the *pAWF* Gateway vector by LR recombinase (Invitrogen).

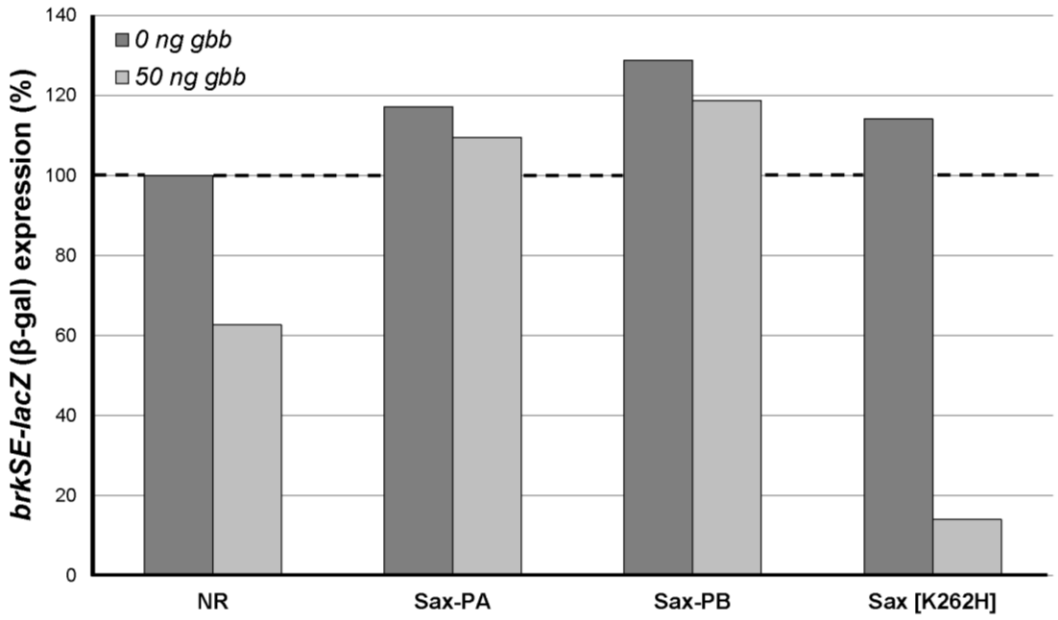


Figure 6.8. Sax-PB isoform inhibits Gbb-signaling. Sax-PB inhibits Gbb-induced repression of the BMP responsive *brkSE-lacZ* reporter as measured by β-Gal activity. Data plotted are the mean (± SEM) of an experiment performed in duplicate. Dashed indicates background β-gal activity set to 100% in cells transfected with only *brkSE-lacZ*.

Results and Conclusions:

Both Sax-PA and Sax-PB inhibited Gbb signaling, whereas Sax^{K262H} enhanced Gbb signaling. These results are inconsistent with the proposal that the dual behavior is divided between the PA and PB isoform. The effect of the PC isoform on Gbb signaling remains to be tested.

Appendix 6.9 – Effect of chimeric type I receptors on Dpp and Scw signaling

Rationale

The chimeric Sax-Tkv receptors analyzed in Chapter 3 revealed that the J-GSD and KD cooperate to determine type I behavior (**Fig. 3.3)**. This conclusion was made based on the chimeric receptors effect on Gbb signaling. To rule out the possibility that these were ligand –specific effects, we tested the effect of the chimeras on Dpp signaling.

Materials and Methods

Cell-based BMP signaling assays using a *lacZ* reporter were performed as previously described (Müller et al., 2003) with some modifications. To investigate the effects of receptors on the BMP signaling activity, we used 100 ng of receptor DNA constructs, 10 ng of *dpp* encoding the Dpp BMP ligand, and 50 ng of *scw* encoding the Scw BMP ligand. S2 cells were transfected at a density of 3.5×10^6 cells/mL with various combinations of DNA using Effectane kit (QIAGEN) and incubated at 25°C for 60 - 72 hours. After incubation, S2 cells (~ 3×10^6 cells) were lysed and β-galactosidase activities were measured by using the Dual-Light system (Applied Biosystems).

Results and Conclusions

Transfection of 10 ng *dpp* was sufficient for strong activation of BMP signaling, which made it difficult to interpret how Sax and Tkv influenced Dpp signaling (i.e. whether it enhanced or mediated signaling. Consistent with our proposal that 2 elements within the intracellular domain are required for determining behavior the TTS, TSS, TST constructs all inhibited Dpp signaling, however TSS displayed the greatest inhibitory

effect. We will need to reduce the amount of *dpp* that is transfected to better address the effect of Sax, Tkv, SST, STT, and STS.

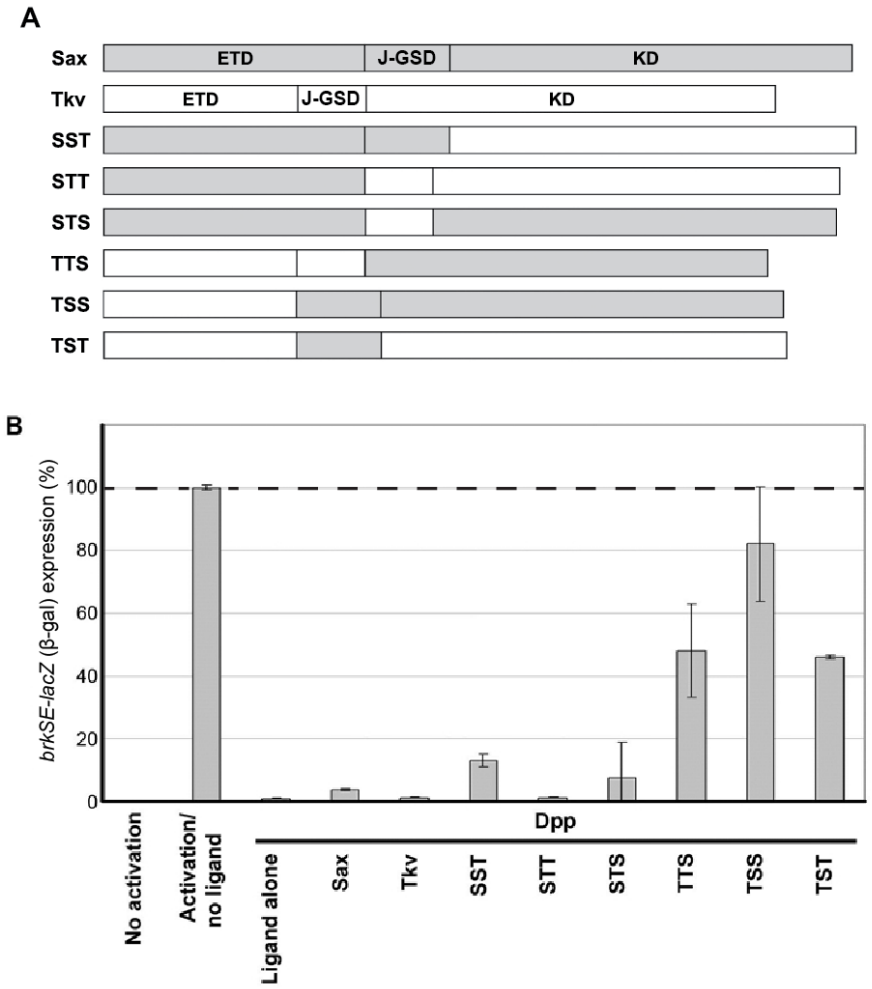


Figure 6.9. Elements within the Sax intracellular domain confer inhibitory behavior. A. Diagrams of Sax, Tkv and Sax-Tkv chimera proteins. Both Sax and Tkv proteins were separated into three domains: ETD (residues 1-199 in Sax, residues 1-148 in Tkv), J-GSD (residues 200-264 in Sax, residues 149-200 in Tkv) and KD (residues 265-570 in Sax, residues 201-509 in Tkv). Gray and white indicate Sax and Tkv, respectively. **B.** Effects of Sax-Tkv chimera proteins on Dpp-signaling. BMP-mediated repression of the *brkSE-lacZ* reporter was measured from β-galactosidase activity. Background BMP signaling measured in S2 cells transfected with the *brkSE-lacZ* alone was set to 100% and is indicated by the dashed line. 10 ng of *dpp* cDNA was co-transfected into *Drosophila* S2 cells with each chimeric construct. After incubation, β-galactosidase activities were measured. Data plotted are the mean (+/- SD) of experiments performed in triplicate.

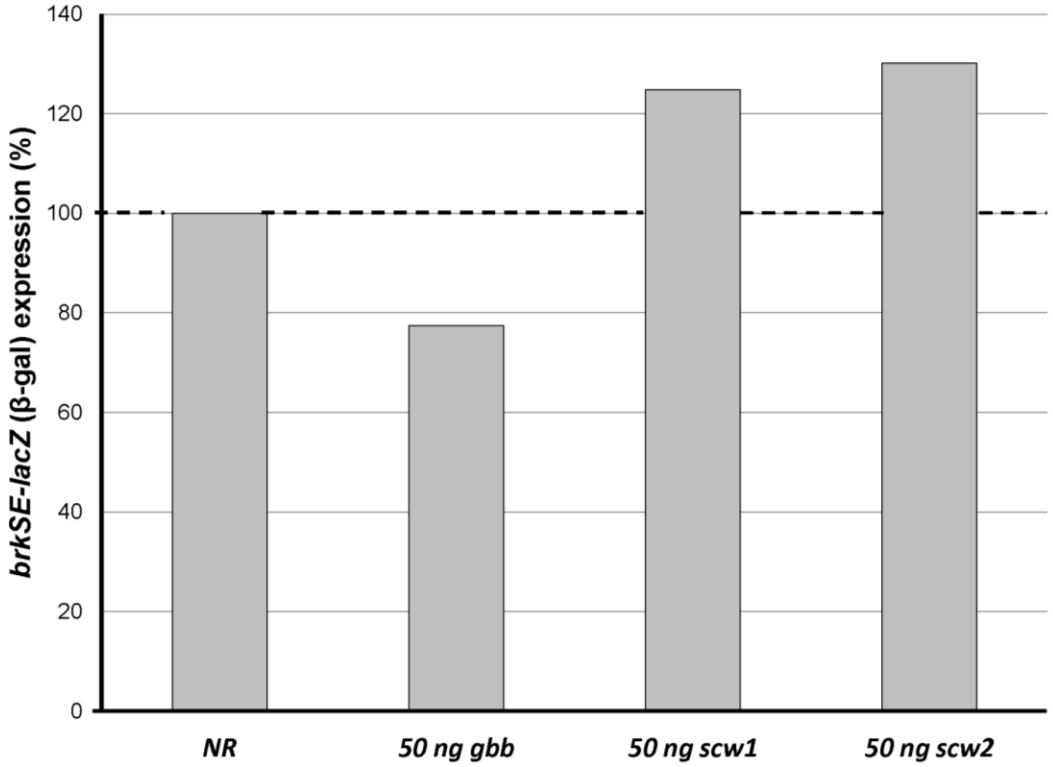


Figure 6.9'. *scw* (50ng) does not induce BMP signaling. Effect *scw* (50ng) transfections on *brkSE-lacZ* expression. Two independent clones of *scw* were used, designated 1 and 2. BMP-mediated repression of the *brkSE-lacZ* reporter was measured from β-galactosidase activity. Background BMP signaling measured in S2 cells transfected with the *brkSE-lacZ* alone was set to 100% and is indicated by the dashed line. After incubation, β-galactosidase activities were measured. Data plotted are the mean of experiments performed in duplicate.

Transfection of *scw,* however, did not activate the pathway. Activation may require higher concentrations of *scw* DNA to be transfected. It is also possible that Scw needs the presence of another ligand, such as Dpp, to signal.

Appendix 6.10 – Determining whether the kinase domain of Tkv (STT) activates the signaling activity of Sax

Rationale

In our model that explains Sax's dual behavior we have proposed that the signaling activity of a receptor complex is dictated by the composition of the type I receptors. Complexes consisting of Tkv:Tkv and Sax:Tkv are competent for signaling, whereas Sax:Sax complexes are unable to transduce signals. It appears that neither Sax molecules in a Sax:Sax combination can phosphorylate Mad and it is unknown whether the Sax kinase is active in the context of a Sax:Tkv complex. It is possible that Tkv can promote/activate Sax kinase function much like ALK5 has been reported to do with ALK1. Another possibility is that Sax's kinase function remains latent even in a Sax:Tkv complex and instead, acts as a silent coreceptor for BMP ligand. However, the observation that Sax^{A} synergizes with Tkv^{A} and the conservation of active site residues in Sax argues in favor of the first possibility.

One way to determine if Sax's kinase activity can be activated by the presence of Tkv is to measure BMP-induced signaling in S2 cells coexpressing Sax and a Tkv kinase dead mutant, which will remove any signaling contribution from Tkv. We reasoned that if BMP signaling level in these cells is intermediate to cells expressing only Sax (Sax:Sax does not signal) and cells coexpressing Sax and wild-type Tkv (Sax:Tkv does signal), then that would suggest that the kinase domain (absent of activity) is sufficient to activate Sax kinase. However, this strategy is complicated by the fact that Tkv binds Dpp preferentially, whereas Sax prefers Gbb. Thus, transfection of either ligand would most likely bias the formation of receptor complexes composed of their high affinity binding partner over heteromeric Sax:Tkv complexes.

To get circumvent this obstacle we decided to exploit the STT chimeras that were generated to dissect the molecular basis of Sax's antagonistic behavior. Since STT carries the Sax extracellular domain it should bind Gbb with equally high affinity as Sax. Therefore, assuming protein levels, Gbb should induce the formation of Sax:Sax, Sax:STT, Sax:STT complexes at an equal ratio. This strategy would at least allow us to address if the kinase domain of Tkv, independent of its ECD) is sufficient promote Sax signaling activity.

Materials and Methods

The following constructs were used in this analysis *pAW gbb 1-1-2* (pKW 299), *pAWF sax (pKW 270), pAWF STT* , *pAWF STT^{G212E}-2, pAWF STT^{G212E}-5, pAWF STT^{G256E}-3,* and *pAWF STT^{G256E}-9*. To generate the *pAWF STT^{G212E}* and *pAWF STT^{G256E}* , I performed QuikChange site-directed mutagenesis (Stratagene) using *pDONR221 STT (T)* as template. To induce the G212E mutation I used the following primers**:**

tkv G212E fwd (prKW 146) 5'-CAAAGGACGATATGAAGAGGTCTGGCTGG-3'

tkv G212E rev (prKW 147) 5'-CCAGCCAGACCTCTTCATATCGTCCTT TG-3'

To induce the G256E mutation, I used the following primers:

tkv G256E fwd (prKW 148) 5'- CGACAATATCTTGGAATTCATTGCCGCCG-3'

tkv G256E rev (prKW 149) 5'- CGGCGGCAATGAATTCCAAGATATTGTCG-3'

The *STT* mutant cDNAs were shuttled from *pDONR221* into the *pAWF* Gateway vector by LR recombinase (Invitrogen).

Transfection of S2 cells and *brkSE-lacZ* signaling assays were performed as previously described. 50ng of each receptor construct was cotransfected with 50 ng *gbb* to determine their affect on Gbb-induced signaling in S2 cells. Transfections were performed in duplicate. *pAWF STT^{G212E}*-2 was selected based on its ability to inhibit Gbb-induced signaling.

To test if STT^{G212E}-2 could activate Sax kinase activity, the ability of 25 ng *sax* cotransfected with 25 ng *STT^{G212E}* to affect Gbb signaling was compared to 25 ng *sax* cotransfeced with25 ng *STT*. 100ng of *gbb* was transfected since 50 ng of *gbb* only mildly activated BMP signaling in the prior assay. of each receptor was transfected for a total of 50ng of receptor DNA. As controls 50ng of *sax, STT,* and *STT^{G212E}* were cotransfected with *gbb*.

Results and Conclusions

Two STT mutants (STT^{G212E} and STT^{G256E}—amino acid position based on Tkv) were generated based on reported glycine mutations that inactivated the kinase activity of TβRI (Weis-Garcia and Massagué, 1996). Two independent clones of each mutant were tested for its effect on Gbb signaling in our *brkSE-lacZ* assay (**Fig. 6.10**). All receptors that were transfected independent of ligand inhibited background BMP signaling. Transfection of *gbb* alone mildly induced signaling (~80% *brkSE-lacZ* repression). Cotransfection of *sax* inhibited Gbb-induced signaling, whereas *STT* enhanced Gbb-induced signaling consistent with previous results. Cotransfection of the STT^{G212E}-2 clone inhibited Gbb signaling, whereas the STT^{G212E}-5 clone did not affect Gbb signaling. Both of the STT^{G256E} clones, however, enhanced Gbb signaling. Based on these results, STT^{G212E}-2 was selected for further analysis.

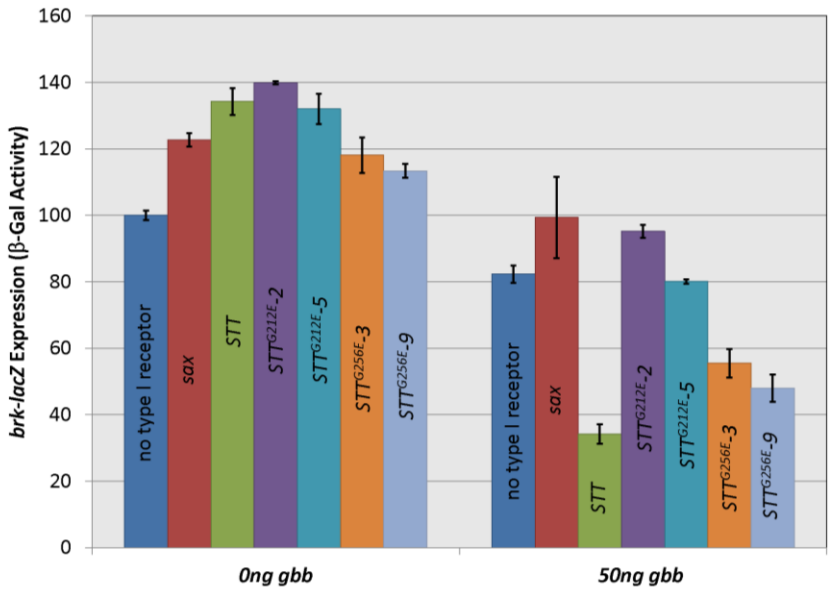


Figure 6.10. Effect of STT mutants on Gbb-induced repression of the BMP responsive *brkSE-lacZ* reporter. Left group, Transfection of receptors alone. All receptors inhibited background levels of BMP signaling. **Right group**, receptors were cotransfected with 50 ng gbb. STT^{G212E}-2 inhibits Gbb signaling, whereas STT^{G212E}-5 does not affect Gbb signaling. Both STT^{G256} clones enhhance Gbb signaling. Data plotted are the mean of an experiment performed in duplicate. Error bars represent high/low measurements.

Different combinations of *sax, STT*, and STT^{G212E} were cotransfected with *gbb* into S2 cells to determine if the presence of the Tkv kinase domain could activate Sax kinase activity. Total transfected receptor DNA was kept constant at 50 ng. Transfection of *gbb* activated BMP signaling, but a p value of 0.055 indicates borderline statistical significance (**Fig. 6.10'**,2 vs 1) . If the Tkv kinase domain activatse Sax, then the level of Gbb-induced signaling in cells coexpressing *sax* and STT^{G212E} (**Fig. 6.10,** 3) should be intermediate to signaling in cells expressing only *sax*(**Fig. 6.10,** 5) and cells coexpressing *sax* and wild-type *STT* (**Fig. 6.10,** 4).

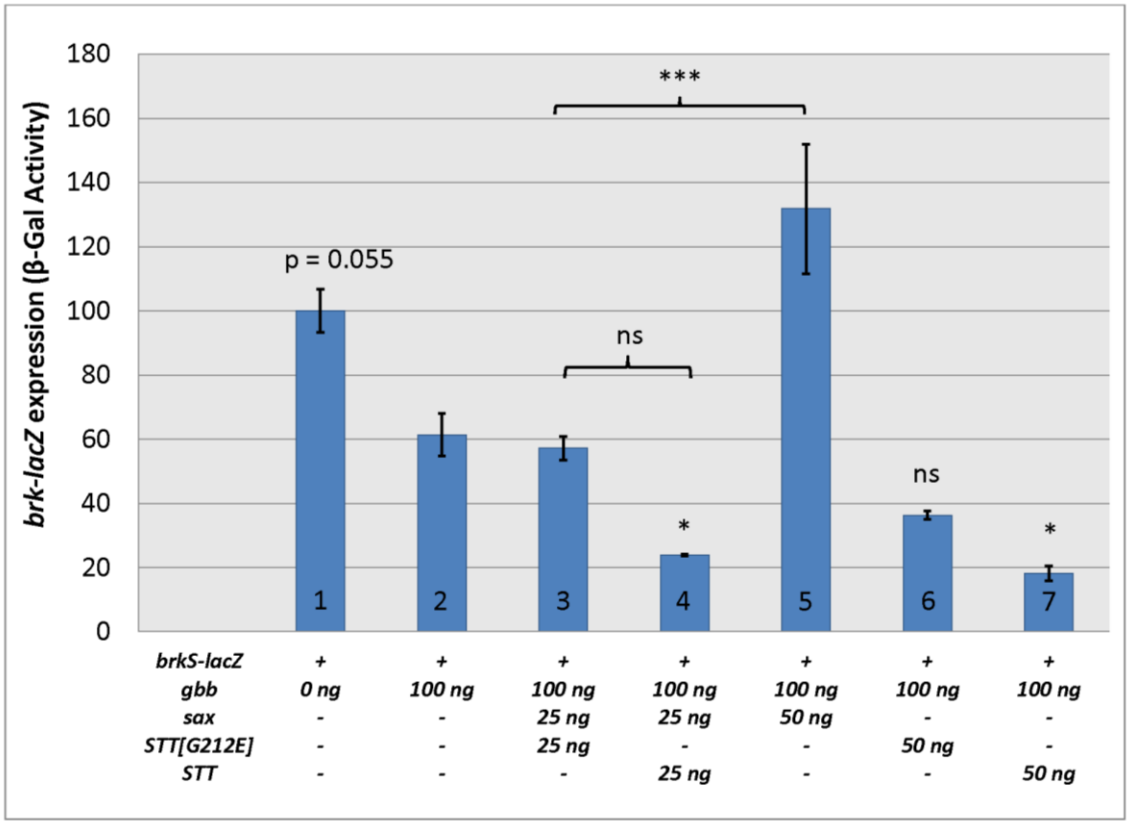


Figure 6.10'. Effect of Tkv kinase domain on the behavior of Sax. Results are inconclusive because in this assay STT^{G212E} is not behaving as a dominant negative, kinase dead receptor. BMP signaling was measured by the repression of a *brkSE-lacZ* reporter. Data plotted are the mean (± SEM) of an experiment performed in triplicate. Statistical significance reported are in relation to *gbb* transfected alone (column 2) unless otherwise indicated. Multiple pairwise comparisons were performed in R using the Tukey HSD test to determine statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

However, we did not observe this in our assay. Although we measured higher Gbb signaling in cells coexpressing *sax* and STT^{G212E} compared to cells expressing *sax* alone (**Fig. 6.10**, 3 vs 5), there was no statistically significant difference when compared to cells coexpressing *sax* and wild-type *STT* (**Fig. 6.10**, 3 vs 4).

As a kinase dead receptor, we expected STT^{G212E} to behave dominant negatively to inhibit Gbb-induced signaling. However, cotransfecting STT^{G212E} with *gbb* did not affect

Gbb-induced signaling either way, calling into question whether *STT*^{G212E} is truly kinase dead. This result may also reflect the decision to increase the amount of transfected *gbb* to 100ng in this assay because in the previous assay transfecting 50 ng of *gbb* activated the pathway modestly. We were concerned that with such modest Gbb-induced signaling we might not accurately observe potential receptor-mediated inhibition.

Therefore, we cannot conclude whether or not the Tkv kinase domain can activate the Sax kinase based on these results because STT^{G212E} is not behaving as a dominant negative kinase dead receptor. However, other mutations can be induced that might abolish STT's kinase activity. For instance, mutating the conserved lysine 232 in TβRI to an arginine (K232R) inactivates the receptors kinase activity. Mutating the corresponding lysine in BMPR1b generates a dominant negative receptor (Zou and Niswander, 1996). Furthermore, mutations associated with *tkv* alleles such as *tkv*^{4} and *tkv*^{7} can be evaluated for their signaling activity in our *brkSE-lacZ* assay and potentially be used to inactivate STT kinase activity.

Appendix 6.11 – ALK2$^{\Delta GS}$ is not constitutively active

Rationale

The GS domain of type I receptors plays an important role in regulating type I receptor kinase activity. Structural studies indicate that in its unphosphorylated state, the GS domain acts as an inhibitory wedge that sits in the cleft of the kinase domain inhibiting kinase activity—a conformation that is presumably kept locked in place by the inhibitor FKBP12, which binds to an LP motif in the GS domain. This inhibition is relieved when ligand facilitates the interaction between type I and type II receptors to form a heteromeric complex in which the type II receptor phosphorylate the GS domain of the type I receptor . Phosphorylation of the serines in the GS domain coincides with release of the FKBP12 molecule and changes the GS domain conformation such that it kicks out from the cleft thereby relieving inhibition of kinase activity. The phosphorylated GS domain also has been shown to participate in binding Smad proteins, which are the substrates for type I receptor phosphorylation (Huse et al., 2001)

In vitro kinase assays reveal that GS domain deletion mutants (ΔGS) of type I receptors have higher basal level of kinase activity than their full –length counterparts, which is consistent with the GS domain's inhibitory function in type I receptor kinase activity (Groppe, personal communication). This suggests that the activity of dGS domain mutants might be ligand-independent since phosphorylation of the GS domain by the type II receptor is facilitated by ligand-binding. To test this in a more biologically relevant system we generated an ALK2 $^{\Delta GS}$ construct and assayed its ability to phosphorylated Mad in S2 cells.

Materials and Methods

Cloning: The ALK$^{\Delta GS}$ construct was generated by Quikchange Site-Directed Mutagenesis using the following primers which remove the GS domain (AA –AA). pDONR ALK2 was used as the template. Following sequencing for confirmation of the deletion, the ALK2$^{\Delta GS}$ was shuttled from the pDONR221 vector to pAWF by LR reaction (Invitrogen).

S2 cell culture maintenance and transfection: S2 cells were cultured under standard conditions (M3 media pH 6.5, suppl with 10% IMS, 2% FBS). 8x10^6 cells (at 4.0x10^6/mL) were used per transfection. Cells were cotransfected with 700 ng pAC FLAG-Mad (pKW338) and 300 ng of one of the following contructs: pAWF ALK2, pAWF ALK2^{R206H}, or pAWF ALK2^{dGS}.

Western Blot analysis: Seven days post-transfection, transfected cells were harvested by centrifuge (5 min spin at 0.4 rcf). Supernatant was discarded and cell pellets were lysed with 0.5 volumes of 2x SDS buffer. Cell lysates were run on a 12% SDS-PAGE gel (standard protocol) and protein transferred onto PVDF membrane by semi-dry transfer. The following antibodies were used for western blot analysis: a-PS3 Rb (1:1000, Epitomics) to detect pMad and a-FLAG M2 (1:1000, Sigma) to detect receptor expression.

Results and Conclusions

pMad was only detected in cells expressing ALK2^{R206H} consistent with the mutant receptor's constitutive activity. Neither ALK2^{WT} nor ALK2 $^{\Delta GS}$ were sufficient to induce phosphorylation of Mad indicating that deleting the GS domain does not confer constitutive activity. This may reflect an inherent difference in the requirements for type I receptor kinase activity *in vitro* and in cells. For one, the *in vitro* kinase assay uses purified truncated type I receptors which lack all sequences upstream of the kinase

domain (i.e. extracellular ligand-binding domain, transmembrane domain, and GS domain). The construct I generated lacks only the GS domain and, therefore, it is possible that the other domains that remain intact may also impact the ability of ALK2$^{\Delta GS}$ to phosphorylate Mad.

Based on these results alone, however, we cannot conclude whether the GS domain is required for Mad phosphorylation. Future experiments should determine whether ALK2$^{\Delta GS}$ can tranduce BMP ligand-induced signaling (e.g. by treating ALK2$^{\Delta GS}$ – transfected S2 cells with BMP7-conditioned media and blotting for pMad) and if the GS domain is required for ALK2-Mad interaction through coimmunoprecipitation studies.

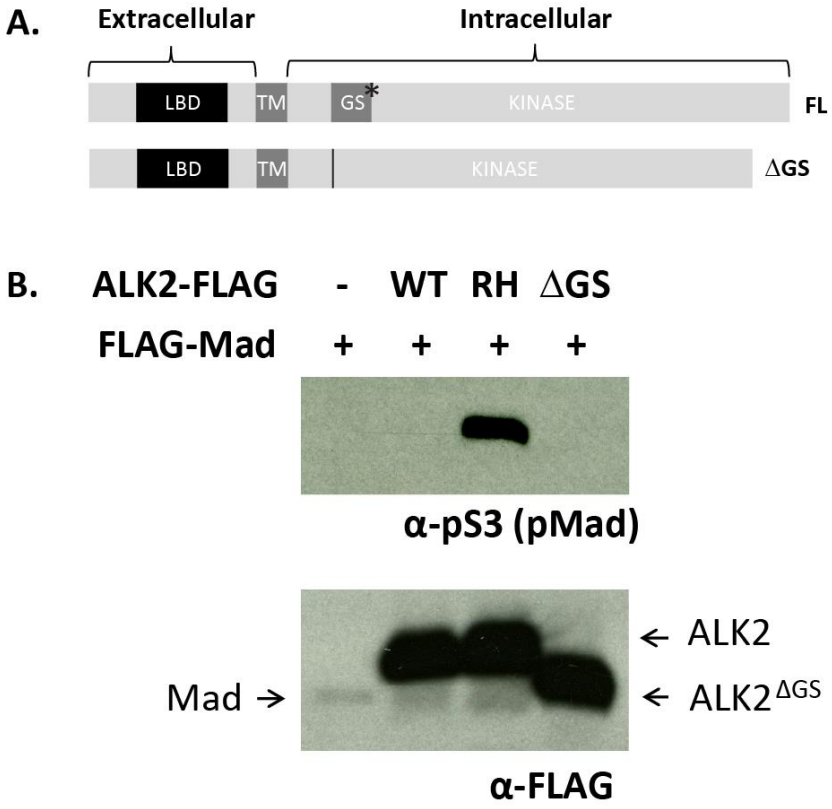


Figure 6.11. ALK2$^{\Delta GS}$ does not induce Mad phosphorylation in S2 cells. A. Diagram comparing full-length ALK2 (FL) and ALK2$^{\Delta GS}$ (ΔGS). * indicates location of the R206H mutation. LBD= ligand-binding domain. TM = transmembrane domain. GS = GS (Glycine-Serine Rich) domain. **B.** Western blot of protein extracts from S2 cells transfected with the indicated constructs. Arrows point to predicted sizes for Mad, ALK2, and ALK2$^{\Delta GS}$. As expected, ALK2^{dGS} runs smaller than the full-length ALK2 protein. RH= R206H.

Appendix 6.12 – Effect of FKBP2 on BMP signaling

Rationale

Fk506-binding protein 1A (FKBP1A) has been shown to inhibit both BMP and TGF-β signaling in mammalian cell culture by binding to the GS domain of the type I receptor thereby preventing its phosphorylation (Chen et al., 1997; Huse et al., 2001; Wang et al., 1996; Wang and Donahoe, 2004). Interestingly, the classic FOP mutation, R206H, disrupts ALK^{R206H} – FKBP1A binding, and this disruption has been proposed to be the molecular mechanism underlying constitutive activity of the $ALK2^{R206H}$ receptor (Groppe et al., 2007). Conflicting reports exist, however, in FKBP1A's ability to inhibit $ALK2^{R206H}$. (Song et al., 2010) report that transfected FKBP1A inhibits $ALK2^{R206H}$-induced expression of the BMP target gene *Alp* in C2C12 cells, whereas transfected FKBP1A was unable to inhibit $ALK2^{R206H}$ signaling as measured by a BMP-responsive element (bre) *luc* reporter in Bovine Aortic Endothelial Cells (BAECs) (van Dinther et al., 2010). This discrepancy in FKBP1A's inhibitory behavior, however, may reflect either cell-specific or even reporter specific behavior.

Score	Expect	Method	Identities	Positives	Gaps
177 bits(450)	6e-58	Compositional matrix adjust.	82/108(76%)	93/108(86%)	0/108(0%)

```
FKBP1A(Hs)  MGVQVVPIAPGDGSTYPKNGQKVTVHYTGTLDDGTKFDSSRDRNKPFKFTIGKGEVIRGW  60
            MGVQV  I+PGDG T+PK GQ   VHYTG L+DG KFDSSRDRNKPFKF +GK EVIRGW
FKBP2(Dm)   MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGW  60

FKBP1A(Hs)  DEGVAQLSVGQRAKLICSPDYAYGSRGHPGVIPPNSTLTFDVELLKVE  108
            +EGVAQ+SVGQRAKL  SPDYAYG+ GHPG+IPP++TL FDVELLK+E
FKBP2(Dm)   EEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE  108
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Figure 6.12. **Sequence alignment of the human protein, FKBP1A, and its Drosophila homolog, FKBP2**. Hs = Homo sapiens. Dm = Drosophila melanogaster. + = highly similar amino acid. The proteins display 76% sequence identity and 86% sequence similarity.

Although the *Drosophila* homolog FKBP2 is well-conserved, its role in BMP signaling remains uncharacterized (**Fig. 6.12).** Here, we address the ability of FKBP2 to inhibit *Drosophila* BMP signaling in cell culture and *in vivo*. Furthermore, we tested the ability of FKBP2 to inhibit and interact with ALK2^{R206H} in S2 cells.
combinations indicated for pAW hBMP7, pAWF ALK2, pAWF ALK2^{R206H}, and pAWH FKBP2.

Materials and Methods

Transfections and *brkSE-lacZ* signaling assays were performed as previously described. S2 cells were cotransfected with pAW gbb (pKW299) and 5 ng, 25 ng, or 50ng of pAWH-FKBP2 respectively to test if FKBP2 could inhibit Gbb-induced signaling in a dose-dependent manner. To test the ability of FKBP2 to inhibit ALK2^{R206H}-mediated signaling, S2 cells were transfected with 30 ng each of the following constructs in the

Fly crosses: *Ms1096-Gal4* males were crossed to *w;UAS-FKBP2 RNAi/Cyo*? virgins. Flies were raised at 25^{o}C. Adult wings were harvested dehydrated in 95% alcohol overnight followed by 3 washes of Xylenes, 1 wash of acetone, and mounted in DPX mountant.

Coimmunoprecitipations: 500 ng of the following receptor constructs: pAWF sax (pKW270), pAWF sax^{K262H} (pKW 231), pAWF ALK2, pAWF ALK2^{R206H} were cotransfected respectively with 500 ng of pAWH FKBP2. Transfected cells were cultured for 7 days at 25^{o}C and then solubilized with Triton X-100 at a final concentration of 0.1%. FLAG-tagged receptors were immunopurified by a-FLAG (M2) antibody bound to protein G Sepharose beads overnight at 4^{o}C. Beads were washed 3x with Wash buffer (Tris pH 7.4) and immunopurified protein complexes were eluted by boiling for 5'. Samples were run on 12% SDS-PAGE gel and analyzed by western blot. Primary

antibodies used were A-FLAG M2 (Sigma 1:1000) and A-HA 3F10 (Roche 1:1000). Light-chain specific anti-mouse IgG secondary antibody was used to avoid detecting heavy chain which runs near the FLAG-tagged receptors.

Results and Conclusions

FKBP2's ability to inhibit BMP signaling was tested in S2 cells using the *brkSE-lacZ* signaling assay **(Fig. 6.12')**. Transfection of *gbb* was sufficient to activate BMP signaling as evidenced by the loss of *brkSE-lacZ* expression. Cotransfection of *FKBP2* relieved *gbb*-induced repression of *brkSE-lacZ* reporter expression in a dose-dependent manner. This indicates that FKBP2 can inhibit ligand-induced BMP signaling.

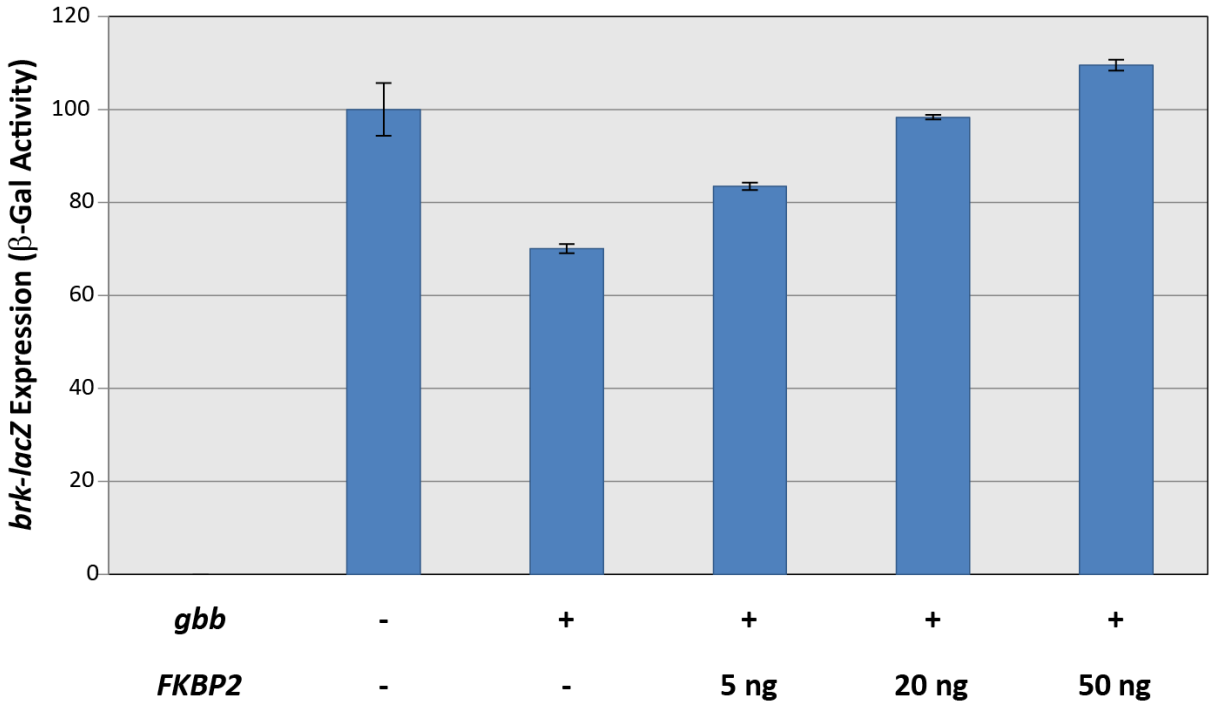

Figure 6.12'. Inhibition of BMP signaling by FKBP2 is dose-dependent. *brkSE-lacZ* signaling assay in S2 cells measures BMP signaling as a loss of B-Gal activity. 100 ng of *pAW gbb* were transfected where indicated (+). *pAWH FKBP2* was transfected at the indicated amounts. Data represent mean (±high/low observation, n=2)

Furthermore, knocking down FKBP2 function by driving FKBP2RNAi in the wing resulted in wing vein phenotypes that are reminiscent of mild *gbb*-overexpression phenotypes (Bangi and Wharton, 2006b; Haerry et al., 1998; Le and Wharton, 2012). Extra vein tissue was observed along longitudinal vein 2 (L2), and at the tip of longitudinal vein 5 (L5) in 23% and 19% of adult wings, respectively. Furthermore, 33% of adult wings displayed PCV "spurs" which have been observed in wings with dysregulated BMP signaling. Given the importance of BMP signaling in wing vein tissue patterning, these results support an inhibitory role for FKBP2 in BMP signaling.

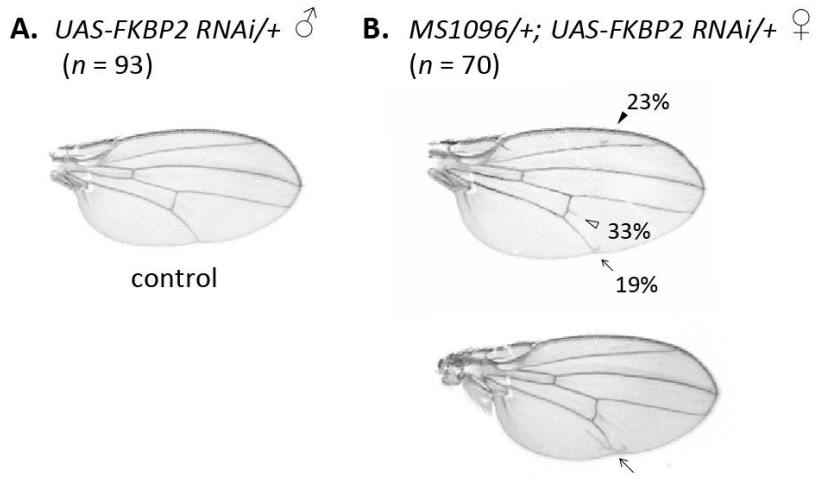

Figure 6.12". FKBP2 RNAi knockdown in the wing results in extra vein tissue. A. UAS-FKBP2/+ control wing displaying wild-type wing vein pattern. **B.** Extra vein tissue observed in adult wings expressing FKBP2-RNAi driven by MS1096-Gal4. Closed arrowhead, extra tissue along longitudinal vein 2 (L2). Open arrowhead, posterior cross vein (PCV) "spur." Arrow, extra tissue at the tip of longitudinal vein 5 (L5). Lower wing displays more severe L5 phenotype. Indicated are the percentages of wings displaying each phenotype.

Given the apparent ability of FKBP2 to inhibit BMP signaling, we next tested whether FKBP2 could inhibit BMP signaling activity induced by ALK^{R206H}. Using the *brkSE-lacZ* signaling assay, FKBP2 was unable to inhibit $ALK2^{R206H}$-mediated repression of reporter activity. This supports a report that the mammalian homolog FKBP1A is unable to inhibit signaling induced by $ALK2^{R206H}$ (van Dinther et al., 2010).

We also observed the ability of *ALK2* to enhance *mBMP7*-induced signaling which further supports that BMP7 binds to the ALK2 receptor (ten Dijke et al., 1994; Greenwald et al., 2003; Le and Wharton, 2012; Macías-Silva et al., 1998). Interestingly, FKBP2 was unable to inhibit signaling induced by *mBMP7* in the presence or absence of transfected *ALK2*. This is in contrast to FKBP2's ability to suppress *gbb*-induced signaling and suggests that the inhibition of BMP signaling by FKBP2 is receptor-specific.

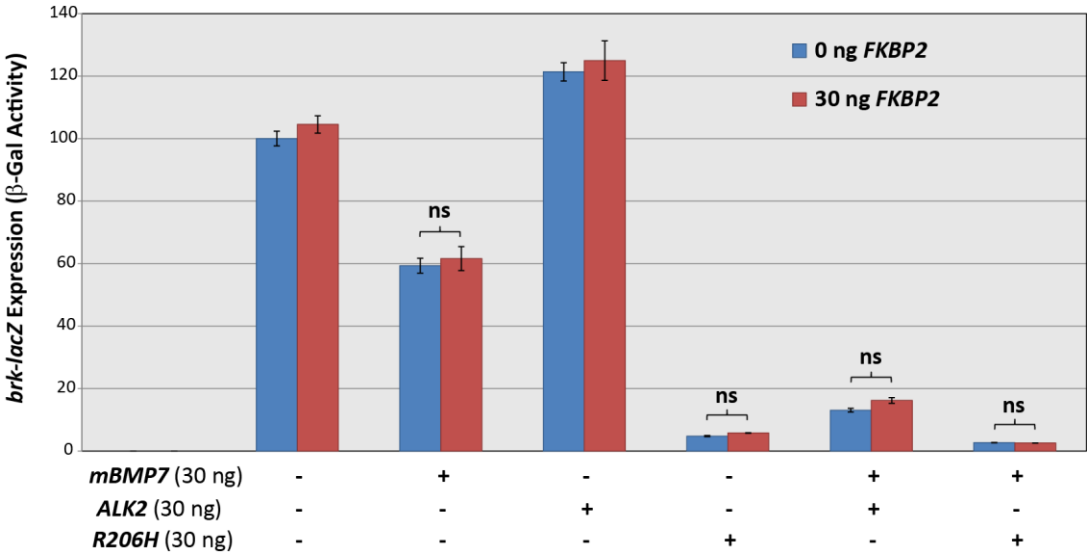

Figure 6.12'''. FKBP2 cannot inhibit BMP signaling induced by ALK2^{R206H} or mBMP7. *brkSE-lacZ* signaling assay in S2 cells measures BMP signaling as a loss of B-Gal activity. Data represent mean (±SEM) of experiment performed in triplicate. Multiple pairwise comparisons were performed in R using the Tukey HSD test. ns = not significant ($p > 0.05$).

Intriguingly, neither the R206H mutation in ALK2 nor the corresponding mutation in Sax, K262H, disrupts binding of FKBP2 **(Fig. 6.12'''')**. When cotransfected in S2 cells, equivalent amounts of FKBP2 coimmunoprecipitated with Sax^{WT}, Sax^{K262H}, ALK2, and ALK2^{R206H}. This is in contrast to published reports indicating that the R206H mutation destabilizes the interaction between ALK2^{R206H} and FKBP2 (Groppe et al., 2011; Shen et al., 2009; Song et al., 2010).

Taken together, these results suggest that FKBP2, like its mammalian homolog FKBP1a, can inhibit BMP signaling. Interestingly, FKBP2's effect appears to be ligand-specific as it can inhibit *gbb*-induced signaling (**Fig. 6.12'**), but not BMP7-induced signaling (**Fig. 6.12'''**). Additionally, it has been shown that the mammalian homolog FKBP1A can inhibit BMP signaling induced by BMP6 but not BMP2 (van Dinther et al., 2010; Kugimiya et al., 2005). However, the apparent ligand-specific effects of FKBP2/FKBP1A might actually be a reflection of the interaction of FKBP1A/FKBP2 with specific type I receptor. Given that ligand-type I receptor pair preferences have been observed (i.e. Gbb has higher affinity for Sax, Dpp has higher affinity for Tkv), FKBP1A/FKBP2 might be exhibiting its inhibitory effects in a type I receptor-specific fashion.

Interestingly, both the BMP ligands and the BMP type I receptors can be classified into groups based on their homology, biological functions, and phylogenetic analysis (Mueller and Nickel, 2012; Newfeld et al., 1999; Nickel et al., 2009) . One major group of BMP ligands is characterized by BMP6,7,8 to which Gbb and Scw belong, whereas Dpp belongs to the BMP2/4 (Fritsch et al., 2010; Newfeld et al., 1999; Padgett et al., 1993; Sampath et al., 1993; Wharton et al., 1991). For the type I receptors, Sax/ALK1/2 constitutes one branch (Chen and Massagué, 1999) , whereas Tkv/ALK3/6 constitutes the other branch (Chen and Massagué, 1999; Newfeld et al., 1999). Furthermore, broadly speaking, the Gbb/BMP6/7/8 branch of ligands displays a preference for signaling through the Sax/ALK1/2 branch of type I receptors and the Dpp/BMP2/4 group of ligands exhibits a preference for the TKV/ALK3/6 branch of type I receptors (ten Dijke et al., 1994; Ebisawa et al., 1999; Haerry et al., 1998; Haerry, 2010; Macías-Silva et al., 1998; Penton et al., 1994).

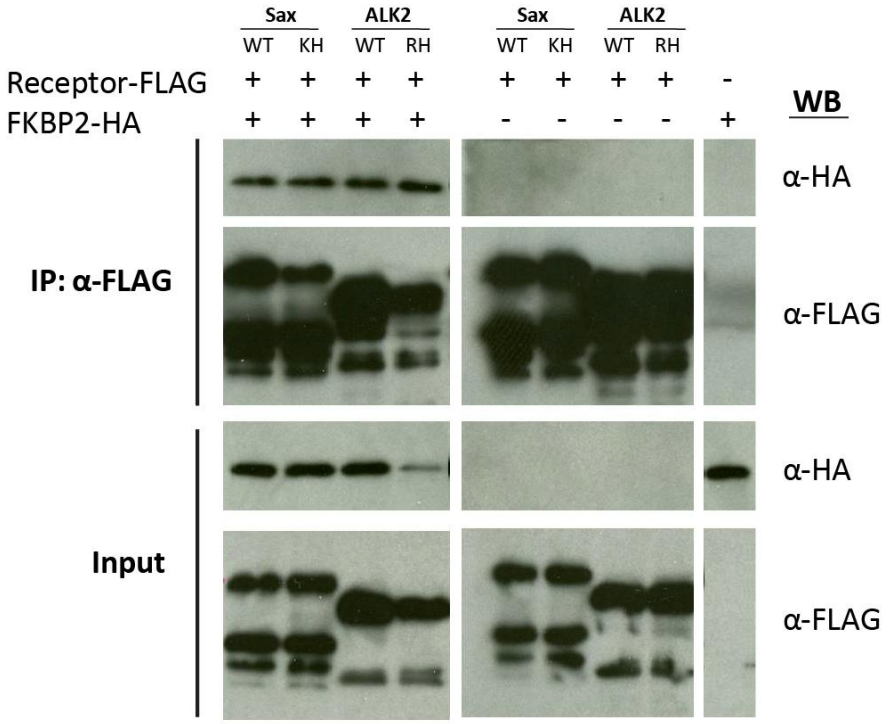

Figure 6.12"". FKBP2 binding to ALK2^{R206H} and Sax^{K262H} is not disrupted. Equivalent amounts of FKBP2-HA coimmunopreciptates with Sax, Sax^{K262H}, ALK2, and ALK2^{R206H}. WT = wild-type, KH = K262H mutation, RH =R206H mutation. Lower molecular weight bands in the α-Flag blots have been previously observed and may correspond to cleavage or degradation products of the Sax and ALK2 receptors. FKBP2 (~12kDA).

One potential model that arises from this information is that FKBP2/FKBP1A can only inhibit BMP signaling that is mediated through the Sax/ALK1/2 group of type I receptors. Our results also demonstrate that FKBP2 cannot inhibit ALK^{R206H} induced BMP signaling, consistent with findings published by (van Dinther et al., 2010). However, our results do not support a model in which the R206H mutation in ALK2 or the corresponding K262H mutation in Sax disrupts FKBP2 binding since equivalent amounts of FKBP2 coimmunoprecipitated with ALK2^{R206H}, Sax^{K262H}, and their wild-type counterparts. This unexpected indicates that ALK2^{R206H} can constitutively signal despite maintaining an interation with the inhibitor FKBP2. It is possible that this reflects an

inherent difference between the *Drosophila* protein FKBP2 and its mammalian homolog FKBP1A, which displays reduced binding to ALK^{R206H}.

Although the neither of mutations disrupted FKBP2 binding, we did not test how BMP ligand treatment would affect the interaction of FKBP2 with either Sax^{K262H} or $ALK2^{R206H.}$ It is conceivable that the K262H and R206H mutations only destabilize the interaction with FKBP2 slightly such that dissociation of FKBP2 would still require binding of BMP ligand to the mutant type I receptor. Therefore, the coimmunoprecipitation study (**Fig. 6.12””**) described here should be repeated with an additional condition in which the transfected cells would be treated with BMP ligand (Gbb for cells transfected with the Sax constructs and BMP7 for the cells transfected with the ALK2 constructs). The results from such an experiment would not only address whether dissociation of FKBP2 form the mutated type I receptors is ligand-dependent, but it could also provide insight into the nature of Sax-mediated signaling. For instance, the ability of Sax^{K262H} to mediate Gbb-induced signaling and the inability of wild-type Sax to do so may be explained if Gbb-binding induces the dissociation of FKBP2 only from Sax^{K262H}, and not wild-type Sax, thereby relieving the inhibition imposed by FKBP2.

Appendix 6.13 – Determining if Sax phosphorylates Mad at only one target serine site.

Rationale

Activation of the TGF-β superfamily signaling pathways requires the phosphorylation of two serines the C-terminus of the R-Smad substrates (Abdollah et al., 1997a; Hoodless et al., 1996; Inoue et al., 1998; Kretzschmar et al., 1997; Souchelnytskyi et al., 1997). The dual behavior of Sax can be explained if Sax is capable of phosphorylating one of these serine targets (**Fig. 6.13**). In this model, Sax:Sax complexes would bind BMP ligand but not be able to transduce signals as a result of incomplete phosphorylation. In Sax:Tkv complexes, however, Sax would cooperate with Tkv to phosphorylate Mad. It is possible that the rate of Mad phosphorylation would be increased by dividing the labor of phosphorylating the serine targets between Sax and Tkv.

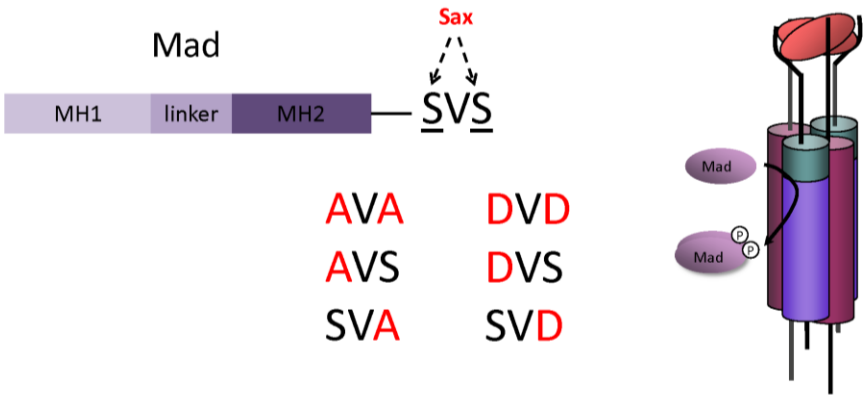

Figure 6.13. Mad C-terminal phosphorylation sites. BMP signaling induces the phosphorylation of a SXS motif at the C-terminus of Smads. Mad is divided into three domains: MH1, linker, and MH2. DNA binding activity resides in the MH1. The linker domain is subject to posttranslational modifications that regulate Smad stability. Transactivation activity and the SXS motif is located in the MH2 domain. Sax may phosphorylate only one of these serine residues. Alanine mutations were induced to abolish phosphorylation sites. Aspartic acid muations were induced as phosphoserine mimetics.

Materials and Methods

S2 cells (8 x 10^6 cells) were separately transfected with 1 μg of pAC-FLAG-Mad^x constructs and either pAWF *tkv* or pAWF *STT*. Five days post transfection, cells transfected with *tkv* or *STT* were collected by centrifuging at 2000rpm for 5 minutes at room temperature. Cell pellets were resuspended 1 volume of S2 cell-conditioned media or 1 volume of Gbb-HA conditioned media and incubated at room temperature rotating for 1.5 hours to allow receptor complex formation. After incubation step, media-treated cells expressing type I receptors and cells expressing $Mad^{X\ constructs}$ were pelleted by centrifuging at 2000rpm for 5 minutes at room temperature and then solubilized using 0.5 volume PBS-1% Triton X-100 at 4^o for 10 minutes. Uncleared lysates were combined at equal volumes +/- 1 μCi γ-^{32}P-ATP. A time course of samples were taken to track the dynamics of Mad phosphorylation. X= WT, AVA.

Results and Conclusions

To determine if Sax can phosphorylate Mad at one serine target we first tried to directly visualize the incorporation of radiolabeled PO_4 (**Fig. 6.13'**). I generated Mad serine-alanine mutations to knockout the serine targets alone and in combination (**Fig. 6.13"**). These mutants would allow me to determine if Sax can phosphorylate only one serine target as well as distinguish if Sax has a preferred target site. This strategy involved separately transfecting S2 cells with the Mad constructs (Mad^x) and different BMP type I receptors (e.g. Tkv, STT, and Sax). The receptor-transfected cells were then treated with Gbb-HA conditioned media to induce signaling complex formation. Next, the Gbb-HA treated cells and the Mad^x transfected cells were solubilized, and the extracts were combined along with ^{32}P-labelled gamma ATP. Samples were taken over time to track the dynamics of phosphorylation by western blotting and autoradiography.

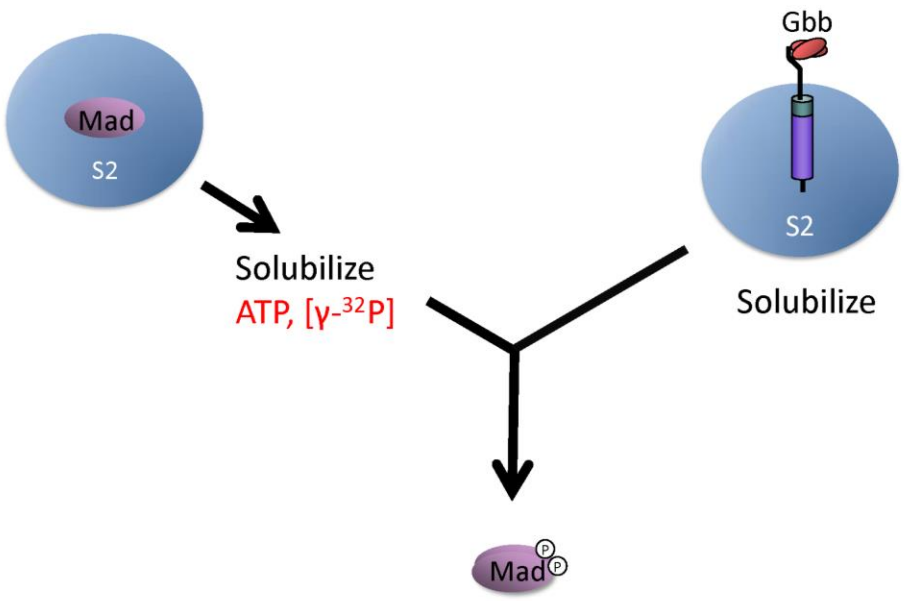

Figure 6.13'. Diagram of radioactive Mad phosphorylation assay. S2 cells are independently transfected with *Mad* or type I receptor cDNA. Type I receptor expressing cells are treated with Gbb-HA conditioned media to induced receptor complex formation. Both cell populations are then solubilized. The Mad lysate is supplemented with ATP [γ-^{32}P] and then mixed with the type I receptor lysate. Samples are taken over time to monitor the dynamics of Mad phosphorylation by autoradiography and western blotting.

In a mock trial without using gamma ATP (^{32}P), I was able to detect rapid phosphorylation of Mad (**Fig 6.13"**). However, I was unable to conclusively detect phosphorylation of Mad either by autoradiography due to significant background. This may require an immunopurification step to reduce background γ-^{32}P signal (**Fig. 6.13"'A**). A "cold" kinase assay was carried out in parallel using the same samples, and weak phosphorylation of Mad^{WT} was detected indicating that the Gbb-treated STT-HA lysates had kinase activity (**Fig. 6.13"'B**). However, the weaker phosphorylation observed in this assay compared to **Fig. 6.13"** may reflect the decision to switch from using Tkv-FLAG (**Fig. 6.13"**) to using STT-HA (**Fig. 6.13"'**). The logic being that STT-HA would improve the phosphorylation of Mad signaling via its higher affinity interaction with Gbb.

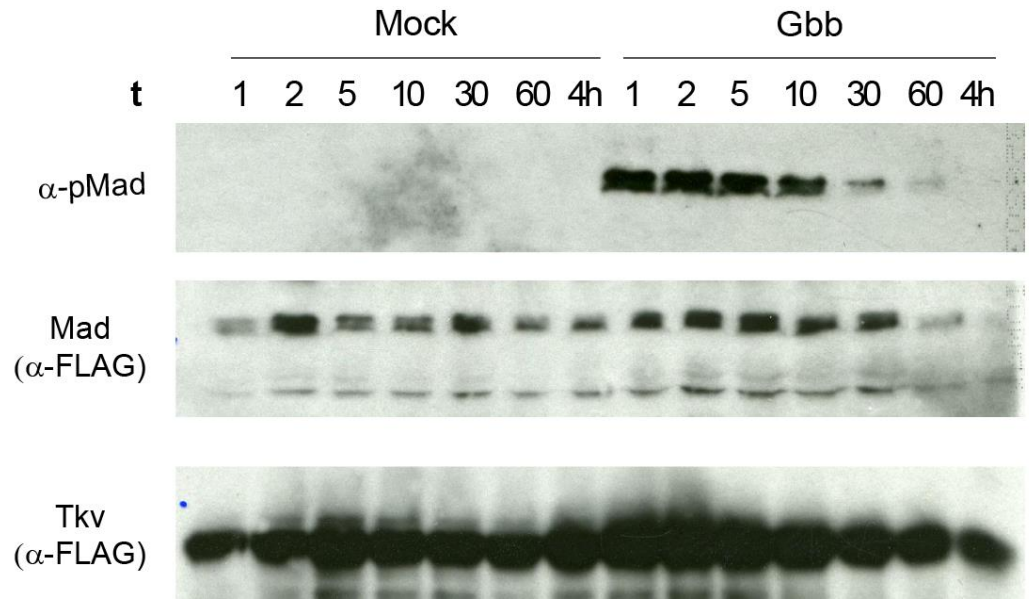

Figure 6.13". Dynamics of Mad phosphorylation. (right) Lysates of Tkv-FLAG-expressing S2 cells treated with GbbHA-conditioned media rapidly induces Mad phosphorylation. The phosphorylation signal begins degrading by 10 minutes. (left)Lysates of Tkv-Flag-expressing S2 cells mock-treated with S2 cell-conditioned media did not induce phosphorylation of Mad. Cells were incubated with S2-conditioned media or GbbHA-conditioned media for 1.5 hours to allow receptor complex formation. t, indicates amount of time that mixed lysates were incubated before sample was taken. Mad and Tkv input for each time point is indicated in the western blots below.

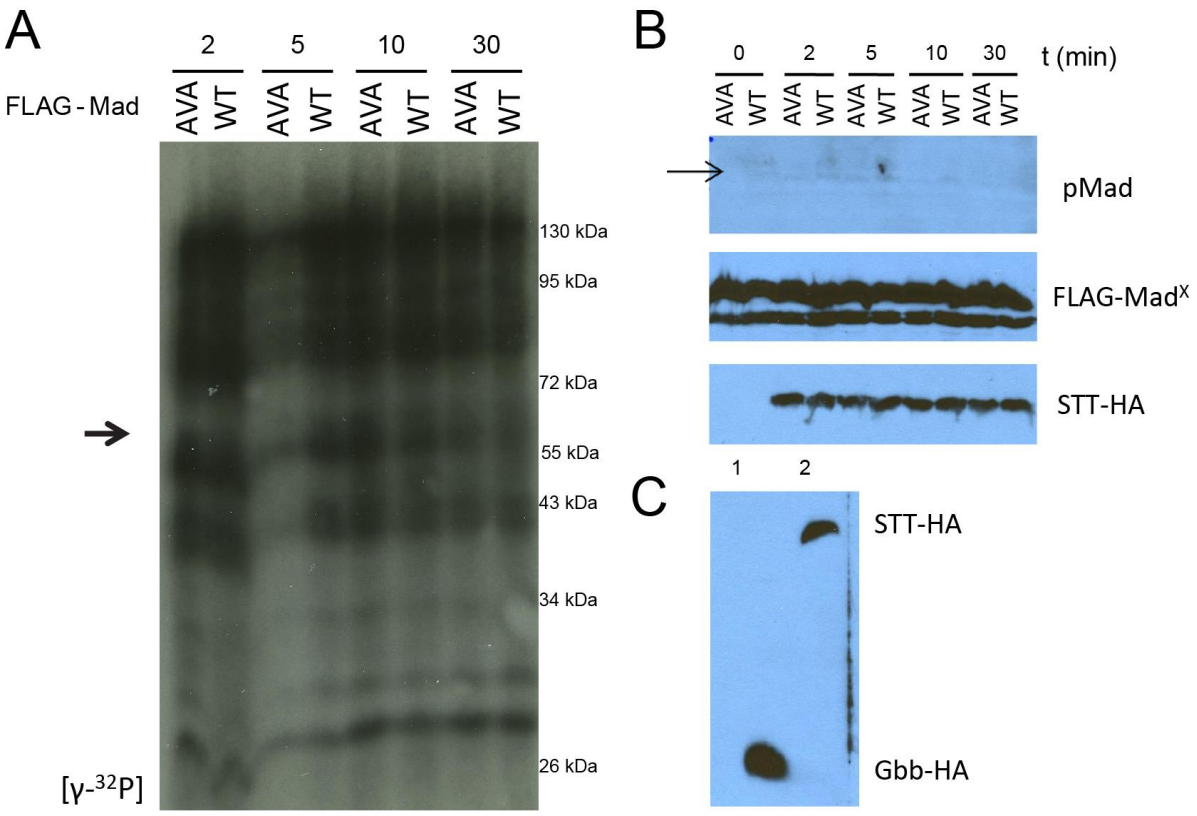

Figure 6.13'''. Radioactive Mad phosphorylation assay. STT-transfected cells were treated with GbbHA conditioned media solubilized and incubated with solubilized lysates from either Mad^{WT}- or Mad^{AVA}-expressing S2 cells. These samples were immediately split and incubated in the presence of [γ-^{32}P-ATP] (**A.**) or absence of [γ-^{32}P-ATP] (**B**.) **A.** No γ-^{32}P-labelled band could be conclusively assigned to a phosphorylated form of either Mad^{WT} or Mad^{AVA} by autoradiography. Arrow indicates predicted size of Mad. **B.** Corresponding samples were used in a "cold" assay without [γ-^{32}P-ATP]. Weak phosphorylation of only Mad^{WT} was detected at the early time points (0, 2, & 5 min) as indicated by arrow. Mad and STT input for each time sample were also measured by western blot. **C.** HA-western blot indicating the presence of GbbHA in GbbHA-conditioned media (1) and the amount of STT-HA present in the input before mixing lysates.

Appendix 6.14 – Determining if Sax can phosphorylate and restore function of Mad aspartic acid mutants.

Rationale

Given that I was unable to detect phosphorylation of Mad in the radioactive kinase assay (**Fig. 6.13'''A**), I took an alternative approach which made use of Mad serine-aspartic acid mutants in single and double mutant combinations (**Fig. 6.13**). Replacing serines with residues that have acidic side chains is often used to produce phosphomimetic mutants. Using these mutants I tested whether Sax could phosphorylate the unchanged serine residues in the phosphomimetic mutant and produce an signaling active $Mad^{pSEr\text{-}V\text{-}D}$ or $Mad^{D\text{-}V\text{-}pSer}$. We hypothesized that we would be able to detect these phosphorylated phosphomimetic mutants by western blotting using existing pSmad antibodies and that the phosphorylated phosphomimetic mutants would be able repress the *brkSE-lacZ* construct in S2 cells.

Materials and Methods

S2 cells (8×10^6 cells) were transfected with 1 µg of pAC-FLAG-Mad^Y constructs. Five days post transfection, transfected cells were colleteded by centrifuging at 2000rpm for 5 minutes at room temperature. Cell pellets were resuspended 1 volume of S2 cell-conditioned media or 1 volume of Gbb-HA conditioned media and incubated at room temperature rotating for 3 hours to stimulation of BMP signaling. After incubation step, protein extracts were prepared with 2x SDS buffer and analyzed by western blotting using standard procedures. The following antibodies were all tested at 1:1000 dilution: α-PS3 (Epitomics, monoclonal Rb), α-pSmad1/5/8 (Cell Signaling, polyclonal Rb), α-PS1 (Ed Laufer, monoclonal Rb), and α-PS1 (Ed Laufer, monoclonal GP).

50 ng of pAC-FLAG-Mad^{Y}, 50 ng of pAW *gbb*, 50 ng of pAWF *sax,* and 50 ng of pAWF *tkv* were used for S2 cell transfections for the *brkSE-lacZ* signaling assays.

Results and Conclusions

Recently, (Nojima et al., 2010) reported that a polyclonal pSmad antibody (Anti-pSmad 1,5,8 Cell Signaling polyclonal Rb) cross reacts with Mad phosphomimetics as they were able to detect a Mad double-phosphomimetic mutants (Mad^{DVD}) by immunohistochemistry as well as immunodetection by western blot (Nojima et al., 2010). I tested four different a-pSmad antibodies (including the polyclonal antibody mentioned above, but I was unable to detect any of the phosphomemitic mutants by western blot (**Fig 6.14**).

Figure 6.14. Gbb-induced phosphorylation of Mad^{SVD} and Mad^{DVS} could not be detected by western blot. Cells transfected with Mad^{WT}, Mad^{DVD}, Mad^{SVD}, or Mad^{DVS} were incubated with GbbHA conditioned media. Protein extracts were analyzed by western blotting using the α-PS3 (Epitomics,monoclonal Rb) antibody. Similar results were obtained using the α-pSmad1/5/8 polyclonal RB antibody from Cell Signaling as well as two antibodies made by Ed Laufer (α-PS1 monoclonal RB & α-PS1 monoclonal GP).

Similarly, I did not detect repression mediated by Mad^{DVD}, Mad^{SVD}, Mad^{DVS} in our *brkSE-lacZ* assay (**Fig. 6.14'**). Gbb-signaling was capable of cooperating with Mad^{WT} to repress *brkSE-lacZ*. This Gbb-Mad^{WT}-mediated repression was enhanced by Tkv and inhibited by Sax consistent with their respective facilitating and antagonistict roles. However, all of the phospho mimetic Mad mutants displayed less activity than Mad^{WT}. Intriguingly, the Mad^{DVD} was not capable of activating the pathway on its own or in combination with Gbb or Tkv. Mad^{DVD} has been used as a "constitutively" phosphorylated form of Mad and in other systems is sufficient to activate the pathway (Kokabu et al., 2011; Nojima et al., 2010).

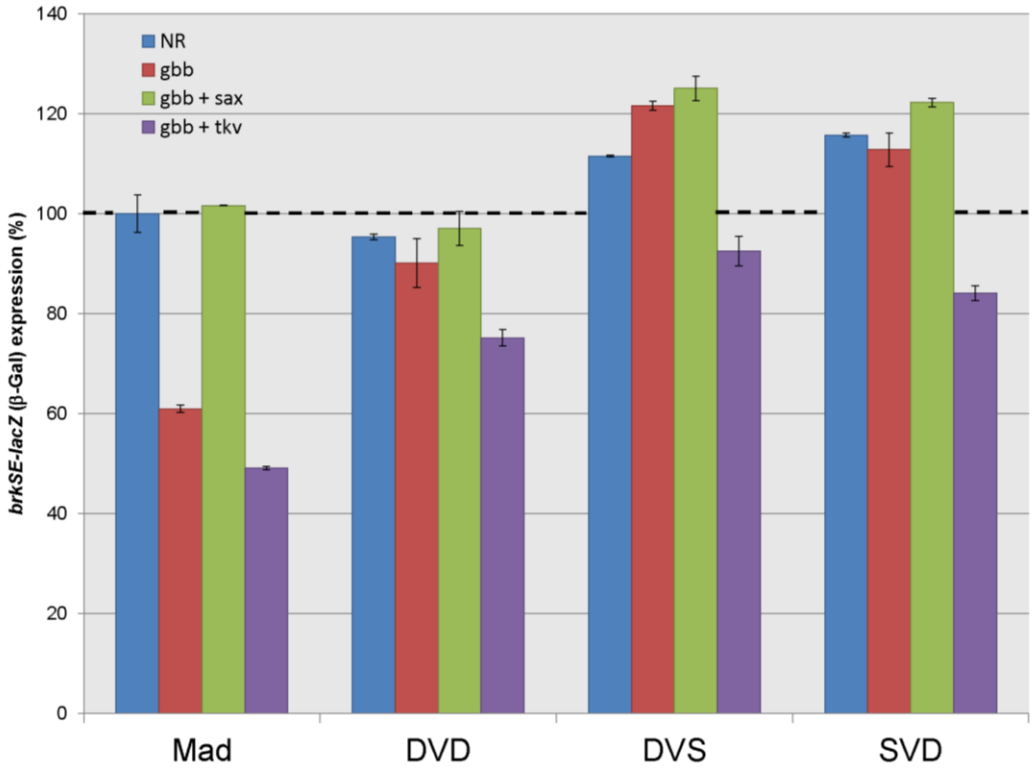

Figure 6.14'. Effect of phosphomimetic aspartic acid mutations on Mad signaling behavior. Mad phosphomimetic mutants were not able to transduce Gbb or Gbb + Tkv signals. *brkSE-lacZ* expression is repressed by BMP signaling. Therefore, loss of β-gal activity serves as a quantitative measure of BMP signaling. Data represent the mean ±SEM (n=3).

We are currently evaluating the use of a different reporter, *2xUbx-lacZ* (Kirkpatrick et al., 2001), to rule out the possibility that these are reporter specific effects. Furthermore, I am generating Mad phosphomimetic mutants using glutamic acid instead of aspartic acid in case there are amino acid specific effects as well. Smad^{EVE} mutants have also been used a constitutively phosphorylated Smad mimic (Fuentealba et al., 2007).

Literature cited

Abdalla SA, Letarte M. 2006. Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. J Med Genet 43:97–110.

Abdollah S, Macías-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL. 1997a. TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. J Biol Chem 272:27678–27685.

Abdollah S, Macı´as-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL. 1997b. TβRI Phosphorylation of Smad2 on Ser465 and Ser467 Is Required for Smad2-Smad4 Complex Formation and Signaling. J Biol Chem 272:27678–27685.

Aberle H, Haghighi AP, Fetter RD, McCabe BD, Magalhães TR, Goodman CS. 2002. wishful thinking Encodes a BMP Type II Receptor that Regulates Synaptic Growth in Drosophila. Neuron 33:545–558.

Adachi-Yamada T, Nakamura M, Irie K, Tomoyasu Y, Sano Y, Mori E, Goto S, Ueno N, Nishida Y, Matsumoto K. 1999. p38 Mitogen-Activated Protein Kinase Can Be Involved in Transforming Growth Factor β Superfamily Signal Transduction in Drosophila Wing Morphogenesis. Mol Cell Biol 19:2322–2329.

Affolter M, Nellen D, Nussbaumer U, Basler K. 1994. Multiple requirements for the receptor serine/threonine kinase thick veins reveal novel functions of TGF beta homologs during Drosophila embryogenesis. Development 120:3105–3117.

Affolter M, Basler K. 2007. The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. Nat Rev Genet 8:663–674.

Akiyama S, Katagiri T, Namiki M, Yamaji N, Yamamoto N, Miyama K, Shibuya H, Ueno N, Wozney JM, Suda T. 1997. Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. Exp Cell Res 235:362–369.

Akiyama T, Kamimura K, Firkus C, Takeo S, Shimmi O, Nakato H. 2008. Dally regulates Dpp morphogen gradient formation by stabilizing Dpp on the cell surface. Dev Biol 313:408–419.

Akiyama T, Marques G, Wharton KA. 2012. A Large Bioactive BMP Ligand with Distinct Signaling Properties Is Produced by Alternative Proconvertase Processing. Sci Signal 5:ra28.

Allendorph GP, Vale WW, Choe S. 2006. Structure of the ternary signaling complex of a TGF-beta superfamily member. Proc Natl Acad Sci U S A 103:7643–7648.

Aoki H, Fujii M, Imamura T, Yagi K, Takehara K, Kato M, Miyazono K. 2001. Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. J Cell Sci 114:1483–1489.

Armes NA, Neal KA, Smith JC. 1999. A Short Loop on the ALK-2 and ALK-4 Activin Receptors Regulates Signaling Specificity but Cannot Account for All Their Effects on EarlyXenopus Development. J Biol Chem 274:7929–7935.

Arora K, Levine MS, O'Connor MB. 1994. The screw gene encodes a ubiquitously expressed member of the TGF-beta family required for specification of dorsal cell fates in the Drosophila embryo. Genes Dev 8:2588–2601.

Ashe HL, Levine M. 1999. Local inhibition and long-range enhancement of Dpp signal transduction by Sog. Nature 398:427–431.

Attisano L, Cárcamo J, Ventura F, Weis FM, Massagué J, Wrana JL. 1993. Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. Cell 75:671–680.

Attisano L, Wrana JL, Montalvo E, Massagué J. 1996. Activation of signalling by the activin receptor complex. Mol Cell Biol 16:1066–1073.

Bagarova J, Vonner AJ, Armstrong KA, Börgermann J, Lai CSC, Deng DY, Beppu H, Alfano I, Filippakopoulos P, Morrell NW, Bullock AN, Knaus P, Mishina Y, Yu PB. 2013. Constitutively Active ALK2 Receptor Mutants Require Type II Receptor Cooperation. Mol Cell Biol 33:2413–2424.

Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V, Tabin CJ. 2006. Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. PLoS Genet 2:e216.

Bangi E. 2005. Genetic analysis of BMP activity gradient formation in the Drosophila wing imaginal disc; Providence, RI: Brown University.

Bangi E, Wharton K. 2006a. Dpp and Gbb exhibit different effective ranges in the establishment of the BMP activity gradient critical for Drosophila wing patterning. Dev Biol 295:178–193.

Bangi E, Wharton K. 2006b. Dual function of the Drosophila Alk1/Alk2 ortholog Saxophone shapes the Bmp activity gradient in the wing imaginal disc. Dev Camb Engl 133:3295–3303.

Bayrak-Toydemir P, McDonald J, Markewitz B, Lewin S, Miller F, Chou L-S, Gedge F, Tang W, Coon H, Mao R. 2006. Genotype-phenotype correlation in hereditary hemorrhagic telangiectasia: mutations and manifestations. Am J Med Genet A 140:463–470.

Bengtsson L, Schwappacher R, Roth M, Boergermann JH, Hassel S, Knaus P. 2009. PP2A regulates BMP signalling by interacting with BMP receptor complexes and by dephosphorylating both the C-terminus and the linker region of Smad1. J Cell Sci 122:1248–1257.

Bennett D, Alphey L. 2002. PP1 binds Sara and negatively regulates Dpp signaling in Drosophila melanogaster. Nat Genet 31:419–423.

Bier E. 2005. Drosophila, the golden bug, emerges as a tool for human genetics. Nat Rev Genet 6:9–23.

Billings PC, Fiori JL, Bentwood JL, O'Connell MP, Jiao X, Nussbaum B, Caron RJ, Shore EM, Kaplan FS. 2008. Dysregulated BMP signaling and enhanced osteogenic differentiation of

connective tissue progenitor cells from patients with fibrodysplasia ossificans progressiva (FOP). J Bone Miner Res Off J Am Soc Bone Miner Res 23:305–313.

Blair SS. 2007. Wing vein patterning in Drosophila and the analysis of intercellular signaling. Annu Rev Cell Dev Biol 23:293–319.

Blitz IL, Cho KWY. 2009. Finding partners: How BMPs select their targets. Dev Dyn 238:1321–1331.

Bocciardi R, Bordo D, Di Duca M, Di Rocco M, Ravazzolo R. 2009. Mutational analysis of the ACVR1 gene in Italian patients affected with fibrodysplasia ossificans progressiva: confirmations and advancements. Eur J Hum Genet EJHG 17:311–318.

Botas J. 2007. Drosophila researchers focus on human disease. Nat Genet 39:589–591.

Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A. 2011. Bone Morphogenetic Proteins: A critical review. Cell Signal 23:609–620.

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Dev Camb Engl 118:401–415.

Brown MA, Zhao Q, Baker KA, Naik C, Chen C, Pukac L, Singh M, Tsareva T, Parice Y, Mahoney A, Roschke V, Sanyal I, Choe S. 2005. Crystal structure of BMP-9 and functional interactions with pro-region and receptors. J Biol Chem 280:25111–25118.

Brummel TJ, Twombly V, Marqués G, Wrana JL, Newfeld SJ, Attisano L, Massagué J, O'Connor MB, Gelbart WM. 1994. Characterization and relationship of Dpp receptors encoded by the saxophone and thick veins genes in Drosophila. Cell 78:251–261.

Capdevila J, Guerrero I. 1994. Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. EMBO J 13:4459–4468.

Casanueva MO, Ferguson EL. 2004. Germline stem cell number in the Drosophila ovary is regulated by redundant mechanisms that control Dpp signaling. Development 131:1881–1890.

De Celis JF, Barrio R. 2000. Function of the spalt/spalt-related gene complex in positioning the veins in the Drosophila wing. Mech Dev 91:31–41.

De Celis JF, Barrio R. 2009. Regulation and function of Spalt proteins during animal development. Int J Dev Biol 53:1385–1398.

De Celis JF. 1997. Expression and function of decapentaplegic and thick veins during the differentiation of the veins in the Drosophila wing. Development 124:1007–1018.

Chaikuad A, Alfano I, Kerr G, Sanvitale CE, Boergermann JH, Triffitt JT, Delft F von, Knapp S, Knaus P, Bullock AN. 2012. Structure of the Bone Morphogenetic Protein Receptor ALK2 and Implications for Fibrodysplasia Ossificans Progressiva. J Biol Chem 287:36990–36998.

Chan MC, Nguyen PH, Davis BN, Ohoka N, Hayashi H, Du K, Lagna G, Hata A. 2007. A Novel Regulatory Mechanism of the Bone Morphogenetic Protein (BMP) Signaling Pathway

Involving the Carboxyl-Terminal Tail Domain of BMP Type II Receptor. Mol Cell Biol 27:5776–5789.

Chang H, Brown CW, Matzuk MM. 2002. Genetic Analysis of the Mammalian Transforming Growth Factor-β Superfamily. Endocr Rev 23:787–823.

Chen D, Ji X, Harris MA, Feng JQ, Karsenty G, Celeste AJ, Rosen V, Mundy GR, Harris SE. 1998a. Differential Roles for Bone Morphogenetic Protein (BMP) Receptor Type IB and IA in Differentiation and Specification of Mesenchymal Precursor Cells to Osteoblast and Adipocyte Lineages. J Cell Biol 142:295–305.

Chen HB, Shen J, Ip YT, Xu L. 2006. Identification of phosphatases for Smad in the BMP/DPP pathway. Genes Dev 20:648–653.

Chen YG, Liu F, Massague J. 1997. Mechanism of TGFbeta receptor inhibition by FKBP12. EMBO J 16:3866–3876.

Chen YG, Massagué J. 1999. Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. J Biol Chem 274:3672–3677.

Chen Y-G, Hata A, Lo RS, Wotton D, Shi Y, Pavletich N, Massagué J. 1998b. Determinants of specificity in TGF-β signal transduction. Genes Dev 12:2144–2152.

Chintapalli VR, Wang J, Dow JAT. 2007. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat Genet 39:715–720.

Clarke TR, Hoshiya Y, Yi SE, Liu X, Lyons KM, Donahoe PK. 2001. Müllerian inhibiting substance signaling uses a bone morphogenetic protein (BMP)-like pathway mediated by ALK2 and induces SMAD6 expression. Mol Endocrinol Baltim Md 15:946–959.

Clevers H. 2006. Wnt/β-Catenin Signaling in Development and Disease. Cell 127:469–480.

Conley CA, Silburn R, Singer MA, Ralston A, Rohwer-Nutter D, Olson DJ, Gelbart W, Blair SS. 2000. Crossveinless 2 contains cysteine-rich domains and is required for high levels of BMP-like activity during the formation of the cross veins in Drosophila. Development 127:3947–3959.

Constam DB, Robertson EJ. 1999. Regulation of Bone Morphogenetic Protein Activity by Pro Domains and Proprotein Convertases. J Cell Biol 144:139–149.

Cook O, Biehs B, Bier E. 2004. brinker and optomotor-blind act coordinately to initiate development of the L5 wing vein primordium in Drosophila. Development 131:2113–2124.

Cui Y, Jean F, Thomas G, Christian JL. 1998. BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development. EMBO J 17:4735–4743.

Cui Y, Hackenmiller R, Berg L, Jean F, Nakayama T, Thomas G, Christian JL. 2001. The activity and signaling range of mature BMP-4 is regulated by sequential cleavage at two sites within the prodomain of the precursor. Genes Dev 15:2797–2802.

Degnin C, Jean F, Thomas G, Christian JL. 2004. Cleavages within the Prodomain Direct Intracellular Trafficking and Degradation of Mature Bone Morphogenetic Protein-4. Mol Biol Cell 15:5012–5020.

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature 425:577–584.

Dewulf N, Verschueren K, Lonnoy O, Morén A, Grimsby S, Vande Spiegle K, Miyazono K, Huylebroeck D, Ten Dijke P. 1995. Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis. Endocrinology 136:2652–2663.

Ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K. 1994. Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 269:16985–16988.

Van Dinther M, Visser N, de Gorter DJJ, Doorn J, Goumans M-J, de Boer J, ten Dijke P. 2010. ALK2 R206H mutation linked to fibrodysplasia ossificans progressiva confers constitutive activity to the BMP type I receptor and sensitizes mesenchymal cells to BMP-induced osteoblast differentiation and bone formation. J Bone Miner Res Off J Am Soc Bone Miner Res 25:1208–1215.

Doctor JS, Jackson PD, Rashka KE, Visalli M, Hoffman FM. 1992. Sequence, biochemical characterization, and developmental expression of a new member of the TGF-β superfamily in Drosophila melanogaster. Dev Biol 151:491–505.

Dorey K, Amaya E. 2010. FGF signalling: diverse roles during early vertebrate embryogenesis. Dev Camb Engl 137:3731–3742.

Dorfman R, Shilo BZ. 2001. Biphasic activation of the BMP pathway patterns the Drosophila embryonic dorsal region. Dev Camb Engl 128:965–972.

Duan X, Liang Y-Y, Feng X-H, Lin X. 2006. Protein Serine/Threonine Phosphatase PPM1A Dephosphorylates Smad1 in the Bone Morphogenetic Protein Signaling Pathway. J Biol Chem 281:36526–36532.

Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T. 1999. Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J Cell Sci 112:3519–3527.

Ehrlich M, Horbelt D, Marom B, Knaus P, Henis YI. 2011. Homomeric and heteromeric complexes among TGF-β and BMP receptors and their roles in signaling. Cell Signal 23:1424–1432.

Feng X-H, Derynck R. 1997. A kinase subdomain of transforming growth factor-[beta] (TGF-[beta]) type I receptor determines the TGF-[beta] intracellular signaling specificity. EMBO J 16:3912–3923.

Feng X-H, Derynck R. 2005. Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol 21:659–693.

Finnson KW, Parker WL, ten Dijke P, Thorikay M, Philip A. 2008. ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. J Bone Miner Res Off J Am Soc Bone Miner Res 23:896–906.

Foletta VC, Lim MA, Soosairajah J, Kelly AP, Stanley EG, Shannon M, He W, Das S, Massagué J, Bernard O. 2003. Direct signaling by the BMP type II receptor via the cytoskeletal regulator LIMK1. J Cell Biol 162:1089–1098.

Franzén P, Heldin CH, Miyazono K. 1995. The GS domain of the transforming growth factor-beta type I receptor is important in signal transduction. Biochem Biophys Res Commun 207:682–689.

Fritsch C, Lanfear R, Ray RP. 2010. Rapid evolution of a novel signalling mechanism by concerted duplication and divergence of a BMP ligand and its extracellular modulators. Dev Genes Evol 220:235–250.

Fritsch C, Sawala A, Harris R, Maartens A, Sutcliffe C, Ashe HL, Ray RP. 2012. Different requirements for proteolytic processing of bone morphogenetic protein 5/6/7/8 ligands in Drosophila melanogaster. J Biol Chem 287:5942–5953.

Fuentealba LC, Eivers E, Ikeda A, Hurtado C, Kuroda H, Pera EM, De Robertis EM. 2007. Integrating Patterning Signals: Wnt/GSK3 Regulates the Duration of the BMP/Smad1 Signal. Cell 131:980–993.

Fujise M, Takeo S, Kamimura K, Matsuo T, Aigaki T, Izumi S, Nakato H. 2003. Dally regulates Dpp morphogen gradient formation in the Drosophila wing. Development 130:1515–1522.

Fukuda T, Kanomata K, Nojima J, Kokabu S, Akita M, Ikebuchi K, Jimi E, Komori T, Maruki Y, Matsuoka M, Miyazono K, Nakayama K, Nanba A, Tomoda H, Okazaki Y, Ohtake A, Oda H, Owan I, Yoda T, Haga N, Furuya H, Katagiri T. 2008. A unique mutation of ALK2, G356D, found in a patient with fibrodysplasia ossificans progressiva is a moderately activated BMP type I receptor. Biochem Biophys Res Commun 377:905–909.

Fukuda T, Kohda M, Kanomata K, Nojima J, Nakamura A, Kamizono J, Noguchi Y, Iwakiri K, Kondo T, Kurose J, Endo K, Awakura T, Fukushi J, Nakashima Y, Chiyonobu T, Kawara A, Nishida Y, Wada I, Akita M, Komori T, Nakayama K, Nanba A, Maruki Y, Yoda T, Tomoda H, Yu PB, Shore EM, Kaplan FS, Miyazono K, Matsuoka M, Ikebuchi K, Ohtake A, Oda H, Jimi E, Owan I, Okazaki Y, Katagiri T. 2009. Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva. J Biol Chem 284:7149–7156.

Furuya H, Ikezoe K, Wang L, Ohyagi Y, Motomura K, Fujii N, Kira J-I, Fukumaki Y. 2008. A unique case of fibrodysplasia ossificans progressiva with an ACVR1 mutation, G356D, other than the common mutation (R206H). Am J Med Genet A 146A:459–463.

Gamer LW, Nove J, Levin M, Rosen V. 2005. BMP-3 is a novel inhibitor of both activin and BMP-4 signaling in Xenopus embryos. Dev Biol 285:156–168.

Gaussin V, Putte TV de, Mishina Y, Hanks MC, Zwijsen A, Huylebroeck D, Behringer RR, Schneider MD. 2002. Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. Proc Natl Acad Sci 99:2878–2883.

Gilboa L, Nohe A, Geissendörfer T, Sebald W, Henis YI, Knaus P. 2000. Bone Morphogenetic Protein Receptor Complexes on the Surface of Live Cells: A New Oligomerization Mode for Serine/Threonine Kinase Receptors. Mol Biol Cell 11:1023–1035.

Goto K, Kamiya Y, Imamura T, Miyazono K, Miyazawa K. 2007. Selective Inhibitory Effects of Smad6 on Bone Morphogenetic Protein Type I Receptors. J Biol Chem 282:20603–20611.

Goumans M-J, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, Karlsson S, ten Dijke P. 2003. Activin Receptor-like Kinase (ALK)1 Is an Antagonistic Mediator of Lateral TGFβ/ALK5 Signaling. Mol Cell 12:817–828.

Gray AM, Mason AJ. 1990. Requirement for activin A and transforming growth factor--beta 1 pro-regions in homodimer assembly. Science 247:1328–1330.

Greenblatt MB, Shim J-H, Glimcher LH. 2010. TAK1 mediates BMP signaling in cartilage. Ann N Y Acad Sci 1192:385–390.

Greenwald J, Fischer WH, Vale WW, Choe S. 1999. Three-finger toxin fold for the extracellular ligand-binding domain of the type II activin receptor serine kinase. Nat Struct Biol 6:18–22.

Greenwald J, Groppe J, Gray P, Wiater E, Kwiatkowski W, Vale W, Choe S. 2003. The BMP7/ActRII extracellular domain complex provides new insights into the cooperative nature of receptor assembly. Mol Cell 11:605–617.

Gregory KE, Ono RN, Charbonneau NL, Kuo C-L, Keene DR, Bächinger HP, Sakai LY. 2005. The Prodomain of BMP-7 Targets the BMP-7 Complex to the Extracellular Matrix. J Biol Chem 280:27970–27980.

Gregson CL, Hollingworth P, Williams M, Petrie KA, Bullock AN, Brown MA, Tobias JH, Triffitt JT. 2011. A novel ACVR1 mutation in the glycine/serine-rich domain found in the most benign case of a fibrodysplasia ossificans progressiva variant reported to date. Bone 48:654–658.

Griffith DL, Keck PC, Sampath TK, Rueger DC, Carlson WD. 1996. Three-dimensional structure of recombinant human osteogenic protein 1: structural paradigm for the transforming growth factor beta superfamily. Proc Natl Acad Sci 93:878–883.

Grimm S, Pflugfelder GO. 1996. Control of the gene optomotor-blind in Drosophila wing development by decapentaplegic and wingless. Science 271:1601–1604.

Groppe JC, Shore EM, Kaplan FS. 2007. Functional modeling of the ACVR1 (R206H) mutation in FOP. Clin Orthop 462:87–92.

Groppe JC, Wu J, Shore EM, Kaplan FS. 2011. In vitro Analyses of the Dysregulated R206H ALK2 Kinase-FKBP12 Interaction Associated with Heterotopic Ossification in FOP. Cells Tissues Organs. http://www.ncbi.nlm.nih.gov/pubmed/21525719.

Groppe J, Hinck CS, Samavarchi-Tehrani P, Zubieta C, Schuermann JP, Taylor AB, Schwarz PM, Wrana JL, Hinck AP. 2008. Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding. Mol Cell 29:157–168.

Gruendler C, Lin Y, Farley J, Wang T. 2001. Proteasomal Degradation of Smad1 Induced by Bone Morphogenetic Proteins. J Biol Chem 276:46533–46543.

Gu Y, Jin P, Zhang L, Zhao X, Gao X, Ning Y, Meng A, Chen Y-G. 2006. Functional analysis of mutations in the kinase domain of the TGF-beta receptor ALK1 reveals different mechanisms for induction of hereditary hemorrhagic telangiectasia. Blood 107:1951–1954.

Gu Z, Reynolds EM, Song J, Lei H, Feijen A, Yu L, He W, MacLaughlin DT, Raaij J van den E, Donahoe PK, Li E. 1999. The type I serine/threonine kinase receptor ActRIA (ALK2) is required for gastrulation of the mouse embryo. Development 126:2551–2561.

Haerry TE, Khalsa O, O'Connor MB, Wharton KA. 1998. Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in Drosophila. Dev Camb Engl 125:3977–3987.

Haerry TE. 2010. The interaction between two TGF-beta type I receptors plays important roles in ligand binding, SMAD activation, and gradient formation. Mech Dev 127:358–370.

Hanks SK, Hunter T. 1995. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J 9:576–596.

Hao J, Ho JN, Lewis JA, Karim KA, Daniels RN, Gentry PR, Hopkins CR, Lindsley CW, Hong CC. 2010. In vivo structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors. ACS Chem Biol 5:245–253.

Hart PJ, Deep S, Taylor AB, Shu Z, Hinck CS, Hinck AP. 2002. Crystal structure of the human TbetaR2 ectodomain--TGF-beta3 complex. Nat Struct Biol 9:203–208.

Hartung A, Bitton-Worms K, Rechtman MM, Wenzel V, Boergermann JH, Hassel S, Henis YI, Knaus P. 2006. Different Routes of Bone Morphogenic Protein (BMP) Receptor Endocytosis Influence BMP Signaling. Mol Cell Biol 26:7791–7805.

Hassel S, Eichner A, Yakymovych M, Hellman U, Knaus P, Souchelnytskyi S. 2004. Proteins associated with type II bone morphogenetic protein receptor (BMPR-II) and identified by two-dimensional gel electrophoresis and mass spectrometry. PROTEOMICS 4:1346–1358.

Hassel S, Schmitt S, Hartung A, Roth M, Nohe A, Petersen N, Ehrlich M, Henis YI, Sebald W, Knaus P. 2003. Initiation of Smad-dependent and Smad-independent signaling via distinct BMP-receptor complexes. J Bone Joint Surg Am 85-A Suppl 3:44–51.

Hata A, Lagna G, Massagué J, Hemmati-Brivanlou A. 1998. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev 12:186–197.

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu Y-Y, Grinnell BW, Richardson MA, Topper JN, Gimbrone Jr. MA, Wrana JL, Falb D. 1997. The MAD-Related Protein Smad7 Associates with the TGFβ Receptor and Functions as an Antagonist of TGFβ Signaling. Cell 89:1165–1173.

Heinecke K, Seher A, Schmitz W, Mueller TD, Sebald W, Nickel J. 2009. Receptor oligomerization and beyond: a case study in bone morphogenetic proteins. BMC Biol 7:59.

Hinck AP. 2012. Structural studies of the TGF-βs and their receptors – insights into evolution of the TGF-β superfamily. FEBS Lett 586:1860–1870.

Ho CC, Bernard DJ. 2009. Bone Morphogenetic Protein 2 Signals via BMPR1A to Regulate Murine Follicle-Stimulating Hormone Beta Subunit Transcription. Biol Reprod 81:133–141.

Lee-Hoeflich ST, Causing CG, Podkowa M, Zhao X, Wrana JL, Attisano L. 2004. Activation of LIMK1 by binding to the BMP receptor, BMPRII, regulates BMP-dependent dendritogenesis. EMBO J 23:4792–4801.

Hoodless PA, Haerry T, Abdollah S, Stapleton M, O'Connor MB, Attisano L, Wrana JL. 1996. MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 85:489–500.

Horton WA, Lunstrum GP. 2002. Fibroblast growth factor receptor 3 mutations in achondroplasia and related forms of dwarfism. Rev Endocr Metab Disord 3:381–385.

Howe J, Sayed M, Ahmed A, Ringold J, Larsen-Haidle J, Merg A, Mitros F, Vaccaro C, Petersen G, Giardiello F, Tinley S, Aaltonen L, Lynch H. 2004. The prevalence of MADH4 and BMPR1A mutations in juvenile polyposis and absence of BMPR2, BMPR1B, and ACVR1 mutations. J Med Genet 41:484–491.

Howe JR, Bair JL, Sayed MG, Anderson ME, Mitros FA, Petersen GM, Velculescu VE, Traverso G, Vogelstein B. 2001. Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. Nat Genet 28:184–187.

Huang T, David L, Mendoza V, Yang Y, Villarreal M, De K, Sun L, Fang X, López-Casillas F, Wrana JL, Hinck AP. 2011. TGF-β signalling is mediated by two autonomously functioning TβRI:TβRII pairs. EMBO J 30:1263–1276.

Huse M, Chen YG, Massagué J, Kuriyan J. 1999. Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. Cell 96:425–436.

Huse M, Muir TW, Xu L, Chen YG, Kuriyan J, Massagué J. 2001. The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. Mol Cell 8:671–682.

Ingham PW, McMahon AP. 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev 15:3059–3087.

Inoue H, Imamura T, Ishidou Y, Takase M, Udagawa Y, Oka Y, Tsuneizumi K, Tabata T, Miyazono K, Kawabata M. 1998. Interplay of Signal Mediators of Decapentaplegic (Dpp): Molecular Characterization of Mothers against dpp, Medea, and Daughters against dpp. Mol Biol Cell 9:2145–2156.

Irish VF, Gelbart WM. 1987. The decapentaplegic gene is required for dorsal-ventral patterning of the Drosophila embryo. Genes Dev 1:868–879.

Isaacs MJ, Kawakami Y, Allendorph GP, Yoon B-H, Belmonte JCI, Choe S. 2010. Bone morphogenetic protein-2 and -6 heterodimer illustrates the nature of ligand-receptor assembly. Mol Endocrinol Baltim Md 24:1469–1477.

Itasaki N, Hoppler S. 2010. Crosstalk between Wnt and bone morphogenic protein signaling: a turbulent relationship. Dev Dyn Off Publ Am Assoc Anat 239:16–33.

Itoh S, Thorikay M, Kowanetz M, Moustakas A, Itoh F, Heldin C-H, ten Dijke P. 2003. Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses. J Biol Chem 278:3751–3761.

Jackson SM, Nakato H, Sugiura M, Jannuzi A, Oakes R, Kaluza V, Golden C, Selleck SB. 1997. dally, a Drosophila glypican, controls cellular responses to the TGF-beta-related morphogen, Dpp. Dev Camb Engl 124:4113–4120.

Javelaud D, Pierrat M-J, Mauviel A. 2012. Crosstalk between TGF-β and hedgehog signaling in cancer. FEBS Lett 586:2016–2025.

Jazwinska A, Rushlow C, Roth S. 1999. The role of brinker in mediating the graded response to Dpp in early Drosophila embryos. Development 126:3323–3334.

Jiang Y, Nohe A, Bragdon B, Tian C, Rudarakanchana N, Morrell NW, Petersen NO. 2011. Trapping of BMP receptors in distinct membrane domains inhibits their function in pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 301:L218–227.

Johnson RL, Rothman AL, Xie J, Goodrich LV, Bare JW, Bonifas JM, Quinn AG, Myers RM, Cox DR, Epstein EH, Scott MP. 1996. Human Homolog of patched, a Candidate Gene for the Basal Cell Nevus Syndrome. Science 272:1668–1671.

Kamiya Y, Miyazono K, Miyazawa K. 2008. Specificity of the inhibitory effects of Dad on TGF-β family type I receptors, Thickveins, Saxophone, and Baboon in Drosophila. FEBS Lett 582:2496–2500.

Kaplan FS, Shen Q, Lounev V, Seemann P, Groppe J, Katagiri T, Pignolo RJ, Shore EM. 2008. Skeletal metamorphosis in fibrodysplasia ossificans progressiva (FOP). J Bone Miner Metab 26:521–530.

Kaplan FS, Xu M, Seemann P, Connor JM, Glaser DL, Carroll L, Delai P, Fastnacht-Urban E, Forman SJ, Gillessen-Kaesbach G, Hoover-Fong J, Köster B, Pauli RM, Reardon W, Zaidi S-A, Zasloff M, Morhart R, Mundlos S, Groppe J, Shore EM. 2009. Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1. Hum Mutat 30:379–390.

Kaplan FS, Glaser DL. 2005. Thoracic Insufficiency Syndrome in Patients With Fibrodysplasia Ossificans Progressiva. Clin Rev Bone Miner Metab 3:213–216.

Kawase E, Wong MD, Ding BC, Xie T. 2004. Gbb/Bmp signaling is essential for maintaining germline stem cells and for repressing bam transcription in the Drosophila testis. Development 131:1365–1375.

Keller S, Nickel J, Zhang J-L, Sebald W, Mueller TD. 2004. Molecular recognition of BMP-2 and BMP receptor IA. Nat Struct Mol Biol 11:481–488.

Khalsa O, Yoon JW, Torres-Schumann S, Wharton KA. 1998. TGF-beta/BMP superfamily members, Gbb-60A and Dpp, cooperate to provide pattern information and establish cell identity in the Drosophila wing. Dev Camb Engl 125:2723–2734.

Kim I-J, Park J-H, Kang HC, Kim K-H, Kim J-H, Ku J-L, Kang S-B, Park SY, Lee J-S, Park J-G. 2003. Identification of a novel BMPR1A germline mutation in a Korean juvenile polyposis patient without SMAD4 mutation. Clin Genet 63:126–130.

Kingsley DM. 1994. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev 8:133–146.

Kirkpatrick H, Johnson K, Laughon A. 2001. Repression of Dpp Targets by Binding of Brinker to Mad Sites. J Biol Chem 276:18216–18222.

Kirsch T, Nickel J, Sebald W. 2000a. BMP-2 antagonists emerge from alterations in the low-affinity binding epitope for receptor BMPR-II. EMBO J 19:3314–3324.

Kirsch T, Sebald W, Dreyer MK. 2000b. Crystal structure of the BMP-2-BRIA ectodomain complex. Nat Struct Biol 7:492–496.

Kitterman JA, Kantanie S, Rocke DM, Kaplan FS. 2005. Iatrogenic Harm Caused by Diagnostic Errors in Fibrodysplasia Ossificans Progressiva. Pediatrics 116:e654–e661.

Kokabu S, Gamer L, Cox K, Lowery J, Tsuji K, Raz R, Economides A, Katagiri T, Rosen V. 2012. BMP3 suppresses osteoblast differentiation of bone marrow stromal cells via interaction with Acvr2b. Mol Endocrinol Baltim Md 26:87–94.

Kokabu S, Ohte S, Sasanuma H, Shin M, Yoneyama K, Murata E, Kanomata K, Nojima J, Ono Y, Yoda T, Fukuda T, Katagiri T. 2011. Suppression of BMP-Smad signaling axis-induced osteoblastic differentiation by small C-terminal domain phosphatase 1, a Smad phosphatase. Mol Endocrinol Baltim Md 25:474–481.

Kotzsch A, Nickel J, Seher A, Sebald W, Müller TD. 2009. Crystal structure analysis reveals a spring-loaded latch as molecular mechanism for GDF-5–type I receptor specificity. EMBO J 28:937–947.

Kretzschmar M, Liu F, Hata A, Doody J, Massagué J. 1997. The TGF-beta family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. Genes Dev 11:984–995.

Kugimiya F, Yano F, Ohba S, Igawa K, Nakamura K, Kawaguchi H, Chung U. 2005. Mechanism of osteogenic induction by FK506 via BMP/Smad pathways. Biochem Biophys Res Commun 338:872–879.

Kutty G, Kutty RK, Samuel W, Duncan T, Jaworski C, Wiggert B. 1998. Identification of a new member of transforming growth factor-beta superfamily in Drosophila: the first invertebrate activin gene. Biochem Biophys Res Commun 246:644–649.

Lavery K, Swain P, Falb D, Alaoui-Ismaili MH. 2008. BMP-2/4 and BMP-6/7 Differentially Utilize Cell Surface Receptors to Induce Osteoblastic Differentiation of Human Bone Marrow-derived Mesenchymal Stem Cells. J Biol Chem 283:20948–20958.

Le VQ, Wharton KA. 2012. Hyperactive BMP signaling induced by ALK2R206H requires type II receptor function in a Drosophila model for classic fibrodysplasia ossificans progressiva. Dev Dyn 241:200–214.

Lee MK, Pardoux C, Hall MC, Lee PS, Warburton D, Qing J, Smith SM, Derynck R. 2007. TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. EMBO J 26:3957–3967.

Lehmann K, Seemann P, Boergermann J, Morin G, Reif S, Knaus P, Mundlos S. 2006. A novel R486Q mutation in BMPR1B resulting in either a brachydactyly type C/symphalangism-like phenotype or brachydactyly type A2. Eur J Hum Genet EJHG 14:1248–1254.

Lehmann K, Seemann P, Stricker S, Sammar M, Meyer B, Süring K, Majewski F, Tinschert S, Grzeschik K-H, Müller D, Knaus P, Nürnberg P, Mundlos S. 2003. Mutations in bone morphogenetic protein receptor 1B cause brachydactyly type A2. Proc Natl Acad Sci 100:12277 –12282.

Letsou A, Arora K, Wrana JL, Simin K, Twombly V, Jamal J, Staehling-Hampton K, Hoffmann FM, Gelbart WM, Massague J, O'Connor MB. 1995. Drosophila Dpp signaling is mediated by the punt gene product: a dual ligand-binding type II receptor of the TGF beta receptor family. Cell 80:899–908.

Li L, Lin M, Wang Y, Cserjesi P, Chen Z, Chen Y. 2011. BmprIa is required in mesenchymal tissue and has limited redundant function with BmprIb in tooth and palate development. Dev Biol 349:451–461.

Lin SJ, Lerch TF, Cook RW, Jardetzky TS, Woodruff TK. 2006. The structural basis of TGF-β, bone morphogenetic protein, and activin ligand binding. Reproduction 132:179–190.

Little SC, Mullins MC. 2009. Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis. Nat Cell Biol 11:637–643.

Lo PC, Frasch M. 1999. Sequence and expression of myoglianin, a novel Drosophila gene of the TGF-beta superfamily. Mech Dev 86:171–175.

Macías-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL. 1998. Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. J Biol Chem 273:25628–25636.

Mahlawat P, Ilangovan U, Biswas T, Sun L-Z, Hinck AP. 2012. Structure of the Alk1 Extracellular Domain and Characterization of Its Bone Morphogenetic Protein (BMP) Binding Properties. Biochemistry (Mosc) 51:6328–6341.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. 2002. The Protein Kinase Complement of the Human Genome. Science 298:1912–1934.

Marom B, Heining E, Knaus P, Henis YI. 2011. Formation of Stable Homomeric and Transient Heteromeric Bone Morphogenetic Protein (BMP) Receptor Complexes Regulates Smad Protein Signaling. J Biol Chem 286:19287–19296.

Marqués G, Musacchio M, Shimell MJ, Wünnenberg-Stapleton K, Cho KW, O'Connor MB. 1997. Production of a DPP activity gradient in the early Drosophila embryo through the opposing actions of the SOG and TLD proteins. Cell 91:417–426.

Marqués G. 2005. Morphogens and synaptogenesis in Drosophila. J Neurobiol 64:417–434.

Marqués G, Bao H, Haerry TE, Shimell MJ, Duchek P, Zhang B, O'Connor MB. 2002. The Drosophila BMP Type II Receptor Wishful Thinking Regulates Neuromuscular Synapse Morphology and Function. Neuron 33:529–543.

Marqués G, Haerry TE, Crotty ML, Xue M, Zhang B, O'Connor MB. 2003. Retrograde Gbb signaling through the Bmp type 2 receptor Wishful Thinking regulates systemic FMRFa expression in Drosophila. Development 130:5457–5470.

Marty T, Müller B, Basler K, Affolter M. 2000. Schnurri mediates Dpp-dependent repression of brinker transcription. Nat Cell Biol 2:745–749.

Marygold SJ, Leyland PC, Seal RL, Goodman JL, Thurmond J, Strelets VB, Wilson RJ, the FlyBase consortium. 2012. FlyBase: improvements to the bibliography. Nucleic Acids Res 41:D751–D757.

Massagué J. 1998. TGF-beta signal transduction. Annu Rev Biochem 67:753–791.

Massagué J, Wotton D. 2000. Transcriptional control by the TGF-beta/Smad signaling system. EMBO J 19:1745–1754.

Massagué J, Seoane J, Wotton D. 2005. Smad transcription factors. Genes Dev 19:2783–2810.

McCabe BD, Hom S, Aberle H, Fetter RD, Marques G, Haerry TE, Wan H, O'Connor MB, Goodman CS, Haghighi AP. 2004. Highwire Regulates Presynaptic BMP Signaling Essential for Synaptic Growth. Neuron 41:891–905.

McCabe BD, Marqués G, Haghighi AP, Fetter RD, Crotty ML, Haerry TE, Goodman CS, O'Connor MB. 2003. The BMP Homolog Gbb Provides a Retrograde Signal that Regulates Synaptic Growth at the Drosophila Neuromuscular Junction. Neuron 39:241–254.

Miraoui H, Marie PJ. 2010. Fibroblast growth factor receptor signaling crosstalk in skeletogenesis. Sci Signal 3:re9.

Miyazono K, Kamiya Y, Morikawa M. 2010. Bone morphogenetic protein receptors and signal transduction. J Biochem (Tokyo) 147:35–51.

Morita K, Flemming AJ, Sugihara Y, Mochii M, Suzuki Y, Yoshida S, Wood WB, Kohara Y, Leroi AM, Ueno N. 2002. A Caenorhabditis elegans TGF-β, DBL-1, controls the expression of LON-1, a PR-related protein, that regulates polyploidization and body length. EMBO J 21:1063–1073.

Moser M, Campbell G. 2005. Generating and interpreting the Brinker gradient in the Drosophila wing. Dev Biol 286:647–658.

Moustakas A, Heldin C-H. 2005. Non-Smad TGF-β signals. J Cell Sci 118:3573–3584.

Moustakas A, Heldin C-H. 2009. The regulation of TGFbeta signal transduction. Dev Camb Engl 136:3699–3714.

Mueller TD, Nickel J. 2012. Promiscuity and specificity in BMP receptor activation. FEBS Lett 586:1846–1859.

Müller B, Hartmann B, Pyrowolakis G, Affolter M, Basler K. 2003. Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. Cell 113:221–233.

Murakami G, Watabe T, Takaoka K, Miyazono K, Imamura T. 2003. Cooperative Inhibition of Bone Morphogenetic Protein Signaling by Smurf1 and Inhibitory Smads. Mol Biol Cell 14:2809–2817.

Murali D, Yoshikawa S, Corrigan RR, Plas DJ, Crair MC, Oliver G, Lyons KM, Mishina Y, Furuta Y. 2005. Distinct developmental programs require different levels of Bmp signaling during mouse retinal development. Development 132:913–923.

Nakao A, Afrakhte M, Morn A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin N-E, Heldin C-H, Dijke P ten. 1997. Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling. Nature 389:631–635.

Nellen D, Affolter M, Basler K. 1994. Receptor serine/threonine kinases implicated in the control of Drosophila body pattern by decapentaplegic. Cell 78:225–237.

Neul JL, Ferguson EL. 1998. Spatially Restricted Activation of the SAX Receptor by SCW Modulates DPP/TKV Signaling in Drosophila Dorsal–Ventral Patterning. Cell 95:483–494.

Newfeld SJ, Chartoff EH, Graff JM, Melton DA, Gelbart WM. 1996. Mothers against dpp encodes a conserved cytoplasmic protein required in DPP/TGF-beta responsive cells. Dev Camb Engl 122:2099–2108.

Newfeld SJ, Wisotzkey RG, Kumar S. 1999. Molecular evolution of a developmental pathway: phylogenetic analyses of transforming growth factor-beta family ligands, receptors and Smad signal transducers. Genetics 152:783–795.

Nguyen M, Parker L, Arora K. 2000. Identification of maverick, a novel member of the TGF-beta superfamily in Drosophila. Mech Dev 95:201–206.

Nguyen M, Park S, Marqués G, Arora K. 1998. Interpretation of a BMP Activity Gradient in Drosophila Embryos Depends on Synergistic Signaling by Two Type I Receptors, SAX and TKV. Cell 95:495–506.

Nickel J, Dreyer MK, Kirsch T, Sebald W. 2001. The crystal structure of the BMP-2:BMPR-IA complex and the generation of BMP-2 antagonists. J Bone Joint Surg Am 83-A Suppl 1:S7–14.

Nickel J, Sebald W, Groppe JC, Mueller TD. 2009. Intricacies of BMP receptor assembly. Cytokine Growth Factor Rev 20:367–377.

Nikaido M, Tada M, Takeda H, Kuroiwa A, Ueno N. 1999a. In vivo analysis using variants of zebrafish BMPR-IA: range of action and involvement of BMP in ectoderm patterning. Development 126:181–190.

Nikaido M, Tada M, Ueno N. 1999b. Restricted expression of the receptor serine/threonine kinase BMPR-IB in zebrafish. Mech Dev 82:219–222.

Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P. 2002. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem 277:5330–5338.

Nojima J, Kanomata K, Takada Y, Fukuda T, Kokabu S, Ohte S, Takada T, Tsukui T, Yamamoto TS, Sasanuma H, Yoneyama K, Ueno N, Okazaki Y, Kamijo R, Yoda T, Katagiri T. 2010. Dual Roles of Smad Proteins in the Conversion from Myoblasts to Osteoblastic Cells by Bone Morphogenetic Proteins. J Biol Chem 285:15577–15586.

O'Connor MB, Umulis D, Othmer HG, Blair SS. 2006. Shaping BMP morphogen gradients in the Drosophila embryo and pupal wing. Dev Camb Engl 133:183–193.

O'Kane CJ. 2003. Modelling human diseases in Drosophila and Caenorhabditis. Semin Cell Dev Biol 14:3–10.

Ogiso Y, Tsuneizumi K, Masuda N, Sato M, Tabata T. 2011. Robustness of the Dpp morphogen activity gradient depends on negative feedback regulation by the inhibitory Smad, Dad. Dev Growth Differ 53:668–678.

Oh H, Irvine KD. 2011. Cooperative regulation of growth by Yorkie and Mad through bantam. Dev Cell 20:109–122.

Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E. 2000. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc Natl Acad Sci U S A 97:2626–2631.

Ohte S, Shin M, Sasanuma H, Yoneyama K, Akita M, Ikebuchi K, Jimi E, Maruki Y, Matsuoka M, Namba A, Tomoda H, Okazaki Y, Ohtake A, Oda H, Owan I, Yoda T, Furuya H, Kamizono J, Kitoh H, Nakashima Y, Susami T, Haga N, Komori T, Katagiri T. 2011. A novel mutation of ALK2, L196P, found in the most benign case of fibrodysplasia ossificans progressiva activates BMP-specific intracellular signaling equivalent to a typical mutation, R206H. Biochem Biophys Res Commun 407:213–218.

Olivieri C, Pagella F, Semino L, Lanzarini L, Valacca C, Pilotto A, Corno S, Scappaticci S, Manfredi G, Buscarini E, Danesino C. 2007. Analysis of ENG and ACVRL1 genes in 137 HHT Italian families identifies 76 different mutations (24 novel). Comparison with other European studies. J Hum Genet 52:820–829.

Onichtchouk D, Chen Y-G, Dosch R, Gawantka V, Delius H, Massague´ J, Niehrs C. 1999. Silencing of TGF-β signalling by the pseudoreceptor BAMBI. Nature 401:480–485.

Padgett RW, Wozney JM, Gelbart WM. 1993. Human BMP sequences can confer normal dorsal-ventral patterning in the Drosophila embryo. Proc Natl Acad Sci U S A 90:2905–2909.

Pandey UB, Nichols CD. 2011. Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. Pharmacol Rev 63:411–436.

Parker L, Stathakis DG, Arora K. 2004. Regulation of BMP and activin signaling in Drosophila. Prog Mol Subcell Biol 34:73–101.

Parker L, Ellis JE, Nguyen MQ, Arora K. 2006. The divergent TGF-beta ligand Dawdle utilizes an activin pathway to influence axon guidance in Drosophila. Dev Camb Engl 133:4981–4991.

Patterson GI, Padgett RW. 2000. TGF beta-related pathways. Roles in Caenorhabditis elegans development. Trends Genet TIG 16:27–33.

Penton A, Chen Y, Staehling-Hampton K, Wrana JL, Attisano L, Szidonya J, Cassill JA, Massagué J, Hoffmann FM. 1994. Identification of two bone morphogenetic protein type I receptors in Drosophila and evidence that Brk25D is a decapentaplegic receptor. Cell 78:239–250.

Perrimon N, Pitsouli C, Shilo B-Z. 2012. Signaling Mechanisms Controlling Cell Fate and Embryonic Patterning. Cold Spring Harb Perspect Biol 4. http://cshperspectives.cshlp.org/content/4/8/a005975.

Persson U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, Engström U, Heldin CH, Funa K, ten Dijke P. 1998. The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. FEBS Lett 434:83–87.

Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785–786.

Petrie KA, Lee WH, Bullock AN, Pointon JJ, Smith R, Russell RGG, Brown MA, Wordsworth BP, Triffitt JT. 2009. Novel mutations in ACVR1 result in atypical features in two fibrodysplasia ossificans progressiva patients. PLoS ONE 4:e5005.

Piccolo S, Agius E, Lu B, Goodman S, Dale L, De Robertis EM. 1997. Cleavage of Chordin by Xolloid Metalloprotease Suggests a Role for Proteolytic Processing in the Regulation of Spemann Organizer Activity. Cell 91:407–416.

Pignolo RJ, Shore EM, Kaplan FS. 2011. Fibrodysplasia Ossificans Progressiva: Clinical and Genetic Aspects. Orphanet J Rare Dis 6:80.

Pignolo RJ, Suda RK, Kaplan FS. 2005. The Fibrodysplasia Ossificans Progressiva Lesion. Clin Rev Bone Miner Metab 3:195–200.

Pyrowolakis G, Hartmann B, Müller B, Basler K, Affolter M. 2004. A Simple Molecular Complex Mediates Widespread BMP-Induced Repression during Drosophila Development. Dev Cell 7:229–240.

Raftery LA, Umulis DM. 2012. Regulation of BMP activity and range in Drosophila wing development. Curr Opin Cell Biol 24:158–165.

Rajagopal R, Huang J, Dattilo LK, Kaartinen V, Mishina Y, Deng C-X, Umans L, Zwijsen A, Roberts AB, Beebe DC. 2009. The type I BMP receptors, Bmpr1a and Acvr1, activate multiple signaling pathways to regulate lens formation. Dev Biol 335:305–316.

Ramel M-C, Hill CS. 2012. Spatial regulation of BMP activity. FEBS Lett 586:1929–1941.

Ramsdell AF, Yost HJ. 1999. Cardiac looping and the vertebrate left-right axis: antagonism of left-sided Vg1 activity by a right-sided ALK2-dependent BMP pathway. Development 126:5195–5205.

Rawson JM, Lee M, Kennedy EL, Selleck SB. 2003. Drosophila neuromuscular synapse assembly and function require the TGF-β type I receptor saxophone and the transcription factor Mad. J Neurobiol 55:134–150.

Ray RP, Wharton KA. 2001. Context-dependent relationships between the BMPs gbb and dpp during development of the Drosophila wing imaginal disk. Development 128:3913–3925.

Reiter LT, Bier E. 2002. Using Drosophila melanogaster to uncover human disease gene function and potential drug target proteins. Expert Opin Ther Targets 6:387–399.

Renlund N, O'Neill FH, Zhang L, Sidis Y, Teixeira J. 2007. Activin receptor-like kinase-2 inhibits activin signaling by blocking the binding of activin to its type II receptor. J Endocrinol 195:95–103.

Robitaille J, MacDonald MLE, Kaykas A, Sheldahl LC, Zeisler J, Dubé M-P, Zhang L-H, Singaraja RR, Guernsey DL, Zheng B, Siebert LF, Hoskin-Mott A, Trese MT, Pimstone SN, Shastry BS, Moon RT, Hayden MR, Goldberg YP, Samuels ME. 2002. Mutant frizzled-4 disrupts retinal angiogenesis in familial exudative vitreoretinopathy. Nat Genet 32:326–330.

Rogulja D, Irvine KD. 2005. Regulation of cell proliferation by a morphogen gradient. Cell 123:449–461.

Rogulja D, Rauskolb C, Irvine KD. 2008. Morphogen control of wing growth through the Fat signaling pathway. Dev Cell 15:309–321.

Ross JJ, Shimmi O, Vilmos P, Petryk A, Kim H, Gaudenz K, Hermanson S, Ekker SC, O'Connor MB, Marsh JL. 2001. Twisted gastrulation is a conserved extracellular BMP antagonist. Nature 410:479–483.

Ruberte E, Marty T, Nellen D, Affolter M, Basler K. 1995. An absolute requirement for both the type II and type I receptors, punt and thick veins, for dpp signaling in vivo. Cell 80:889–897.

Sampath TK, Rashka KE, Doctor JS, Tucker RF, Hoffmann FM. 1993. Drosophila transforming growth factor beta superfamily proteins induce endochondral bone formation in mammals. Proc Natl Acad Sci U S A 90:6004–6008.

Sampath TK, Muthukumaran N, Reddi AH. 1987. Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. Proc Natl Acad Sci 84:7109–7113.

Sanyal A, Jo Oursler M, Clemens VR, Fukumoto T, Fitzsimmons JS, O'Driscoll SW. 2002. Temporal expression patterns of BMP receptors and collagen II (B) during periosteal chondrogenesis. J Orthop Res 20:58–65.

Saremba S, Nickel J, Seher A, Kotzsch A, Sebald W, Mueller TD. 2008. Type I receptor binding of bone morphogenetic protein 6 is dependent on N-glycosylation of the ligand. FEBS J 275:172–183.

Satow R, Kurisaki A, Chan T, Hamazaki TS, Asashima M. 2006. Dullard Promotes Degradation and Dephosphorylation of BMP Receptors and Is Required for Neural Induction. Dev Cell 11:763–774.

Scheufler C, Sebald W, Hülsmeyer M. 1999. Crystal structure of human bone morphogenetic protein-2 at 2.7 Å resolution. J Mol Biol 287:103–115.

Schmierer B, Hill CS. 2007. TGFβ–SMAD signal transduction: molecular specificity and functional flexibility. Nat Rev Mol Cell Biol 8:970–982.

Schupbach T, Wieschaus E. 1989. Female Sterile Mutations on the Second Chromosome of Drosophila Melanogaster. I. Maternal Effect Mutations. Genetics 121:101–117.

Schwank G, Dalessi S, Yang S-F, Yagi R, de Lachapelle AM, Affolter M, Bergmann S, Basler K. 2011. Formation of the long range Dpp morphogen gradient. PLoS Biol 9:e1001111.

Sebald W, Nickel J, Zhang J-L, Mueller TD. 2004. Molecular recognition in bone morphogenetic protein (BMP)/receptor interaction. Biol Chem 385:697–710.

Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH, Gelbart WM. 1995. Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics 139:1347–1358.

Serpe M, Umulis D, Ralston A, Chen J, Olson DJ, Avanesov A, Othmer H, O'Connor MB, Blair SS. 2008. The BMP-Binding Protein Crossveinless 2 Is a Short-Range, Concentration-Dependent, Biphasic Modulator of BMP Signaling in Drosophila. Dev Cell 14:940–953.

Shen Q, Little SC, Xu M, Haupt J, Ast C, Katagiri T, Mundlos S, Seemann P, Kaplan FS, Mullins MC, Shore EM. 2009. The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization. J Clin Invest 119:3462–3472.

Shi W, Chang C, Nie S, Xie S, Wan M, Cao X. 2007. Endofin acts as a Smad anchor for receptor activation in BMP signaling. J Cell Sci 120:1216–1224.

Shibuya H, Iwata H, Masuyama N, Gotoh Y, Yamaguchi K, Irie K, Matsumoto K, Nishida E, Ueno N. 1998. Role of TAK1 and TAB1 in BMP signaling in early Xenopus development. EMBO J 17:1019–1028.

Shim J-H, Greenblatt MB, Xie M, Schneider MD, Zou W, Zhai B, Gygi S, Glimcher LH. 2009. TAK1 is an essential regulator of BMP signalling in cartilage. EMBO J 28:2028–2041.

Shimmi O, O'Connor MB. 2003. Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in Bmp signals during dorsoventral patterning of the Drosophila embryo. Dev Camb Engl 130:4673–4682.

Shimmi O, Ralston A, Blair SS, O'Connor MB. 2005a. The crossveinless gene encodes a new member of the Twisted gastrulation family of BMP-binding proteins which, with Short gastrulation, promotes BMP signaling in the crossveins of the Drosophila wing. Dev Biol 282:70–83.

Shimmi O, Umulis D, Othmer H, O'Connor MB. 2005b. Facilitated Transport of a Dpp/Scw Heterodimer by Sog/Tsg Leads to Robust Patterning of the Drosophila Blastoderm Embryo. Cell 120:873–886.

Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho T-J, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M, Morhart R, Rogers JG, Smith R, Triffitt JT, Urtizberea JA, Zasloff M, Brown MA, Kaplan FS. 2006. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet 38:525–527.

Singer MA, Penton A, Twombly V, Hoffmann FM, Gelbart WM. 1997. Signaling through both type I DPP receptors is required for anterior-posterior patterning of the entire Drosophila wing. Development 124:79–89.

Sivasankaran R, Vigano MA, Müller B, Affolter M, Basler K. 2000. Direct transcriptional control of the Dpp target omb by the DNA binding protein Brinker. EMBO J 19:6162–6172.

Song G-A, Kim H-J, Woo K-M, Baek J-H, Kim G-S, Choi J-Y, Ryoo H-M. 2010. Molecular consequences of the ACVR1(R206H) mutation of fibrodysplasia ossificans progressiva. J Biol Chem 285:22542–22553.

Souchelnytskyi S, Tamaki K, Engström U, Wernstedt C, Dijke P ten, Heldin C-H. 1997. Phosphorylation of Ser465 and Ser467 in the C Terminus of Smad2 Mediates Interaction with Smad4 and Is Required for Transforming Growth Factor-β Signaling. J Biol Chem 272:28107–28115.

Suzuki Y, Yandell MD, Roy PJ, Krishna S, Savage-Dunn C, Ross RM, Padgett RW, Wood WB. 1999. A BMP homolog acts as a dose-dependent regulator of body size and male tail patterning in Caenorhabditis elegans. Development 126:241–250.

Szuperák M, Salah S, Meyer EJ, Nagarajan U, Ikmi A, Gibson MC. 2011. Feedback regulation of Drosophila BMP signaling by the novel extracellular protein Larval Translucida. Development 138:715–724.

Tanimoto H, Itoh S, ten Dijke P, Tabata T. 2000. Hedgehog Creates a Gradient of DPP Activity in Drosophila Wing Imaginal Discs. Mol Cell 5:59–71.

Terracol R, Lengyel JA. 1994. The thick veins gene of Drosophila is required for dorsoventral polarity of the embryo. Genetics 138:165–178.

Tsuneizumi K, Nakayama T, Kamoshida Y, Kornberg TB, Christian JL, Tabata T. 1997. Daughters against dpp modulates dpp organizing activity in Drosophila wing development. Nature 389:627–631.

Twombly V, Bangi E, Le V, Malnic B, Singer MA, Wharton KA. 2009. Functional Analysis of saxophone, the Drosophila Gene Encoding the BMP Type I Receptor Ortholog of Human ALK1/ACVRL1 and ACVR1/ALK2. Genetics 183:563–579.

Urist MR. 1965. Bone: Formation by Autoinduction. Science 150:893–899.

Veraksa A, Del Campo M, McGinnis W. 2000. Developmental patterning genes and their conserved functions: from model organisms to humans. Mol Genet Metab 69:85–100.

Visser JA, Olaso R, Verhoef-Post M, Kramer P, Themmen AP, Ingraham HA. 2001. The serine/threonine transmembrane receptor ALK2 mediates Müllerian inhibiting substance signaling. Mol Endocrinol Baltim Md 15:936–945.

Vuilleumier R, Springhorn A, Patterson L, Koidl S, Hammerschmidt M, Affolter M, Pyrowolakis G. 2010. Control of Dpp morphogen signalling by a secreted feedback regulator. Nat Cell Biol 12:611–617.

Walsh DW, Godson C, Brazil DP, Martin F. 2010. Extracellular BMP-antagonist regulation in development and disease: tied up in knots. Trends Cell Biol 20:244–256.

Wang T, Li BY, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T, Donahoe PK. 1996. The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. Cell 86:435–444.

Wang T, Donahoe PK. 2004. The immunophilin FKBP12: a molecular guardian of the TGF-beta family type I receptors. Front Biosci J Virtual Libr 9:619–631.

Wartlick O, Mumcu P, Kicheva A, Bittig T, Seum C, Jülicher F, González-Gaitán M. 2011a. Dynamics of Dpp signaling and proliferation control. Science 331:1154–1159.

Wartlick O, Mumcu P, Jülicher F, Gonzalez-Gaitan M. 2011b. Understanding morphogenetic growth control - lessons from flies. Nat Rev Mol Cell Biol 12:594–604.

Weber D, Kotzsch A, Nickel J, Harth S, Seher A, Mueller U, Sebald W, Mueller TD. 2007. A silent H-bond can be mutationally activated for high-affinity interaction of BMP-2 and activin type IIB receptor. BMC Struct Biol 7:6.

Webster MK, Donoghue DJ. 1996. Constitutive activation of fibroblast growth factor receptor 3 by the transmembrane domain point mutation found in achondroplasia. EMBO J 15:520–527.

Wehner L-E, Folz BJ, Argyriou L, Twelkemeyer S, Teske U, Geisthoff UW, Werner JA, Engel W, Nayernia K. 2006. Mutation analysis in hereditary haemorrhagic telangiectasia in

Germany reveals 11 novel ENG and 12 novel ACVRL1/ALK1 mutations. Clin Genet 69:239–245.

Weis-Garcia F, Massagué J. 1996. Complementation between kinase-defective and activation-defective TGF-beta receptors reveals a novel form of receptor cooperativity essential for signaling. EMBO J 15:276–289.

Wharton KA, Cook JM, Torres-Schumann S, de Castro K, Borod E, Phillips DA. 1999. Genetic analysis of the bone morphogenetic protein-related gene, gbb, identifies multiple requirements during Drosophila development. Genetics 152:629–640.

Wharton KA, Ray RP, Gelbart WM. 1993. An activity gradient of decapentaplegic is necessary for the specification of dorsal pattern elements in the Drosophila embryo. Development 117:807–822.

Wharton KA, Thomsen GH, Gelbart WM. 1991. Drosophila 60A gene, another transforming growth factor beta family member, is closely related to human bone morphogenetic proteins. Proc Natl Acad Sci 88:9214–9218.

Wharton K, Derynck R. 2009. TGFbeta family signaling: novel insights in development and disease. Dev Camb Engl 136:3691–3697.

Wieser R, Wrana JL, Massagué J. 1995. GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. EMBO J 14:2199–2208.

Wine-Lee L, Ahn KJ, Richardson RD, Mishina Y, Lyons KM, Crenshaw EB. 2004. Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord. Development 131:5393–5403.

Wong WKP, Knowles JA, Morse JH. 2005. Bone Morphogenetic Protein Receptor Type II C-Terminus Interacts with c-Src. Am J Respir Cell Mol Biol 33:438–446.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. 1988. Novel regulators of bone formation: molecular clones and activities. Science 242:1528–1534.

Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. 1994a. Mechanism of activation of the TGF-beta receptor. Nature 370:341–347.

Wrana JL, Tran H, Attisano L, Arora K, Childs SR, Massagué J, O'Connor MB. 1994b. Two distinct transmembrane serine/threonine kinases from Drosophila melanogaster form an activin receptor complex. Mol Cell Biol 14:944–950.

Wu MY, Hill CS. 2009. Tgf-beta superfamily signaling in embryonic development and homeostasis. Dev Cell 16:329–343.

Xia L, Jia S, Huang S, Wang H, Zhu Y, Mu Y, Kan L, Zheng W, Wu D, Li X, Sun Q, Meng A, Chen D. 2010. The Fused/Smurf Complex Controls the Fate of Drosophila Germline Stem Cells by Generating a Gradient BMP Response. Cell 143:978–990.

Xie J, Murone M, Luoh S-M, Ryan A, Gu Q, Zhang C, Bonifas JM, Lam C-W, Hynes M, Goddard A, Rosenthal A, Jr EHE, de Sauvage FJ. 1998. Activating Smoothened mutations in sporadic basal-cell carcinoma. Nature 391:90–92.

Xie T, Finelli AL, Padgett RW. 1994. The Drosophila saxophone gene: a serine-threonine kinase receptor of the TGF-beta superfamily. Science 263:1756–1759.

Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K. 1999. XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1–TAK1 in the BMP signaling pathway. EMBO J 18:179–187.

Yoon BS, Ovchinnikov DA, Yoshii I, Mishina Y, Behringer RR, Lyons KM. 2005. Bmpr1a and Bmpr1b have overlapping functions and are essential for chondrogenesis in vivo. Proc Natl Acad Sci U S A 102:5062–5067.

Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Bouxsein ML, Hong DW, McManus PM, Katagiri T, Sachidanandan C, Kamiya N, Fukuda T, Mishina Y, Peterson RT, Bloch KD. 2008a. BMP type I receptor inhibition reduces heterotopic [corrected] ossification. Nat Med 14:1363–1369.

Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, Lin HY, Bloch KD, Peterson RT. 2008b. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 4:33–41.

Zakin L, De Robertis EM. 2010. Extracellular regulation of BMP signaling. Curr Biol 20:R89–R92.

Van der Zee M, Fonseca RN da, Roth S. 2008. TGFβ signaling in Tribolium: vertebrate-like components in a beetle. Dev Genes Evol 218:203–213.

Zhang Y, Feng X, We R, Derynck R. 1996. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. Nature 383:168–172.

Zhou XP, Woodford-Richens K, Lehtonen R, Kurose K, Aldred M, Hampel H, Launonen V, Virta S, Pilarski R, Salovaara R, Bodmer WF, Conrad BA, Dunlop M, Hodgson SV, Iwama T, Järvinen H, Kellokumpu I, Kim JC, Leggett B, Markie D, Mecklin JP, Neale K, Phillips R, Piris J, Rozen P, Houlston RS, Aaltonen LA, Tomlinson IP, Eng C. 2001. Germline mutations in BMPR1A/ALK3 cause a subset of cases of juvenile polyposis syndrome and of Cowden and Bannayan-Riley-Ruvalcaba syndromes. Am J Hum Genet 69:704–711.

Zou H, Niswander L. 1996. Requirement for BMP Signaling in Interdigital Apoptosis and Scale Formation. Science 272:738–741.

Zúñiga JE, Groppe JC, Cui Y, Hinck CS, Contreras-Shannon V, Pakhomova ON, Yang J, Tang Y, Mendoza V, López-Casillas F, Sun L, Hinck AP. 2005. Assembly of TbetaRI:TbetaRII:TGFbeta ternary complex in vitro with receptor extracellular domains is cooperative and isoform-dependent. J Mol Biol 354:1052–1068.