Analysis of Endoderm Patterning and Pancreatic Organogenesis in *Xenopus laevis*

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This dissertation by Cassandra K. Bilogan is accepted in its present form by the Department of Molecular Biology, Cellular Biology and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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Chapter 1: Introduction

HISTORICAL PERSPECTIVE

Xenopus laevis was first described in Francois Marie Daudin's *Histoire naturelle des rainettes, des grenouilles, et des crapauds* at the beginning of the nineteenth century (Daudin, 1802). For most of that century the interest in studying *Xenopus* in Europe was aimed at the investigation of the natural history of imperial fauna. At the turn of the century, focus had begun to shift towards fertilization and rearing of *Xenopus* in laboratories for the study of embryonic development. Establishment of laboratory colonies of *Xenopus* in Europe and North America became more common in the 1930s with new uses of *Xenopus* in reproductive physiology research and its implementation as the most efficient early diagnosis of pregnancy at the time (reviewed in Gurdon and Hopwood, 2000)

The convenience of being able to be used throughout the year and availability of countless embryos from induced *Xenopus* ovulations allowed for ample working material for developmental biologists to perform microsurgery and biochemical studies. Dominance of *Xenopus* in research over other amphibia took off in the 1960s with the development of homozygous 0-nucleated frog that was used as a genetic marker in nuclear and germ-cell transplantation experiments (Blackler and Fischberg, 1961; Fischberg et al., 1958; Gurdon et al., 1958). Around this time, a shift in research towards an emphasis in molecular biology from cellular levels occurred, and *Xenopus* emerged as a worthy model system to study the molecular analysis of early animal development because of the ability to combine experimental embryological methods with molecular and genetic techniques (reviewed in Gurdon and Hopwood, 2000).

BENEFITS AND DRAWBACKS OF *XENOPUS LAEVIS* AS A MODEL SYSTEM

The reason *Xenopus* has played significant roles in elucidating mechanisms of early development is due to the experimental benefits of this model system. The eggs and embryos can be produced in large numbers by means of a simple hormone injection. They also develop externally, are relatively large in size with identifiable blastomeres and can be cultured in a simple salt solution. The above provide an advantage over other model systems, such as chick and mouse, which are not as bountiful in comparison. *Xenopus* embryos are also easily manipulated; gain- or loss-of-function experiments can be easily performed by tissue-targeted injections of mRNA for overexpression or knockdown by morpholino (MO) or transcription activator-like effector nucleases (TALENs) (Lei et al., 2012; Summerton and Weller, 1997). Another avenue to study gene function in *Xenopus* is transgenesis (K. L. Kroll and Amaya, 1996). Addition of chemicals to the salt solution at any point during development can be used to manipulate signaling pathways and cell autonomous interactions can be studied by tissue grafting. These techniques have led to many important contributions to the study of early development. The ability to manipulate *Xenopus* embryos has also led to the description of stage-specific fate maps and states of developmental commitment of cells to different lineages (Dale and Slack, 1987; Heasman et al., 1984). Experiments using the animal cap of the blastula embryo can be used to study embryonic induction, as it will respond to the appropriate signals to produce a variety of tissues (Jones and Woodland, 1986). These experiments led to the identification of the role of growth factors in the TGF-ß and FGF

families in providing the signals for embryonic induction (reviewed in Beck and Slack, 2001).

Although *Xenopus* is a very powerful model organism, one drawback is the relatively long generation time of 1-2 years. For years *Xenopus laevis* was also deemed unfit for experimental genetics given its pseudotetraploid genome (Bisbee et al., 1977), as the varying levels of functional redundancy between many of the *X. laevis* alloalleles impeded genetic analysis. However for the past decade a closely related diploid species, *Xenopus tropicalis,* has been used for transgenesis and genetic screens. *X. tropicalis* genetics have been underexploited likely because of the common misconception that genetics cannot be done in frogs. This is untrue as a number of groups have used forward screens to identify mutations in *X. tropicalis* and have been successful in reproducing disease models. Interestingly, more than half of the identified phenotypes do not have a zebrafish correlate, suggesting an advantage to using frog genetics, especially for insight into limb and heart development (Abu-Daya et al., 2012; 2011; 2009; Geach and Zimmerman, 2010; Goda et al., 2006; Grammer et al., 2005; Hayes et al., 2007). Genome sequencing and the advancement of high-throughput sequencing not only aid in the identification of forward screen mutations but also position *Xenopus* as an excellent model system for comparative screens. The ability to easily manipulate gene function and isolate a large amount of tissue has led to the identification of numerous new genes and a better understanding of gene regulatory networks (Bilogan and Horb, 2012a; Gurdon and Hopwood, 2000; Hooker et al., 2012; Oropez and Horb, 2012; Pearl et al., 2011; Sinner et al., 2006; Tan et al., 2013; Wessely et al., 2004).

ENDODERM FORMATION IN *XENOPUS*

Maternal Determinants and the Initiation of Asymmetry

Development of the prospective germ layers is initiated prior to fertilization within the single cell *Xenopus* oocyte where maternal determinants are patterned in a gradient along the animal-vegetal axis (reviewed in Heasman, 2006). The endoderm germ layer arises from the vegetal pole (Fig. 1a). One of the best characterized maternally inherited factors and most important in endoderm development is the T-box transcription factor *VegT* (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1999; J. Zhang and King, 1996). VegT is sufficient to initiate ectopic endoderm and mesoderm formation by microinjection into naïve ectoderm cells (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; J. Zhang and King, 1996). Maternal *VegT* is also required for endoderm formation as knockdown of VegT in the oocyte prior to fertilization results in embryos that contained primarily ectodermal structures and lingering mesoderm tissues where the endoderm should be (Xanthos et al., 2001; J. Zhang et al., 1998). Analysis of VegT-depleted embryos revealed that VegT is required for the expression of most zygotic endoderm and mesoderm genes such as Nodal-related genes, Mix/Bix family, Gata family and Sox 17 family of transcription factors (Xanthos et al., 2001; J. Zhang et al., 1998; Zorn and Wells, 2007). Exogenous expression of Nodal-related genes into VegT-depleted embryos can restore proper endoderm and mesoderm formation (Kofron et al., 1999; Xanthos et al., 2001).

Other vegetally localized maternal transcripts showed to play a role in endoderm formation include the TGFβ family member *Vg1* and the RNA-binding protein *Bicaudal-C* (*Bic-C*). *Vg1* mRNA is inherited by the most vegetal cells of the embryo similarly to *VegT* mRNA (Weeks and Melton, 1987). In Vg1-depleted embryos Smad2 phosphorylation is reduced in late blastula embryos, suggesting Vg1 is required for mesoderm induction (Birsoy et al., 2006). Vg1 is also required for expression of early zygotic genes, particularly the BMP and Wnt antagonists Chordin, Cerberus, Noggin and Dickkopf (Birsoy et al., 2006). Upon fertilization *Bic-C* moves from its vegetal localization to the future dorsal side of the *Xenopus* embryo (Wessely and De Robertis, 2000). Overexpression of *Bic-C* mRNA specifically induced dorsal endoderm (Wessely and De Robertis, 2000). More recently *Bic-C* expression was identified in renal epithelial cells and loss of function resulted in a polycystic kidney disease phenotype (Tran et al., 2007; 2010). The organization and establishment of the germ layers in *Xenopus* is dependent on maternally localized transcripts and proteins, which are not only necessary to pre-pattern the embryo but also regulate zygotic gene expression at the mid-blastula transition (MBT) (reviewed in Heasman, 2006).

Formation and Patterning of the Early Endoderm

At fertilization, sperm entry leads to the movement of the cortical cytoplasm away from the sperm-entry point. Membrane bound vesicles containing vegetally localized determinants are transported away from sperm-entry and this leads to the activation of the canonical Wnt/β-catenin signaling pathway on the dorsal side (Fan and Sokol, 1997; Ishibashi et al., 2008; Watabe et al., 1995). As a result, β-catenin accumulates in the

nucleus (Fig. 1a), where it interacts with Tcf/Lef transcription factors to directly stimulate the transcription of Wnt/β-catenin target genes such as the homeodomain (HD) transcription factors Siamois (Sia) and Twin (Twn) and the secreted factor Nodal-related-3 (Xnr3) (Brannon et al., 1997; Carnac et al., 1996; Harland and Gerhart, 1997; Heasman, 2006; McKendry et al., 1997).

After maternal Wnt11/β-catenin (mWnt11/β-catenin) signaling initiates dorsal asymmetry, Nodal-related signals are activated in a dorsal-ventral gradient in the future endoderm and mesoderm by the combination of the β-Catenin signal and other maternal genes (Fig. 1b) (De Robertis et al., 2000). Nodal ligands are members of the TGFβ family of secreted growth factors. In *Xenopus* there are five nodal-related genes (*xnr1,2,4,5,6*) and they are all involved in mesoderm and endoderm development. (Agius et al., 2000; Yang et al., 2002; Zorn and Wells, 2007). These ligands bind to transmembrane serine-threonine kinase receptors formed by a heterodimer between Alk4 or Alk7 and ActRIIA or ActRIIB, and an EGF-CFC family coreceptor. Once bound, Alk4/7 phosphorylates the cytosolic protein Smad2 or Smad3, which then binds Smad4 and translocates into the nucleus where they complex with transcription factors FoxH1 or Mixer to stimulate transcription of genes required for endodermal and mesodermal fates (X. Chen et al., 1997; Ring et al., 2002; Wardle and Smith, 2006; Zorn and Wells, 2009). By the time mWnt11/β-catenin signaling activates Nodal signaling, embryos have reached a blastula stage, and since there is little cell mixing prior to gastrulation, the animal-vegetal gradient of germ layer fate is maintained. The endodermal precursors are localized to the vegetal half of the embryo, these cells induce overlying equatorial tissue into mesodermal

precursors, while the animal tissue above the blastocoel cavity gives rise to the ectoderm (Zorn and Wells, 2007).

This combination of mWnt11/β-catenin and Nodal signaling forms the organizer in the dorsal margin of the embryo. During gastrulation, the organizer patterns all three germ layers by promoting dorsal-anterior fates though the expression of transcription factors such as Goosecoid (Gsc), Otx2 and Lim1. Expression of *gsc* is directly regulated by Wnt signaling effectors, Sia and Twin as well as Nodal signals through directly binding of FoxHI and Smad2/3 to the *gsc* promoter (Bae et al., 2011; Blythe et al., 2009; Laurent et al., 1997). Similarly both Nodal and Wnt signaling pathways directly activate transcription of *otx2* and *lim1*. Cell fate induction is also mediated by the expression of a number of secreted Wnt, TGFβ and BMP antagonists, such as Chordin, Noggin, Crescent and Cerberus, that inhibit of ventral-posterior fates (Fig. 1c). Initial transcription of *chordin* and *cerberus*, and likely many of the other secreted factors is directly activated by the cooperation between both Sia and Twin and the Nodal effectors FoxHI and Smad2/3 (Engleka and Kessler, 2001; Reid et al., 2012; Watabe et al., 1995). The secreted antagonists act by inhibiting BMP4 and zygotic Wnt8 (zWnt8) ligands expressed in the ventral marginal zone. BMP4 and zWnt8 promote ventral–posterior fates and restrict dorsal–anterior fates, in part by inducing the expression of the HD transcriptional repressors Vent1 and Vent2, which inhibit organizer gene expression (Fig. 1c) (De Robertis et al., 2000; Harland and Gerhart, 1997; Kessler, 1997; Laurent et al., 1997; Rankin et al., 2011; Sasai et al., 1995).

Nodal signaling is not only necessary and sufficient for endoderm and mesoderm development, but the specification of one germ layer over the other appears to be dose-dependent, with low doses inducing mesoderm and higher doses inducing endoderm (Agius et al., 2000; Osada et al., 2000; Zorn and Wells, 2009). Late blastula endoderm development is dependent on Nodal signaling to reinforce endodermal fate. Nodal signaling acts through an autoregulatory loop to increase transcription of the Nodal ligand and also maintains the expression of zygotic endoderm transcription factors of the Mix-like, Gata4/5/6 and Sox17 families that were initially activated by maternal VegT (Zorn and Wells, 2007). The epistatic relationships of these Nodal targets appears to be a complex network where they regulate each other's expression, as well as overlapping and distinct downstream targets required for endoderm development (Sinner et al., 2006; Zorn and Wells, 2009; 2007). Unlike Nodal signaling, the mWnt11/β-catenin signaling pathway is not absolutely required for endoderm formation. In the absence of β-catenin, anterior endoderm development and patterning is lost, but the fate of the posterior endoderm is not affected. This appears to be due to delayed *Xnr* expression and lower dorsal *Xnr* expression levels (Xanthos et al., 2002). While many studies have focused on the function of endoderm for mesoderm induction and patterning, we still lack the understanding of how the endoderm germ layer is patterned separately from the mesoderm during gastrulation.

The function of the Spemann organizer in germ layer formation and patterning is dependent on the proper spatial and temporal transcriptional activation of specific transcription factors and secreted proteins. To date, most studies have focused on how

these factors are regulated at transcriptional level and how they function to regulate transcription of their targets (Engleka and Kessler, 2001; Laurent et al., 1997; Rankin et al., 2011; Reid et al., 2012; Watabe et al., 1995). It is known that cells within the organizer domain receive signals from both the Wnt and Nodal pathways, and increasing evidence suggests that most of the organizer gene promoters are directly activated by both Wnt and Nodal inputs (Reid et al., 2012). Therefore another level of regulation must be required to establish the spatially and temporally distinct transcription domains of these organizer genes. I recently identified a role for Staufen2 (Stau2), a conserved double-stranded RNA-binding protein (dsRBP), in patterning along the anterior-posterior (A-P) axis of the endoderm (Chapter 2) (Bilogan and Horb, 2012b). While Stau2 appears to be ubiquitously expressed within the gastrula stage embryo, Stau2 knockdown in the developing dorsal endoderm and mesoderm led to an endoderm-only loss of patterning phenotype (Chapter 3). Stau2 appears to be required to maintain Wnt/β-catenin pathway signaling during gastrulation, providing evidence that the endoderm and mesoderm are patterned separately during gastrulation (Chapter 3).

Signaling Pathways Involved in A-P Endoderm Patterning

The foregut endoderm gives rise to the esophagus, trachea, lungs, thyroid, stomach, liver and the pancreas. During gastrulation, the endodermal organ domains become patterned along the A-P axis and this is reflected in their final location in the adult body (Chalmers and Slack, 2000). Partitioning of the endoderm into organ domains is specified by the combination of the endodermal transcription factors Mix, Gata, Sox17 and the integration of signaling pathways along the A-P axis (Heasman, 2006; Zorn and Wells, 2009). While

the endoderm develops initial A–P identity during gastrulation, stable regional specification of the endoderm only occurs at tailbud stages, as the endoderm remains competent to respond to signals secreted from the adjacent mesodermal tissues until then (Horb and Slack, 2001).

Throughout development as the endoderm becomes patterned into foregut and hindgut progenitor domains and then more specifically segregated into organ lineages, the same signaling pathways are used reiteratively and the same cells can respond differently depending on the stage (Zorn and Wells, 2009). As the endoderm is defined into discrete organ lineages, distinct Wnt/β-catenin signals are involved in the specification and development of the anterior endoderm organs (Z. Zhang et al., 2013; Zorn and Wells, 2009). In contrast to its requirement to define the dorsal axis of the embryo, inhibition of Wnt/β-catenin signaling at neural stages was thought to be essential for proper foregut development (McLin et al., 2007). New data support a revised model of endoderm patterning with different Wnt signaling thresholds along the A–P axis. High Wnt activity is still required to promote hindgut fate, however a low essential threshold of Wnt signaling is necessary to maintain foregut progenitors (Z. Zhang et al., 2013). There is evidence suggesting that some Wnt ligands can also regulate cytoskeleton dynamics, cell polarity and cell shape changes during gut morphogenesis through the activation of a non-canonical β-catenin-independent Wnt/JNK pathway in the endoderm, which signals via Rho-family GTPases and Jun-N-terminal-kinase (JNK) (Y. Li et al., 2008). It was recently demonstrated that low levels of both Wnt/β-catenin and Wnt/JNK signaling directs foregut progenitor fate, proliferation and morphogenesis (Z. Zhang et al., 2013).

FGF signaling is vegetally restricted prior to gastrulation, which appears to play a critical role in the development of the mesoderm and endoderm cells. However, prolonged inhibition of FGF signaling after gastrulation suppresses the maintenance and differentiation of the endoderm (Cha et al., 2008). FGF4 signaling from the mesoderm also promotes hindgut endoderm by the repression of anterior genes (McLin et al., 2007). FGF signaling appears to be required for proper lung and liver development, as loss of FGF signaling suppressed lung and liver development, while pancreatic and duodenal marker expression was expanded (Rankin et al., 2012; Shifley et al., 2012). BMP signaling during gastrulation promotes ventral-posterior fates while repressing dorsal-anterior cells fates (Rankin et al., 2011). After gastrulation BMP signaling is essential to maintain foregut progenitors (Kenny et al., 2012). Later, patterning of the anterior endoderm towards a pancreatic fate has been shown to require the inhibition of BMP signaling (Spagnoli and Brivanlou, 2008). Retinoic acid (RA) is also required for proper endoderm regionalization as it regulates the boundaries between foregut and hindgut expression domains. Loss of RA during gastrulation leads to a later reduction in pancreatic markers (Bayha et al., 2009; Y. Chen et al., 2004). While multiple signaling pathways are required throughout development to pattern the endoderm and direct the formation of specific organ lineages, it is unclear how the effects of FGF, Wnt, BMP, and RA are integrated during endoderm patterning.

RNA-BINDING PROTEINS

From the moment mRNA is transcribed in the nucleus, RNA-binding proteins (RBPs) assemble on the nascent transcripts and form large messenger ribonucleoprotein particles (mRNPs). mRNA is contained in these mRNPs throughout its life cycle and RBPs have been shown to play roles in transcription, 5'end capping, mRNA splicing, 3'end processing, nuclear export, localization, mRNA stability and translation (Glisovic et al., 2008; Müller-McNicoll and Neugebauer, 2013). Each mRNA is bound by multiple RBPs and individual RBPs can target hundreds to thousands of mRNA transcripts. Although all RBPs bind RNA, they do so with different RNA sequence specificity and affinities (Glisovic et al., 2008). RBPs contain at least one, but more often, multiple RNA-binding domains (RBDs). This modular design functions to recognize a much longer stretch of nucleotides or multiple RNAs that would not be possible with a single domain (Lunde et al., 2007). To add even more complexity, the RBPs usually interact with other proteins to form multi-protein complexes. Throughout the maturation and transport of the bound transcripts it is thought that mRNPs continuously change their composition (Müller-McNicoll and Neugebauer, 2013).

RNA-Binding Domains

The diverse functions of RBPs are built from RBDs present in various arrangements within the protein. Many RBDs are evolutionarily conserved and have been well characterized, as their function are critical to diverse cellular processes. The following is a list of some well-known RBDs. RNA recognition motif (RRM), also known as RNA-binding domain (RBD) and RNP domain, is the most common and best characterized of

the RBDs. In eukaryotes, RRM proteins function at all levels of post-transcriptional control from pre-mRNA processing to translational regulation and degradation (Maris et al., 2005). The K-homology (KH) domain was originally characterized in the heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Siomi et al., 1993). A single point mutation in the KH domain consensus sequence of fragile X mental retardation protein (FMRP) alters its RNA-binding abilities and causes fragile-X mental retardation syndrome (De Boulle et al., 1993). Zinc finger (ZnF) proteins are classically known for their DNA-binding abilities but have more recently been shown to bind to RNA as well (Lunde et al., 2007). This domain was originally characterized in the transcription factor TFIIIA, which contains nine CCHH zinc fingers that either bind DNA or RNA, with the exception of finger 5 that binds both (Clemens et al., 1993; Lu et al., 2003; Searles et al., 2000). The PAZ and PIWI domains are found in a large number of proteins that function in RNA processing during RNA interference (RNAi) and microRNA (miRNA) biogenesis. (Lunde et al., 2007). Finally, the double-stranded RNA binding domain (dsRBD) is conserved from bacteria to humans and is composed of 70-90 amino acids that form a small αβ domain. dsRBDs are a unique domain as they bind secondary structure of RNA with very little sequence specificity. The specificity of at least some dsRBDs is mediated in part by an N-terminal helix that binds to irregular helical elements in A-form RNA such as stem-loops and mismatched base bulges (Lunde et al., 2007; Tian et al., 2004). Staufen (Stau) is a well-conserved dsRBP that contains five dsRBDs and has been demonstrated to function in mRNA localization and translational control in *Drosophila, Xenopus* oocytes and vertebrate neurons.

mRNA Localization and RNA-Binding Proteins in *Xenopus*

Localization of mRNA allows for the establishment of cell polarity, which is crucial for many developmental processes such as embryonic patterning, cell fate specification and cell movements (reviewed in Medioni et al., 2012). The purpose of mRNA localization is to ultimately regulate temporal and spatial protein expression. Asymmetric distribution of RNAs during oogenesis is essential for germ layer patterning and axis specification in *Xenopus.* The *Xenopus* oocyte has served as an important model system for understanding RNA localization, as a small number of maternal RNAs have been identified to localize to the animal or vegetal pole and the functional significance of their localization is reflected in developmental defect phenotypes (Houston and King, 2000; King et al., 2005; J. Zhang et al., 1998). Two mechanistically different pathways operate during oogenesis to localize distinct RNA to the vegetal cortex, the early or METRO (message transport organizer) pathway and the late pathway. Early pathway localization occurs during early oogenesis (stage I-II), maternal transcripts involved in germ cell determination, such as *Xcat2 (nanos1)* and *Xdazl* (Houston and King, 2000; Lai et al., 2011) are concentrated in the vegetal cortex through an association with the mitochondrial cloud (MC) (Chang et al., 2004; Heasman et al., 1984). Localization of germline RNAs to the MC appears to occur by a diffusion/entrapment mechanism, whereby smooth ER within the METRO region traps the germline RNA, though whether this association is direct remains to be determined (Chang et al., 2004; Kloc et al., 1996).

In late oogenesis, the late pathway functions to localize germ layer determinants to the vegetal cortex such as, *Vg1* and *VegT* mRNA, which encode a TGFβ signaling molecule

and a T-box transcription factor, respectively. These factors will later be inherited by the vegetal blastomeres during cleavage and are crucial for endoderm and mesoderm specification during embryogenesis (Birsoy et al., 2006; Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Weeks and Melton, 1987; J. Zhang et al., 1998; J. Zhang and King, 1996). Late pathway RNAs are initially expressed throughout the cytoplasm of the early oocyte, however they are excluded from the MC, which contains early pathway RNAs. During mid-oogenesis, late pathway RNA transport is directed along microtubules towards the vegetal cortex and remain tightly anchored to the cortical cytoplasm until the end of oogenesis (reviewed in King et al., 2005).

Proper localization of cytoplasmic RNAs relies on *cis*-acting localization elements (LEs), generally found in the 3'UTR of the localized message (reviewed in Kloc et al., 2002b). Detailed studies have identified LEs for several early and late pathway RNAs (Bubunenko et al., 2002; Chang et al., 2004; Kwon et al., 2002; Mowry and Melton, 1992). The early pathway RNA, *Xcat2,* requires two distinct elements for proper localization, MCLS and GGLE, both of which are found in the 3'UTR. The MCLS element is necessary and sufficient for *Xcat2* entrapment into the MC and the GGLE enables *Xcat2* inclusion into the germinal granules, though it requires the prior localization of the transcript into the MC (Chang et al., 2004; Kloc et al., 2002a). LEs identified in late pathway RNAs, *VegT*, *Vg1* and *Xvelo* do not share any evident sequence similarities (Bubunenko et al., 2002; Claussen and Pieler, 2004; Kwon et al., 2002; Mowry and Melton, 1992), however *Vg1* and *VegT* LEs appear to direct vegetal localization through the same molecular mechanism (Bubunenko et al., 2002; Kwon et al.,

2002). It has been proposed that LEs form conserved secondary and tertiary structures that are bound by RBPs (reviewed in Martin and Ephrussi, 2009; Pratt and Mowry, 2013).

Recognition of the LEs by multiple *trans*-acting protein factors leads to the formation of RNP complexes that are required for transport of RNA to their destined cytoplasmic localization (King et al., 2005; Mowry, 1996). RNP granules can contain multiple mRNA species and molecular motors, resulting in the transport different transcript to the same cellular location. Previously it was thought that specific RNP formation directing localization only occurred within the cytoplasm, however RBPs can associate with target RNAs within the nucleus. Evidence suggests that localization-specific RNP formation may be initiated in the nucleus and can regulate transport of RNA to its destination in the cytoplasm (King et al., 2005; Kress et al., 2004). Within the *Xenopus* oocyte, extensive work on late pathway *Vg1* RNA localization has provided insight into the complex mechanism involved in RNA localization. *Vg1* RNA associates with several proteins termed VgRBPs, two of which, Vg1RBP/vera and VgRBP60/hnRNP1, bind to parts of the LE sequences in the *Vg1* 3'UTR and function in its localization (Cote et al., 1999; Deshler, 1997; Deshler et al., 1998; Havin et al., 1998; T. T. Kroll et al., 2002; Mowry, 1996; Schwartz et al., 1992; Yoon and Mowry, 2004; Zhao et al., 2001). Both Vg1RBP/vera and VgRBP60/hnRNP1 first associate with *Vg1* as well as *VegT* transcripts within the oocyte nucleus (Kress et al., 2004). Nuclear export of Vg1-RNPs corresponds with modifications to the protein complex; though VgRBP60/hnRNP1 remains associated with *Vg1*, the RBP is phosphorylated during export of the RNP and this may lead to the

recruitment of other factors, such as Prrp and Stau (King et al., 2005; Kress et al., 2004; Yoon and Mowry, 2004; Zhao et al., 2001). Stau may play a role in Vg1 localization as it interacts with both *Vg1* mRNA and Kinesin I and expression of a mutant Stau disrupts *Vg1* localization (Fig. 3d) (Yoon and Mowry, 2004). At its final destination in the vegetal cortex, VgRBP60/hnRNP1 remains colocalized with *Vg1* RNA (Cote et al., 1999; Lewis et al., 2004). Once localized to the vegetal cortex Vg1 translation is repressed until late oogenesis (Tannahill and Melton, 1989; Wilhelm et al., 2000). Understanding the mechanisms involved in *Xenopus* vegetal localization pathways is essential to comprehend embryonic development.

mRNA localization was also shown to be required for axonal growth cone steering decisions in studies performed in growing *Xenopus* and murine axons. The selection of axonally localized mRNAs appears to depend on external signals and developmental cues (reviewed in Medioni et al., 2012). mRNA localization also plays a role in cell migration, studies in *Xenopus* migrating neural cells have demonstrated that Vg1RBP, identified in *Vg1* mRNA localization in the oocyte, asymmetrically accumulates within neural cells and is required for their migrations (Yaniv et al., 2003). Interestingly, many well-studied RBPs identified in oocyte development are expressed later in development, however our understanding of their function is limited. As most cells are polarized, mRNA localization is likely required for proper cell function and RBP function would be crucial. It will be intriguing to determine how RBPs and mRNA localization pathways utilized later in development compare to those studied in *Xenopus* oocyte vegetal localization.

RNA-Binding Proteins in *Xenopus* Endoderm Development

The specification and patterning of the endoderm germ layer into organ lineages and discrete organs depends on an intricate gene regulatory network of factors and signaling molecules. In *Drosophila* development, RBPs play a vital role in specification and patterning as almost 70% of mRNAs within the embryo are localized (Lécuyer et al., 2007). While many of these RBPs are conserved in vertebrates and have even been implicated in mRNA localization in *Xenopus* oocytes, the role of RBPs during *Xenopus* endoderm development is poorly understood.

VegT is a T-box transcription factor, encoded by a maternal mRNA and is localized to the vegetal pole of *Xenopus* oocyte (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; J. Zhang and King, 1996). VegT is essential for the formation of the endoderm germ layer and induction of the mesoderm (J. Zhang et al., 1998). While we understand its requirement to be localized in the oocyte, the mechanism by which VegT is regulated within the developing embryo is poorly understood. Recently an RBP, XSeb4R has been shown to positively regulate *VegT* mRNA by interacting with its 3'UTR leading to transcript stabilization and translational stimulation (Souopgui et al., 2008). Loss of XSeb4R function showed that it is required for proper endodermal and mesodermal germ layer formation, likely through the regulation of *VegT* and other unknown mRNA targets (Souopgui et al., 2008).

Another maternal mRNA, *Vg1*, which encodes a TGF-β family growth factor involved in endoderm and mesoderm induction (Birsoy et al., 2006; Thomsen and Melton, 1993).

Vg1 mRNA is vegetally localized in the *Xenopus* oocyte by the RBPs including Stau, PTB/hnRNP1, 40LoVe and Vg1RBP/vera (Cote et al., 1999; Czaplinski and Mattaj, 2006; Deshler, 1997; Thomsen and Melton, 1993; Weeks and Melton, 1987; Yoon and Mowry, 2004). This protein has biphasic expression; initially as a maternal component and then later as it is expressed zygotically. First, maternal Vg1RBP accumulates in the vegetal pole of the *Xenopus* oocyte and plays a role in the specification of the endodermal germ layer through localization of *Vg1* mRNA. Later, Vg1RBP is expressed in a variety of tissues including endodermally derived organs and the surrounding mesoderm (Spagnoli and Brivanlou, 2006). Zygotic activity of Vg1RBP has been shown to be required for the specification of the pancreatic endoderm and proper expression of *insulin* during development (Spagnoli and Brivanlou, 2006). This function requires an interaction between Vg1RBP and *shirin* 3'UTR, however the Shirin protein is not required for pancreatic specification. Interestingly, this interaction may function to pattern the pancreatic region by protecting Vg1RBP from degradation, as oppose to the RBP regulating *shirin* expression (Spagnoli and Brivanlou, 2006).

RBP families are conserved throughout evolution and have been identified to play critical roles in many aspects of development in several organisms, however our understanding of RBPs during endoderm development is lacking. A better understanding of the role these proteins play during development will help unravel how complex gene regulatory networks are being regulated post-transcriptionally.

STAUFEN

Comparison of Stau Orthologs

Stau is a dsRBP originally identified in *Drosophila* for its role in oocyte patterning by localizing *bicoid* and *oskar* mRNA to anterior and posterior poles, respectively (Ferrandon et al., 1994; St Johnston, 1995; St Johnston et al., 1991; St Johnston et al., 1989). In vertebrates there are two Stau orthologs, Stau1 and Stau2, which are coded by two separate genes, and each Stau gene produces multiple isoforms through alternative splicing. In Stau orthologs from *Drosophila* to humans, high sequence identity is found between corresponding dsRBDs, and their overall structures and relative position within the proteins are well conserved (Fig. 2) (Wickham et al., 1999). All known Stau proteins contain an insert in dsRBD2, which in *Drosophila* was determined to prevent its potential to bind dsRNA *in vitro* (Allison et al., 2004; Duchaîne et al., 2002; Micklem et al., 2000; Wickham et al., 1999; Yoon and Mowry, 2004). The most striking difference between orthologs is that mammalian Stau1 does not contain dsRBD1, however *Xenopus* Stau1 does contain dsRBD1 (Allison et al., 2004; Wickham et al., 1999; Yoon and Mowry, 2004). In human Stau1, dsRBD2 and dsRBD5 are short domains compared to the consensus (Wickham et al., 1999). In addition, mammalian Stau1 does not contain the long N-terminal sequence of the *Drosophila* protein, in a yeast two-hybrid assay the amino-half of the *Drosophila* Stau was shown to interact to Oskar protein (Breitwieser et al., 1996). The vertebrate Stau1 does contain a tubulin-binding domain (TBD) between dsRBD4 and dsRBD5 that does not appear to be found in the *Drosophila* protein (Wickham et al., 1999).

The other Stau ortholog, Stau2 has many conserved features in common with Stau1, vertebrate Stau2 also contains the TBD between dsRBD4 and dsRBD5 identified in mammalian Stau1 (Fig. 2). Mammalian Stau2 differs from Stau1 as it contains the corresponding *Drosophila* dsRBD1; however the domain is not fully conserved in all three isoforms. Stau2^{62} contains for the full-length dsRBD1, Stau2^{59} and Stau2^{52} only contain the C-terminal half of the domain (Duchaîne et al., 2002). Furthermore, a truncated form of dsRBD5 is found in Stau2, which contains only the C-terminal domain in a rearranged form (Duchaîne et al., 2002). Three main functions of Stau proteins have been characterized; mRNA localization, translational regulation and mRNA decay; the latter has only been illustrated in mammalian cells. Both Stau orthologs have been demonstrated to perform all of these functions, however their roles do not appear to be fully redundant (Dugre-Brisson et al., 2005; Y. K. Kim et al., 2005; Kretz et al., 2013; St Johnston et al., 1991; Yoon and Mowry, 2004).

mRNA Localization

Drosophila

Most cells are polarized with cellular components such as organelles, transcripts and proteins asymmetrically partitioned throughout the cell that fulfill specific biological functions (Lécuyer et al., 2007). This asymmetric distribution of cellular proteins is highly dependent on the localization of mRNA for regionalized translation. Patterning in the *Drosophila* embryo is generated by the asymmetric distribution of maternal transcripts within the oocyte along the A-P axis and the dorsal-ventral (D-V) axis. It has been well established that asymmetry along the A-P axis is initiated by the restricted

expression of *bicoid* mRNA to the anterior of the oocyte and *oskar* mRNA to the posterior (reviewed in Kugler and Lasko, 2009).

Stau, which was identified in *Drosophila,* was the first RBP shown to be involved in mRNA localization (St Johnston et al., 1991). Within the oocyte, Stau is required for proper A-P axis formation. Stau protein is initially expressed throughout the cytoplasm, later it accumulates at the anterior pole, and finally moves around the cortex to the posterior pole (Ferrandon et al., 1994). Anterior expression of *bicoid* is required for development of the *Drosophila* head and thorax, and its proper localization is a multistep process that involves multiple proteins including ESCRT-II, Exuperantia, Swallow and Stau (Ferrandon et al., 1994; Irion and St Johnston, 2007; Schnorrer et al., 2000). Stau function was initially suggested to be required only to anchor *bicoid* mRNA to the anterior pole of the oocyte (Ferrandon et al., 1994), while Swallow was required to link *bicoid* mRNA to the dynein motor (Schnorrer et al., 2000). However, advancements in microscopy have allowed recent studies to visualize components of mRNPs, identifying particles containing Stau, dynein and *bicoid* mRNA at the oocyte anterior, suggesting Stau, not Swallow plays a direct role in dynein-dependent *bicoid* mRNA transport (Fig. 3a) (Weil et al., 2009). Stau interacts with the *bicoid* 3'UTR, specifically a highly structured 400 nucleotide segment that appears to form dimers between single-stranded RNA loops, forming RNA-RNA interactions that can interact with Stau (Ferrandon et al., 1994; 1997).

Stau is also required for the proper localization of *oskar* mRNA to the posterior end of the *Drosophila* oocyte (Fig. 3b) (Ferrandon et al., 1994). Posterior localization of *oskar* as well as *nanos* mRNA is required for the assembly of the pole plasm and for abdomen patterning and germ cells formation in the embryo (reviewed in Kugler and Lasko, 2009). Transport of this complex to the posterior appears to occur along microtubules using Kinesin I (Brendza et al., 2000; Clark et al., 1994), and Stau transport to the posterior pole seems to require an RNA-interaction (Ferrandon et al., 1994). Many proteins have been shown to be involved in the assembly of *oskar* mRNA into mRNPs and to localize it to the posterior pole, this complex also appears to be assembled in a stepwise manner. First, as the mRNA is being transcribed the hnRNP family member RBP Hrp48 is thought to bind *oskar*, next components of the exon junction complex (EJC), Mago-Y14 and eIF4AIII associate with the RNA as it is spliced. Finally, another EJC component, Barentsz is recruited as the *oskar* complex is exported from the nucleus, the *oskar* complex is then bound by Stau in the cytoplasm (Hachet and Ephrussi, 2001; Huynh et al., 2004; Mohr et al., 2001; Palacios et al., 2004; van Eeden et al., 2001; Yano et al., 2004). Recent data using *in vivo* quantitative imaging of *oskar* and Stau colocalization revealed that Stau does not associate with *oskar* as the transcript travels from the nurse cell and through the anterior oocyte, and is likely only involved in localization once *oskar* is already in the posterior half of the oocyte (Mhlanga et al., 2009).

Within the embryo, Stau has been shown to bind to the 3'UTR of *prospero (pros)* and localize it to the basal cortex of neuroblast (NB) cells (Fig. 3c) (P. Li et al., 1997). In *Drosophila,* there are two determinants of cell fate Numb and Pros that are

asymmetrically localized to the basal cortex of NB cells. After cell division they are specifically separated into the ganglion mother cells (GMC), which are the daughter cells (P. Li et al., 1997). Pros is a HD protein that activates genes required for GMC fate and repress NB fate (Doe et al., 1991, Vaessin et al., 1991). In the absence of Stau, NBs mostly show uniform *pros* localization, though a low frequency of *pros* mRNA is still basally localized, suggesting Stau is not the only factor able to localize *pros* mRNA (Doe et al., 1998). Basal localization of the *pros*/Stau complex involves the binding of Miranda, which acts as an adapter to tether the complex to the basal cortex (Matsuzaki et al., 1998; Shen et al., 1998). Miranda appears to be able to directly bind *pros* mRNA, however its basal localization appears to require the binding of Miranda to the dsRBD5 of Stau (Ikeshima-Kataoka et al., 1997; Irion et al., 2006; Shen et al., 1997). In *Drosophila*, Stau-mediated mRNA localization is required in both oocyte and embryonic development for proper cell fate specification.

Mammals

The role of Stau in mRNA transport in mammalian cells was suggested when Stau expression was determined to be restricted to the somatodendritic region of mature rat hippocampal neurons (Kiebler et al., 1999). Stau was shown to associate in RNA-containing granules and travel within dendrites in a microtubule-dependent manner (Fig. 3e) (Köhrmann et al., 1999; Monshausen et al., 2001; Tang et al., 2001). Transport of dendritic mRNA via Stau1 or Stau2 particles appears to be mediated by kinesin motor proteins and dendritic targeting requires the TBD in the Stau C-terminal region (Jeong et al., 2007; Mallardo et al., 2003). Isoforms of the Stau1 and Stau2 orthologs form distinct

particles that are differently localized within the dendrite and may have different functions (Mallardo et al., 2003). Neuronal activity appears to regulate Stau-mediated mRNA transport, as depolarization increased the amount of Stau-containing mRNP complexes in dendrites (K.-C. Kim and H. K. Kim, 2006). The MAPK pathway may modulate this depolarization-dependent activity, as an interaction between Stau2 and ERK1/2 is required for transport of Stau2-containing mRNP complexes in the distal dendrites (Nam et al., 2008). It is likely that synaptic activity may influence the selected RNAs that are transported to the sites of demand (Mikl et al., 2011).

A biological role for dendritic mRNA transport has been identified in long-term memory formation. Stau mRNA transport has been shown to be critical in regulation of synaptic strength, spine morphogenesis and maintenance of functional connectivity changes underlying hippocampus-dependent learning and memory (Dubnau et al., 2003; Goetze et al., 2006; Lebeau et al., 2011; 2008). In Stau1 mutant mice, which express a truncated Stau1 protein lacking a functional dsRBD3, hippocampal neurons develop reduced dendritic trees, fewer synapses and display decreases in dendritic mRNPs containing Stau1 and *β-actin* mRNA (Vessey et al., 2008). Stau proteins have also been identified in sensory neuronal axons, potentially allowing neurons to rapidly alter their function by local translation of different sensory-specific mRNAs (Price et al., 2006).

Since the identification of Stau as a key player in mRNA localization within dendrites, many studies have focused on determining the composition of Stau-containing mRNPs and understanding how this protein can selectively target certain transcripts to specific

subcellular localizations. One level of selection is through its interaction with protein partners. Some mRNA transport machinery appears to be conserved during evolution, while novel protein partners have also been identified (Macchi et al., 2003; Monshausen et al., 2005). At the nucleus and into the cytoplasm, Stau2 forms a complex with EJC proteins Y14, Mago and Barentsz (Macchi et al., 2003; Monshausen et al., 2005). The Stau-Y14/Mago association is conserved with the *oskar* mRNA transport pathway in *Drosophila* (Monshausen et al., 2005). In mammalian cells, Barentsz behaves like a nucleo-cytoplasmic shuttling protein in an RNA-dependent manner (Macchi et al., 2003). Stau also contains a phosphatase interacting motif and can interact with Protein Phosphatase-1 in hippocampal neurons, raising the possibility that Stau function may be regulated by phosphorylation (Monshausen et al., 2002).

Other efforts made to identify proteins present in mRNPs containing Stau2 have led to the identification of other mRNA binding proteins (FMRP, PABPC1, hnRNP H1, YB1 and hsc70), as well as elements of the cytoskeleton and regulators of cytoskeleton (tubulin, actin, cdc42 and rac) and motor proteins (dynein, kinesin and myosin Va) (Maher-Laporte et al., 2010; Ohashi et al., 2002; Villacé et al., 2004). Stau1 has been shown to self-associate through dsRBD2 and dsRBD5 (Martel et al., 2010). Recently a novel motif called Stau-swapping motif (SSM) and the dsRBD5 were shown to be responsible for protein homo-dimerization (Gleghorn et al., 2013). The two SSM α-helices of one molecule interact primarily through a hydrophobic patch with the two RBD5 α-helices of a second molecule (Gleghorn et al., 2013). Each newly identified protein partner provides insight into how Stau-containing mRNPs are transported and

locally expressed according to the cell's needs. These could also aid in our understanding of how Stau interacts with protein partners.

Within dividing neural stem cells, Stau's role in asymmetric localization to regulate a balance between stem cell maintenance versus differentiation appears to be conserved from *Drosophila* to mammals (Kusek et al., 2012; Vessey et al., 2012). Within radial glial precursor cells, Stau2 has been found to complex in granules with Pumilio2 and DDX1 proteins and conserved mRNAs *β-actin* and *prox1* (mammalian *prospero*). Knockdown of Stau2 leads to premature differentiation of radial glial precursors into neurons and mislocalization of *prox1* mRNA (Vessey et al., 2012).

While Stau proteins are mostly found in the cytoplasm, the protein can enter both the nucleus and the nucleolus (Macchi et al., 2004; Martel et al., 2006; Miki and Yoneda, 2004). Nuclear trafficking of Stau proteins appears to be a highly regulated process that likely involves several determinants (Martel et al., 2006). In myotonic dystrophy (DM1), Stau1 interacts with mutant DM1-related mRNAs through its dsRBD3, it promotes their nuclear export and translation of aberrantly spliced mRNAs leading to the pathology. Overexpression of Stau1 can rescue the alternative splicing of two key pre-mRNAs in DM1, uncovering a novel role for Stau1 in splicing regulation and its potential as a therapeutic target (Ravel-Chapuis et al., 2012).

Xenopus

Both Stau homologs have been identified in *Xenopus* throughout development, though their function in mRNA localization has only been studied within oocytes (Allison et al., 2004; Yoon and Mowry, 2004). While Stau1 appears to be more abundant in oocytes, the levels of both Stau1 and Stau2 proteins peak during mid-oogenesis and decline after stage IV (Allison et al., 2004; Yoon and Mowry, 2004). During oogenesis development, Stau proteins are dynamically expressed, at early stages exhibiting uniform cytoplasmic staining then at late oogenesis expression becomes cortically restricted and asymmetrically concentrated at the vegetal cortex (Allison et al., 2004; Yoon and Mowry, 2004). The timing of the vegetal concentration of Stau correlates with that of the late pathway RNA localization (stage III-IV). Stau containing mRNPs were shown to contain late pathway vegetally localized *Vg1* and *VegT* mRNA, and Stau has also been shown to interact with *Vg1* RBPs, hnRNP1 and Vg1RBP/vera as well as a Kinesin I (Fig. 3d) (Kress et al., 2004; Yoon and Mowry, 2004). Interestingly, Stau does not appear to interact with RNAs localized by the early pathway, such as *Xcat2* or *Xwnt11*, or non-localized RNAs (Yoon and Mowry, 2004). Overexpression of the *Vg1* localization elements (VLE) RNA recruits endogenous Stau to vegetally localized mRNPs (Yoon and Mowry, 2004). This vegetal Stau localization was abolished when a dominant-negative Stau (DN-Stau), lacking dsRBD1 and dsRBD5, was overexpressed along with the VLE (Yoon and Mowry, 2004). Failure of DN-Stau to vegetally localize with VLE RNA may be caused by the inability of the DN-Stau to interact with certain components of the localization machinery since DN-Stau cannot interact with hnRNP1, however its ability to associate with Vg1RBP was not affected by the DN-Stau (Yoon and Mowry, 2004).

Similar to reports in dendritic cells, the *Xenopus* Stau homologs appear to localize in different mRNP complexes within the oocyte (Allison et al., 2004). During meiotic maturation, when RNAs such as *Vg1* mRNA are released from their tight localization at the vegetal cortex, Stau proteins are transiently phosphorylated by the MAPK pathway. While Stau protein localization is not affected by phosphorylation, its association with endoplasmic reticulum (ER) at the vegetal cortex is lost (Allison et al., 2004). It is not known whether phosphorylation alters Stau function, though it may modulate Stau's RNA binding ability, its protein-protein interactions or cytoskeletal and ER interactions that are required for anchoring (Allison et al., 2004).

Translational Control

Stau's role in translational control in *Drosophila* appears to be linked to mRNA localization. In vertebrates, Stau containing mRNPs associate with ribosomes, suggesting a role in translation (Kiebler et al., 1999; Marión et al., 1999; Wickham et al., 1999). To date, most studies have focused on identifying mRNA targets and protein partners associated with mRNPs while our understanding of the mechanism used by Stau for translational regulation is limited. Whereas mRNA localization appears to be regulated by Stau interacting with the 3'UTR of it's targets (Ferrandon et al., 1994; P. Li et al., 1997), multiple mechanisms have been identified in Stau regulated translational control. First, a report has shown that Stau1^{55} can stimulate translation by binding to dsRNA at the 5'end of reporter transcripts (Dugre-Brisson et al., 2005). More recently, the long non-coding RNA (lncRNA) TINCR has been shown to complex with Stau1 in somatic tissue and mediate stabilization of differentiation mRNAs to ensure their expression

(Kretz et al., 2013). Third, Stau has been identified as a host protein that is selectively incorporated into HIV particles (Chatel-Chaix et al., 2004). Within these particles, Stau1 along with the nonsense-mediated mRNA decay (NMD) factor Upf1 appear to be required for HIV-1 RNA stability and can positively influence HIV-1 RNA translatability (Ajamian et al., 2008). Finally, a study showed that the effect of tethering Stau2 to the 3'UTR of an mRNA was cell-type dependent, while there was little change in target expression in HeLa cells, mRNA and protein levels of the targets were increased in 293F cells (Mikl et al., 2011). These data suggest that the mechanism for Stau regulated translational control may be cell-type specific.

mRNA Decay

In contrast to its role in HIV, mammalian Stau1 and Stau2 have been shown to dimerize and associate with the NMD factor Upf1 to mediate mRNA decay of target transcripts (Fig. 3f). This occurs independently of splicing and most of the other NMD factors (Cho et al., 2012; Y. K. Kim et al., 2005; Park et al., 2013). In fact, Stau1 binding to Upf1 competes with NMD factor Upf2 binding to Up1, therefore as the efficiency of Stau1 binding to Upf1 increases, the efficiency of NMD decrease (Maquat and Gong, 2009). This new process was named Stau-mediated mRNA decay (SMD) and requires a Stau binding site (SBS) which is a conserved 19 base pair stem with a 100-nucleotide apex either formed in the secondary structure of the targets 3'UTR or through the interaction of ½ of the SBS (½SBS) in the targets 3'UTR and an Alu element in a lncRNA (Gong and Maquat, 2011; Y. K. Kim et al., 2005). As Alu elements exist only within the human genome, recent evidence showed that B and identifier (ID) short interspersed elements

(SINEs) in mice lncRNAs can base pair to ½SBS in mice target 3'UTRs (Wang et al., 2013).

This relatively novel mechanism of regulation appears to play a role in cell differentiation (Cho et al., 2012; Gleghorn et al., 2013; Gong et al., 2009; Y. K. Kim et al., 2007). In myogenesis, Pax3 is a critical gene involved in maintenance of myoblasts, while myogenin is required for the differentiation of myoblasts into myotubes. It was observed that the efficiency of SMD increases as C2C12 myoblasts differentiate into myotubes, and that Pax3 is an SMD target while myogenin is an NMD target. These data suggest the competitive interaction of Upf1 with Stau1 and Upf2 can contribute to myogenesis (Gong et al., 2009; Y. K. Kim et al., 2007). Another biological role for SMD has been identified in adipogenesis, where Stau targets a novel substrate anti-adipogenic Kruppel-like factor 2 (Klf2) for SMD leading to accelerated adipogenesis (Cho et al., 2012). Whether SMD is conserved in all organisms remains to be determined.

Stau Function During Development

While Stau proteins have been identified in most vertebrates, little is known about their biological function in early vertebrate development. Both Stau homologs are expressed in mouse and zebrafish germ cells where they are required to maintain *vasa* expression and for the survival of zebrafish primordial germ cell (Bateman et al., 2004; Ramasamy et al., 2006; Saunders et al., 2000). Knockdown studies in mouse embryonic stem (mES) cells suggest that Stau is required for cell lineage differentiation (Gautrey et al., 2008). Recently, Stau2 was shown to associate with basal determinants and asymmetrically

segregate into Tbr2+ neuroblast daughter cells in the developing mouse cortex. Knockdown of Stau2 in neuroblast cells stimulated their differentiation to periventricular neurons (Kusek et al., 2012). Stau1 is also prominently expressed in adult mouse gastric epithelium and in Caco-2 intestinal epithelial cells where asymmetrically distributed proteins and mRNA may help epithelial progenitors establish differential communication with neighboring cells in their niche (Gautrey et al., 2005; Mills et al., 2002). Finally, in chick embryos, Stau2 is involved in regulation of cell proliferation within the developing eye through the regulation of *hes1* and *sox2* expression (Cockburn et al., 2012).

In *Xenopus*, Stau has been demonstrated to vegetally co-localize with VLE RNA and can interact biochemically with vegetal determinants, *Vg1* and *VegT* mRNA, in oocytes (Yoon and Mowry, 2004). In Chapter 2, I demonstrate that *stau2* is expressed within all three germ layers of the gastrula embryo but expression becomes restricted to neural tissue and the anterior endoderm by tailbud stages. The data presented in Chapter 2 identified a new function for Stau2 in patterning of the endoderm (Bilogan and Horb, 2012b) In Chapter 3, I demonstrate that Stau2 is required for maintenance of the Wnt/β-catenin signaling pathway during gastrulation and I identified novel Stau2 mRNA targets during endoderm development.

RNA-Binding Domains

Since the identification of Stau as a dsRBP, numerous studies have attempted to elucidate the functional role of the five dsRBDs, many of which have yielded conflicting results. This is likely due to the experimental design, for example by overexpressing individual

dsRBPs *in vitro* or using different dsRNA targets. In *Drosophila*, dsRBD1, dsRBD3 and dsRBD4 can bind dsRNA *in vitro*, while dsRBD2 is required for microtubule-dependent localization of *oskar* mRNA and dsRBD5 is involved in de-repression of *oskar* mRNA once it is localized (Micklem et al., 2000). Another study that mutated the RNA-binding activity of dsRBD3 showed it is required *in vivo* to localize *bicoid* and *oskar* mRNAs (Ramos et al., 2000). In *C. elegans*, no single Stau1 dsRBD appeared to be required for binding dsRNA, suggesting more than one dsRBD may bind to the same target RNA (LeGendre et al., 2013). However, using an RNA binding assay, mammalian Stau1 dsRBD3 and dsRBD4 were independently sufficient to bind *bicoid* RNA while dsRBD2 and dsRBD5 were not (Wickham et al., 1999). In a similar experiment, individual dsRBDs from Stau2 were tested for their ability to bind to *bicoid* 3'UTR, only dsRBD3 bound strongly while dsRBD4 bound weakly (Duchaîne et al., 2002). Yoon and Mowry (2004) showed that expression of *Xenopus* Stau lacking dsRBD1 and dsRBD5 in oocytes blocked vegetal localization *in vivo*. While the mutant Stau retained the ability to bind Vg1RBP, interaction with hnRNP1 was disrupted.

RNA Targets of RBPs

RBPs interact with mRNA targets and mediate their transport, stability, translation and cellular localization. Eukaryotes encode a large number of RBPs and while each RBD family has conserved characteristics, every RBP has a unique RNA-binding ability and protein-protein interaction characteristics. RBPs regulate multiple RNA targets and our knowledge of the complete list of mRNA targets that are bound by RBPs is lacking (Glisovic et al., 2008; Lee, 2006). Identification of all the mRNA targets of a given RBP

would provide insight into how an RBP controls development, as well as help identify the target sequences and/or structures that it binds to and how it controls its targets (Lee, 2006). Stau RBPs are expressed throughout most cell types and appear to regulate a wide array of mRNA targets and functions. Identification of direct Stau mRNA targets has proven to be difficult due to the lack of sequence specificity required for dsRNA binding and indirect target identification through protein-protein interactions.

A handful of studies have begun to provide insight into Stau mRNA targets using microarray analysis. The first high-throughput analysis of mammalian Stau mRNA targets was performed using a microarray against mRNA isolated from Stau-containing complexes from HEK293T cells transfected with Stau1^{55}-HA, Stau2^{59}-HA or Stau2^{62}-HA (Furic et al., 2008). More recently, genome-wide approaches to identify endogenous Stau2-bound mRNAs in embryonic rat brains and embryonic mouse cortex were performed (Kusek et al., 2012; Maher-Laporte and DesGroseillers, 2010). These studies each identified thousands of potential mRNA targets, and clustering of the identified genes into biological processes identified a wide spectrum of functions from transcription, RNA metabolism, intracellular transport and translation (Furic et al., 2008; Kusek et al., 2012; Maher-Laporte and DesGroseillers, 2010). Microarray analysis of endogenous Stau1-mRNPs in *C. elegans* identified mRNAs involved in embryonic, larval and reproductive biology, which are consistent with previously characterized roles in developmental patterning in *Drosophila* oocytes and embryos (LeGendre et al., 2013). However these differ from the diverse biological functions identified from mammalian Stau mRNA targets (Furic et al., 2008; Maher-Laporte and DesGroseillers, 2010).

Stau proteins have been demonstrated to play a role in a variety of cellular functions and it is likely that these functions are cell-specific (Mikl et al., 2011). Stau has been shown to regulate cell specification and differentiation in many species from *Drosophila* to mammals, and how Stau regulates its mRNA targets may be unique in each case. Therefore comparison of identified Stau targets from different tissues and developmental stages may not be useful to fully understand how Stau elicits its function, as Stau may perform cellular- or temporal-specific functions throughout development. I recently demonstrated that Stau2 is required for patterning of the developing *Xenopus* endoderm (Chapter 2) (Bilogan and Horb, 2012b). To determine how Stau2 may be mediating endoderm patterning I set out to identify mRNA targets within the endoderm when Stau2 function is required using RNA-seq (Chapter 3, Fig. 3). Focusing on a particular function or cell type may provide a better understanding of how Stau can regulate mRNA localization, stability and translational control of many different targets.

PANCREAS DEVELOPMENT IN *XENOPUS*

The vertebrate pancreas is a complex endodermal organ composed of exocrine and endocrine components. In *Xenopus*, as with other vertebrates, the pancreas arises from separate dorsal and ventral pancreatic buds. The dorsal pancreatic bud arises first at the Nieuwkwoop and Faber stage of *Xenopus laevis* development (NF) 35/36 (Nieuwkoop and Faber, 1967), while the ventral pancreas develops from two separate ventral buds at NF37/38. The two ventral buds fuse together around NF40, this is different than in mammals, where one of the ventral buds actually regresses. The dorsal and ventral pancreatic buds then fuse to form a discrete organ comprised of endocrine and exocrine tissues (Kelly and Melton, 2000; Pearl et al., 2009).

The endocrine pancreas is composed of 5 different cell types, α, β, δ, ε and PP cells that secrete Glucagon, Insulin, Somatostatin, Ghrelin, and Pancreatic Polypeptide hormones into the bloodstream. The exocrine pancreas consists of both acinar and ductal cells that secrete and transport digestive enzymes, such as Amylase, Trypsinogen and Elastase into the duodenum. In *Xenopus*, the exocrine and endocrine cells initially develop in separate pancreatic anlagen. Prior to fusion, endoderm markers are restricted to the dorsal bud, while exocrine cells are found in the ventral buds, however after fusion the cells types slowly become expressed across the entire organ (Kelly and Melton, 2000; Pearl et al., 2009)

The transcriptional regulatory network involved in pancreatic cell fate specification and differentiation of both endocrine and exocrine lineages is complex. Many transcription

factors have been identified to play critical roles during pancreas development, however their exact function and downstream targets remains unknown. Two of the earliest acting transcription factors that are considered key genes in pancreas development are Pdx1 and Ptf1a. Pdx1 is a ParaHox gene expressed in the endoderm at the level of the future dorsal and ventral pancreatic buds as well as the duodenum, prior to overt morphogenesis (Wright et al., 1989). Later in development, Pdx1 becomes most highly expressed in β-cells and required for their specification over other pancreatic endocrine cell fates. In *Xenopus,* Pdx1 and Ptf1a are both necessary and sufficient for pancreas development (Afelik et al., 2006; Horb et al., 2003; Jarikji et al., 2007) In fact, ectopic expression of both can simultaneously convert posterior endoderm into pancreatic tissue (Afelik et al., 2006).

Functional Analysis of Ptf1a in *Xenopus laevis*

The basic helix-loop-helix (bHLH) protein pancreas-specific transcription factor 1a (PTF1a) is required for proper development of the pancreas, retina and nervous system and is one of the earliest pancreas-specific markers (Fujitani et al., 2006; Glasgow et al., 2005; Kawaguchi et al., 2002; Roux et al., 1989; Sellick et al., 2004). Loss of Ptf1a function in mice and humans results in the complete absence of acinar cells and a large reduction of *insulin* expression (Kawaguchi et al., 2002; Krapp et al., 1996; Sellick et al., 2004), whereas in zebrafish it is required mainly for exocrine pancreas development (Lin et al., 2004; Zecchin et al., 2004). In *Xenopus*, *ptf1a* expression is detected as early as NF27/30 in the developing dorsal and ventral pancreatic anlagen (Afelik et al., 2006). It is expressed in tadpole acinar cells until the climax of metamorphosis when the *Ptfla*

gene is shut off (Pearl et al., 2009). This expression is similar to that seen in mice, where Ptf1a is expressed in all pancreatic progenitors at the earliest stages of pancreas development, but becomes localized to acinar cells shortly thereafter (Kawaguchi et al., 2002; Krapp et al., 1996).

Within the pancreas, Ptf1a plays a biphasic role in development; early Ptf1a is required for the specification of foregut endodermal cells to adopt a pancreatic fate, and Ptf1a is expressed throughout the pancreatic multipotent progenitor cells (MPCs) (Kawaguchi et al., 2002). Over time, Ptf1a expression becomes restricted to acinar cells and directly promotes expression of terminal exocrine differentiation genes such as Elastase and Carboxypeptidase 1 (Cockell et al., 1989; Krapp et al., 1996). The switch from Ptf1a's function in pancreas specification is linked to co-regulatory proteins in the trimeric PTF1 complex, while Ptf1a initially complexes with the vertebrate Suppressor of Hairless homolog Rbpj, during exocrine differentiation Ptf1a preferentially interacts with another homolog Rbpjl (Fig. 4) (Masui et al., 2007). The genes targeted by Ptf1a in exocrine differentiation are well defined, however it is not specifically known how Ptf1a regulates pancreatic specification.

Two studies examined the function of *Xenopus* Ptf1a in early pancreas development and found Ptf1a to be both necessary and sufficient for pancreas development. Both studies found that knockdown of Ptf1a resulted in a complete loss of acinar cells; however, striking differences regarding endocrine cell development were found (Afelik et al., 2006; Jarikji et al., 2007). In the first study, a single morpholino (MO) was used to

knockdown Ptf1a, and early expression of *insulin* at NF35 was unaffected, while the late expression of both *insulin* and *glucagon* was lost at NF48 (Afelik et al., 2006). The second study, using two MOs to target distinct regions of the *ptf1a* transcript, found the exact opposite: knockdown of Ptf1a resulted in an absence of early *insulin* expression, while low levels of *insulin* were detected at later stages (Jarikji et al., 2007). The reason for this discrepancy is currently unclear. Nevertheless, these studies show that the function of Ptf1a in *Xenopus* pancreas development is quite similar to that observed in other vertebrates where Ptf1a is required for development of all acinar cells and a subset of endocrine cells.

Overexpression of Ptf1a, on the other hand, was found to be sufficient to promote ectopic pancreatic cell fates. Injection of *ptf1a* mRNA into the two dorsal-vegetal blastomeres at the eight-cell stage (targeting the anterior endoderm) resulted in the conversion of stomach/duodenum to pancreas (Afelik et al., 2006; Jarikji et al., 2007). Using a hormone-inducible version (Ptf1a-GR), it was found that when Ptf1a was activated at NF27 or earlier, it was able to promote ectopic pancreatic cell fates, whereas when activated after NF36, it had no effect (Afelik et al., 2006). Activation of Ptf1a between NF30 and NF35 did not convert stomach/duodenum to pancreas, but did result in an enlarged pancreas. In contrast, a super-active form of Ptf1a (Ptf1a-VP16) was found to have greater activity than the unmodified Ptf1a in naïve endoderm. In addition to being able to convert prospective stomach/duodenum to pancreas as seen with unmodified Ptf1a, Ptf1a-VP16 was also able to convert prospective liver to pancreas. However, only the acinar cell fate was promoted (Jarikji et al., 2007). Transgenic overexpression of

Ptf1a-VP16 in *Xenopus* tadpoles was sufficient to convert liver to acinar cells (but had no effect in the stomach or duodenum), while Ptf1a converted stomach/duodenum to endocrine and acinar cell fates (but had no effect in liver) (Jarikji et al., 2007). These results confirm earlier data in mice where it was shown that loss of Hes1 induces ectopic expression of Ptf1a in the stomach, duodenum, and common bile duct resulting in the production of ectopic patches of pancreatic tissue (Fukuda et al., 2006).

Few studies have focused on the downstream targets activated by Ptf1a in pancreatic MPCs; these would provide insight into the gene regulatory network involved in pancreatic specification. To date, a handful of direct downstream targets have been identified, known pancreas factors Pdx1, Nkx6.1, Onecut1 and Mnx1 are direct targets of Ptf1a, and Ptf1a promotes their expression within pancreatic progenitors (Miyatsuka et al., 2007; Thompson et al., 2012; Wiebe et al., 2007). Comparison of differentially regulated E10.5 pancreas progenitors in Ptf1a knockout (KO) mice to those of controls lead to the identification of novel direct targets such as BMP7, Nr5a2, RhoV and P2rx1 (Holmstrom et al., 2011; Thompson et al., 2012).

I recently performed a comparison of differently regulated genes in *X. laevis* embryos overexpressing *ptf1a* mRNA to those of controls (Bilogan and Horb, 2012a) (Chapter 4). I identified over 1000 genes over two time-points in early pancreatic specification that are likely involved in the gene regulatory network downstream of Ptf1a in pancreas development (Bilogan and Horb, 2012a) (Chapter 4). A better understanding of the regulatory network involved in Ptf1a directed pancreas development would provide

insight it how mature pancreatic cells could be altered to regain multipotency to produce β-cells.

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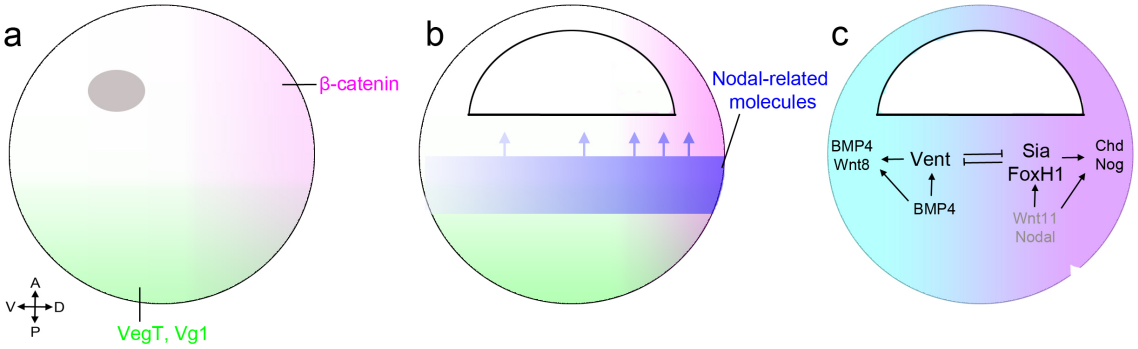

Figure 1. Patterning of the *Xenopus* embryo A: The oocyte is patterned along the animal-vegetal axis prior to fertilization; VegT and Vg1 are vegetally localized transcripts. After cortical rotation, β-Catenin levels accumulate on the future dorsal side of the embryo. **B:** At the midblastula stage, higher β-Catenin levels on the dorsal side of the embryo, together with the vegetally located factors, generate a gradient of Nodal-related molecules expressed in the endoderm. In turn, this gradient induces the formation of overlying mesoderm. **C:** Model of regulatory mechanisms for pattern formation at gastrulation. The organizer secretes a cocktail of factors that refine the initial patterning and promote dorsal-anterior fates. In the dorsal half, Wnt and Nodal signaling initiate the expression of Gsc, Otx2 and Lim1 as well as secreted proteins Chordin (Chd) and Noggin (Nog). In the ventral half, BMP4 signaling leads to the expression of Vent1/2. Zygotic Wnt8 is also expressed in the ventral center half of the embryo. Together these pathways promote ventral-posterior fates. Adapted from (De Robertis et al., 2000; Sander et al., 2007)

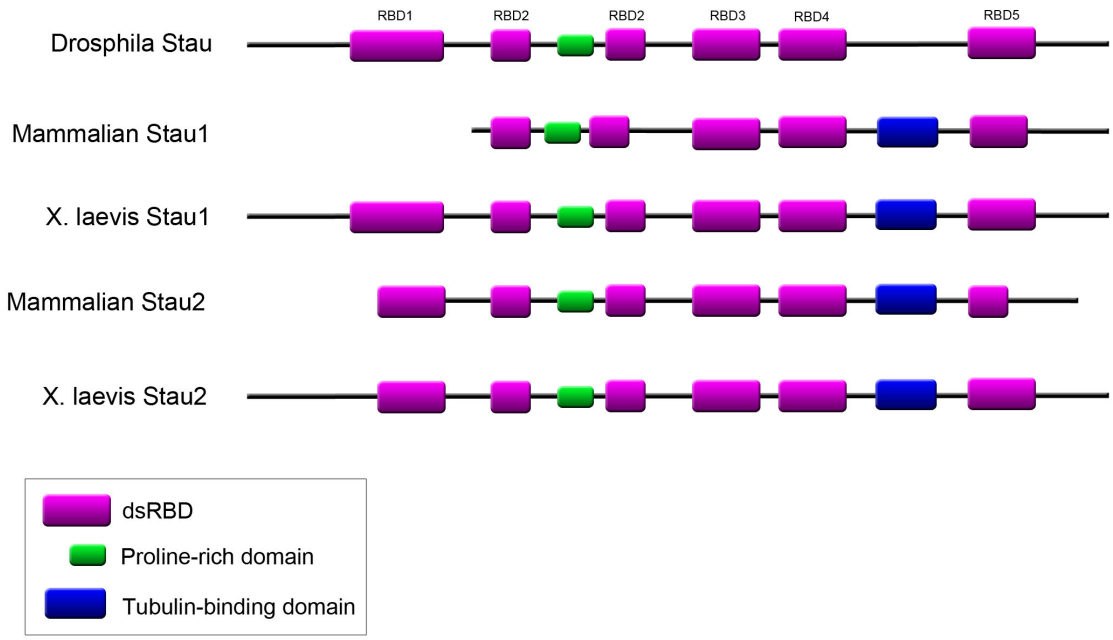

Figure 2. Characterization of Stau proteins. Schematic representation of *Drosophila*, *Xenopus*, and human (mammalian) Stau proteins**.** Adapted from (Allison et al., 2004; Micklem et al., 2000; Wickham et al., 1999).

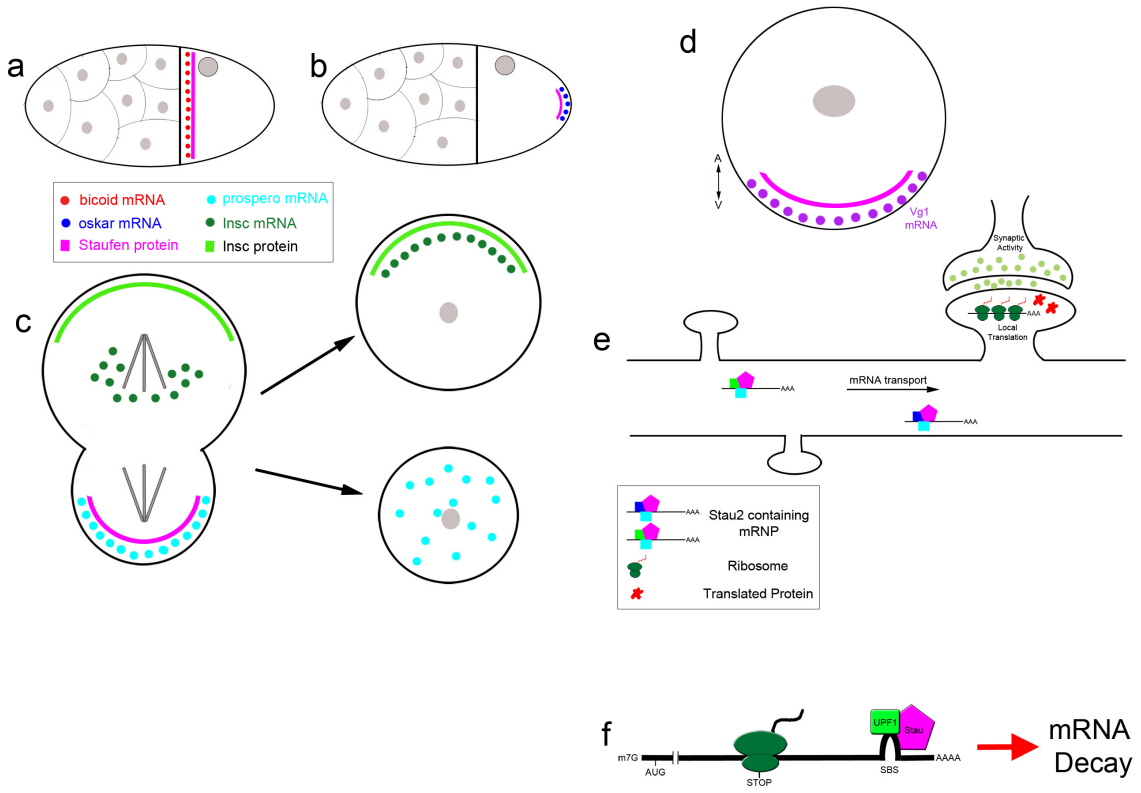

Figure 3. Known functions of Stau proteins. A-B: A stage 9 *Drosophila* oocyte is depicted, with accessory nurse cells shown on the left. **A:** *bicoid* mRNA (red) along with Stau protein (pink) is localized to the anterior margin of the oocyte. **B:** *oskar* mRNA (blue) is localized to the posterior pole with Stau protein. **C:** Asymmetrically dividing *Drosophila* embryo neuroblast (NB) cell, division leads to the regeneration of a NB and produces a smaller cell, the ganglion mother cell (GMC). *Prospero* mRNA (turquoise) is basally localized in the diving NB along with Stau protein, *prospero* mRNA is segregated into the GMC. *Inscuteable* mRNA (dark green) and protein (light green) localize at the apical side of the NB and are segregated into the regenerated NB. **D:** Asymmetric *Vg1* mRNA localization to the vegetal cortex (dark purple) of a *Xenopus* oocyte. **E:** Schematic of part of a dendrite. mRNA is transported along the dendrite within mRNPs and can lead to local translation within dendritic spines. **F:** In mammals, SMD targets mRNA provided it contains an SBS downstream of the normal termination codon. When translation terminates sufficiently upstream of SBS-bound Stau, UPF1 binds Stau and this triggers mRNA decay. AUG, translation initiation codon; STOP, normal translation termination codon. Adapted from (Maquat and Gong, 2009; Medioni et al., 2012)

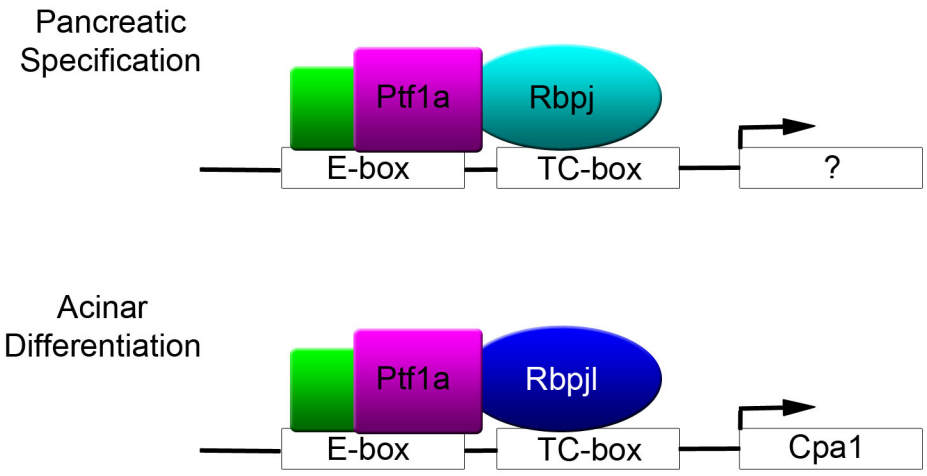

Figure 4. Distinct Ptf1a binding partners during pancreas development. A model for the two PTF1 complexes and specific DNA binding. During pancreatic specification, Ptf1a complexes with Rbpj in nascent pancreatic tissue. During acinar cell differentiation, Ptf1a complexes with Rbpjl and promotes expression of digestive enzyme genes.

Chapter 2: *Xenopus* Staufen2 is Required for Anterior Endodermal Organ Formation

Bilogan CK and Horb ME. 2012. *Genesis* 50:251-9

ABSTRACT

Defining the regulatory molecular networks involved in patterning the developing anterior endoderm is essential to understanding how the pancreas, liver, stomach and duodenum are discretely specified from each other. In this study, we analyzed the expression and function of the double-stranded RNA-binding protein Staufen2 in *Xenopus laevis* endoderm. We found that *staufen2* was broadly expressed within the developing endoderm beginning at gastrulation becoming localized to the anterior endoderm at later stages. Through morpholino-mediated knockdown, we demonstrate that Staufen2 function is required for proper formation of the stomach, liver and pancreas. We define that its function is required during gastrulation for proper patterning of the dorsal-ventral axis and that it acts to regulate expression of BMP signaling components.

INTRODUCTION

The vertebrate endoderm contributes to the epithelia lining the respiratory and digestive tract and the organs associated with these systems. This germ layer is derived from cells located in the vegetal pole of the early *Xenopus* embryo (Dale and Slack 1987). nitiation of its development requires the maternal T domain transcription factor, VegT, which activates expression of many signaling molecules and transcription factors necessary for subsequent endoderm formation (Lustig et al. 1996; Stennard et al. 1996; Zhang and King 1996; Horb and Thomsen 1997; Xanthos et al. 2001). To date, many studies have focused on how regional specification of the endoderm is regulated and recent advances have provided a deeper understanding to how signaling molecules and gene regulatory networks control endoderm development (Sinner et al. 2006; McLin et al. 2007; Pan et al. 2007; Pearl et al. 2009; Zorn and Wells 2009). However, increasing evidence suggests a vital role for post-transcriptional control during differentiation and specification of the endoderm (Mayer and Fishman 2003; Spagnoli and Brivanlou 2006; Horb and Horb 2010).

RNA binding proteins play a vital role during embryogenesis in the regulation of gene expression through translational control, subcellular localization or mRNA stability (Kuersten and Goodwin 2003). Double-stranded RNA binding proteins (dsRBP) are present in all eukaryotes, suggesting they play a critical function in controlling gene expression within the cell (Saunders and Barber 2003; Tian et al. 2004). Staufen-related proteins are one family of dsRBPs that have been shown to play crucial roles during development. Originally identified in *Drosophila,* Staufen was shown to be essential for

anterior-posterior axis formation, by acting to localize *oskar* and *bicoid* mRNAs to their respective poles (St Johnston et al. 1989; St Johnston et al. 1991; Ferrandon et al. 1994; St Johnston 1995). It was also shown to participate in embryonic neuroblast differentiation by mediating asymmetric segregation of *prospero* mRNA (Broadus et al. 1998; Schuldt et al. 1998; Shen et al. 1998).

In vertebrates, there are two Staufen homologs, Staufen1 and Staufen2 that are expressed in a variety of tissues, including primordial germ cells and neural tissue. Mouse *Staufen1* and *Staufen2* are expressed in germ cells during oogenesis and embryogenesis (Saunders et al. 2000). Similarly, zebrafish *staufen1* and *staufen2* are expressed in primordial germ cells and necessary for their proper migration and survival (Ramasamy et al. 2006). Staufen proteins have been identified in RNA-containing granules that co-localize to microtubules and function in mRNA transport within the dendrites (Buchner et al. 1999; Roegiers and Jan 2000; Macchi et al. 2003; Kanai et al. 2004; Kim et al. 2005a). Staufen1 has also been implicated in a new mRNA decay mechanism (Kim et al. 2005b). In *Xenopus*, Staufen1 and Staufen2 proteins are vegetally localized in a complex containing *Vg1* mRNA, and may regulate proper localization of *Vg1* mRNA within the oocyte (Allison et al. 2004; Yoon and Mowry 2004). In the adult, Staufen1 and 2 expression is largely confined to the testis and ovary, though Staufen2 protein was also detected less abundantly in adult spleen, pancreas and intestine (Allison et al. 2004).

Here we report that *Xenopus staufen2* is expressed early in the developing endoderm and that its function was required for proper formation of anterior organ derivatives

(pancreas, stomach and liver). We demonstrate that knockdown of Staufen2 inhibited the expression of the BMP antagonist Chordin resulting in early disruption of dorsal-ventral axis specification. Our results suggest that Staufen2 has a unique role in patterning and development of the anterior endoderm during embryogenesis.

METHODS

Plasmids, RT-PCR and Whole Mount In Situ Hybridization

For all constructs, primers were designed based on full-length sequence information obtained from GenBank, and the PCR products were cloned into pCRII (Invitrogen). The GenBank accession number for Staufen2 is BC046732.1. RT-PCR was performed on isolated whole guts for amylase and NF35 whole embryos for insulin, and normalized to EF1α as described (Jarikji et al. 2007). Whole-mount in situ hybridizations with single probes were performed as described using BM Purple (Horb et al. 2003). Antisense digoxigenin probe for Staufen2 was synthesized from Staufen2 in pCRII linearized with *XbaI* and transcribed with SP6 RNA polymerase. Probes for other pancreatic markers were prepared as previously described (Horb et al. 2003; Jarikji et al. 2007). Complete information for each clone is available upon request. For whole-mount in situ analysis in the endoderm at tail bud and early tadpole stages was prepared as described (Horb et al. 2009).

Antisense Morpholino

Antisense morpholino oligonucleotides were designed by Gene Tools, LLC. The antisense morpholinos were designed in the 5′UTR. Morpholinos were injected into the dorsal vegetal blastomeres at the eight-cell stage. For functional analysis, we selected only those samples where the morpholino targeted the entire anterior part of the gut. Targeting to the stomach, liver and pancreas was confirmed by monitoring fluorescence from labeled oligonucleotides, only after isolation of whole guts from injected embryos.

The sequences of the antisense morpholinos used was Staufen2 5′-CCGGGCTGCTCACACCTCCAACATT-3, and the sequence of the mismatch morpholino used was Staufen2-mismatch 5’-TGGCTCTGCGTTAATCTCTGCGATC-3’ The specificity of the morpholinos was tested by in vitro transcription/translation reactions using a FLAG antibody to detect the Staufen2 protein as described (Horb et al. 2009).

RESULTS

Staufen2 is Required for Anterior Endoderm Organogenesis

In *Xenopus*, *staufen2* was shown to be expressed almost exclusively in reproductive tissues, but low levels were also detected in pancreas and spleen (Allison et al. 2004). However, whether it was expressed within the endoderm during development was not examined. We initially identified *staufen2* as one gene that was down regulated in Ptf1a knockdown embryos, suggesting that it was expressed in the developing endoderm, and we therefore examined its endodermal expression in detail. Initial expression of *staufen2* was detected during gastrulation throughout the developing endoderm. Expression was punctate throughout the endoderm, including within the dorsal marginal zone (Fig. 1a). During neurulation, expression remained punctate throughout the endoderm, and was also detected in the archenteron roof as well as the mesoderm (Fig. 1b arrowheads). At later tadpole stages, expression was localized to the anterior endoderm, with expression also detected in the spinal cord (Fig. 1c,d). At NF40, when the anterior endoderm has been patterned into discrete organs, *Staufen2* continued to be expressed in the stomach, liver and pancreas (Fig. 1e), and expression remained restricted to these regions at later stages as well (Fig. 1f). No expression was detected in posterior endoderm tissues at any stage. The restricted expression pattern of *Staufen2* within the anterior endoderm during development suggested a possible role in differentiation of endodermal organs.

To investigate whether Staufen2 was necessary for endoderm development we created a knockdown phenotype using antisense morpholinos. The antisense morpholino was designed to the 5′UTR and injected (40ng) into the dorsal-vegetal blastomeres at the eight-cell stage targeting the anterior endoderm. Embryos injected with antisense Staufen2 morpholino developed normally during the first 3 days, without any overt defects. Beginning at NF40 we noticed a lack of discernable outgrowth of the stomach, liver and pancreas (Fig. 2). This phenotype was observed in 100% of embryos where the morpholino was targeted to the anterior endoderm (n=162). The fact that the embryos only displayed a phenotype at the stage when discrete organs are first observed in tadpoles suggested that functional Staufen2 was required for specification or patterning of the anterior endoderm.

To define which organs were affected by the knockdown of Staufen2 protein, we examined the expression of pancreas, stomach and liver markers. Within the pancreas, both endocrine and exocrine markers were considerably reduced. At NF44 expression of *insulin*, which is normally punctate throughout the pancreas, was significantly diminished (Fig. 2a,b). We have previously observed a similar phenotype in Ptf1a knockdown embryos, where there was no apparent pancreatic tissue, yet insulin-expressing cells are detected. Since morpholino knockdown is not 100% effective, it is possible that an endocrine cell fate can still be initiated from the residual, lower level of Staufen2 (Jarikji et al. 2007). Expression of *glucagon*, which is expressed in both pancreatic and gastrointestinal endocrine cells was also substantially reduced (Fig. 2c,d). Similarly, expression of the exocrine marker *XPDIp* was almost entirely lost (Fig. 2e,f).

We confirmed these results using quantitative reverse-transcription polymerase chain reaction (RT-PCR); *amylase* expression was almost completely abolished whereas *insulin* expression was reduced more than 2-fold (Fig. 2k,l). In addition to the pancreas, we found expression of stomach marker, *tm4sf3*, to be significantly reduced in the anterior endoderm, while expression of the liver maker hex was absent in Staufen2 morphants (Fig. 2g-j). Taken together, these results demonstrate that Staufen2 function was required for proper development of pancreas, stomach and liver prior to NF40.

To confirm that the observed phenotype was directly related to the inhibition of endogenous Staufen2 we attempted to rescue the morpholino-induced phenotype through co-injection of a *flag-Staufen2* mRNA lacking the 5′UTR sequence to which the morpholino was designed. Co-injection of 2000 pg of *flag-Staufen2* was sufficient to rescue the differentiation of the endoderm as judged by expression of *ptf1a* (Fig. 2m-o). In control embryos, the stomach, liver and pancreas were all of normal size and abundant *ptf1a* expression was detected in the pancreas (Fig. 2m). In the morpholino-injected embryos, the expression of *ptf1a* was reduced in 100% of the injected samples (Fig. 2n, n=20). In embryos injected with the morpholino and *flag-Staufen2* mRNA the morphology of the stomach, liver and pancreas were restored along with the expression of *ptf1a* in 69% of the injected embryos (Fig. 2o, n=45). In vitro transcription and translation confirmed that the morpholino blocked translation of *staufen2* mRNA, while the mismatch did not (Fig. 2p). These results demonstrate that the morpholino-induced phenotype was directly due to the loss of Staufen2.

Staufen2 is Required for the Patterning of the Developing Endoderm

To determine at which stage Staufen2 function was required for endoderm development, we studied the effect of Staufen2 knockdown at earlier stages, before overt morphogenesis of the organs has occurred. At NF35, we examined whether the anterior endoderm was properly patterned and specified by determining whether expression of *pdx1* and *ptf1a* was affected. In Staufen2 knockdown embryos we found a complete absence of both *pdx1* and *ptf1a* expression, indicating that the pancreas and duodenum was not properly induced (Fig. 3a-d). Similarly, we found expression of the endocrine progenitor marker, *insm1*, and the beta cell marker *insulin* to be diminished, reflecting the reduction of endocrine cells observed in older tadpoles (Fig. 4a,b, data not shown). Expression of the early acinar cell marker, *XPDIp* was also completely lost in the developing endoderm of the knockdown embryo (Fig. 4c,d). As we also observed a lost of stomach and liver organs, we studied the expression of *frp5* and *hnf6,* early markers for the stomach and liver, respectively, to determine whether Staufen2 function was also required for their specification. We found expression of *frp5* and *hnf6* to be almost completely absent within the developing anterior endoderm at NF35 (Fig. 4e-h). The loss of early expression of genes required for the development of stomach, liver and pancreas indicated that patterning of the anterior endoderm into discrete organ domains did not properly occur, and suggested that Staufen2 function was required at a much earlier stage.

Staufen2 Acts Upstream of the Endoderm Transcription Factor Network

We next examined whether Staufen2 function was required at earlier stages for the commitment of cells to an endodermal fate or for early anterior-posterior patterning of the endoderm. To determine if commitment to an endoderm fate was affected, we examined whether expression of the general endoderm marker *sox17beta* expression was disrupted. In control embryos *sox17beta* was expressed in a punctate pattern throughout the endoderm, and no change was seen in Staufen2 knockdown embryos (Fig. 5a,b). This confirmed that commitment to an endodermal cell fate was not dependent on Staufen2 function and that perhaps it was early anterior-posterior (AP) patterning that was affected. To ascertain whether AP patterning was altered, we examined whether expression of key anterior endoderm markers *gata4*, *sizzled, foxA2, hnf6,* and *hex* were reduced in Staufen morphants. In Staufen2 knockdown embryos, expression of *gata4*, *sizzled, foxA2, hnf6,* and *hex* were all lost within the anterior endoderm (Fig. 5c-h, data not shown). These results demonstrated that Staufen2 was required at gastrula and neurula stages when the endoderm is specified into anterior and posterior domains.

Staufen2 Regulates Early Endoderm Patterning Through the BMP Pathway

Expression of endogenous *staufen2* within the dorsal marginal zone and perturbed endoderm axis development suggests it may be involved in the formation or function of the organizer. To define the mechanism by which Staufen2 inhibits anterior endoderm development, we analyzed gastrula stage embryos for altered expression of opposing signaling pathway components involved in the organizer activity. In embryos injected

with Staufen2 morpholino, there was a loss of dorsal expression of *chordin* at NF10 (Fig. 6a,b). Chordin is secreted from the Spemann organizer and acts as a BMP antagonist, to keep BMP signaling levels lowest near the organizer. Conversely, expression of *bmp4* and *vent2*, a downstream target of BMP4, were upregulated in the anterior endoderm of Staufen2 knockdown embryos (Fig. 6c,d, data not shown). To determine whether loss of Staufen2 is acting specifically on *chordin* expression or whether it has a general effect on the organizer we studied the expression of *cerberus*, a multivalent inhibitor secreted by the organizer and known to antagonize Nodal, BMP and Wnt. *Cerberus* expression however was unaffected in embryos lacking Staufen2 (Fig. 6e,f). Taken together, these results indicate that Staufen2 is necessary during gastrulation to ensure the expression of *chordin* within the developing embryo, leading to the appropriate establishment of the BMP signaling gradient.

DISCUSSION

In this study, we identified a new function for Staufen2 in endoderm specification. We showed that *Staufen2* expression within the endoderm and mesoderm occurred early in development beginning at gastrulation, and is expressed in the dorsal marginal zone. By performing loss-of-function experiments, we demonstrated that Staufen2 is required for expression of *chordin*. Knockdown of Staufen2 resulted in increased expression of BMP4 signaling components and altered regionalization of the developing anterior endoderm. This led to an almost complete loss of pancreas, stomach and liver differentiation, which ultimately led to hypoplasia or aplasia of these organs. Based on these results, we conclude that Staufen2 is an indispensable mediator of anterior endoderm patterning in *X. laevis*.

The role of RNA binding proteins in endoderm development has not been profoundly investigated, though increased focus towards understanding the molecular networks involved in the development of the endoderm has led to a recent studies detailing RNA binding protein function. Our lab recently identified another RNA binding protein involved in endoderm development, BrunoL1, which was shown to regulate proliferation of endoderm cells through translational control of *cyclin A2* mRNA (Horb and Horb 2010). Another study in *Xenopus* showed that Vg1RBP was essential for pancreas development (Spagnoli and Brivanlou 2006), while in zebrafish, *nil per os (npo)* was shown to be required for gut morphogenesis and exocrine pancreas development (Mayer and Fishman 2003). While these results suggest that modulating the activity of target

RNAs may control organogenesis in part, what these targets are and how they are involved in endoderm development remains to be elucidated.

It is unclear how Staufen2 mediates its effect on *chordin* expression, but data from other organisms showed that Staufen2 acts to regulate mRNA localization and translation. In zebrafish, Staufen proteins seem to be required for migration and survival of primordial germ cells through mRNA localization (Ramasamy et al. 2006). It is possible that in *Xenopus* endoderm development Staufen2 may be involved in the asymmetric localization of specific mRNAs, which in turn regulate activation of Chordin, though we do not necessarily agree with this hypothesis. In mammals, Staufen1 protein has been shown to be apically distributed within differentiated Caco-2 intestinal cells (Gautrey et al. 2005). Finally, in *Drosophila*, interaction between Staufen and the 3'UTR of *propero* is required for basal localization during embryonic neuroblast differentiation (Schuldt et al. 1998; Shen et al. 1998).

Alternatively, Staufen1 has been shown to mediate mRNA decay for conditional down-regulation of genes encoding mRNAs that contain a STAU1 binding site in their 3'UTR (Kim et al. 2005b; Gong et al. 2009). In C2C12 myoblast cells, Stau1-mediated mRNA decay (SMD) increases in efficiency as these cells differentiate into myotubes, and *pax3* mRNA, which encodes a transcription factor that inhibits myogenesis, has been identified as a target of SMD, therefore its down-regulation allows for myogenesis to occur (Epstein et al. 1995; Gong et al. 2009). While Staufen2 has yet to be studied as a mediator of mRNA decay, it may be possible that instead of playing a role in the

localization and translation of mRNA required for endoderm development, Staufen2 may be required to down-regulate genes that are involved in the specification and regionalization of the endoderm.

We have shown here that Staufen2 is required for the proper expression of *chordin* during gastrulation. Although we have not conclusively addressed whether the loss of Chordin is directly responsible for the observed foregut defects, there is increasing evidence suggesting regionalization of the endoderm occurs as the germ layers are being formed at this early stage in development, and it was previously shown that knockdown of Chordin resulted in reduced expression of *pdx1* (Zorn et al. 1999; Wells and Melton 2000; Spagnoli and Brivanlou 2008). Recently, the inhibition of BMP signaling within the dorsal endoderm was shown to be required for specification of the of the pancreatic field. Knockdown of Chordin, leads to dorsal overexpression of BMP, and ultimately in a loss of Pdx1 expression (Spagnoli and Brivanlou 2008). Although it has been shown that this inhibition is endogenously mediated throughout endoderm development by Chordin and TGIF2, we have identified a novel role of Staufen2 that appears to be upstream of these factors. We believe that Staufen2 acts to mediate translation of specific targets at this early stage rather than asymmetric localization or mRNA decay. We are currently attempting to identify Staufen2 mRNA targets and ascertain whether this is indeed the case.

In conclusion, we have shown that Staufen2 function is essential for proper development of anterior endodermal organs. In particular, we showed that knockdown of Staufen2 resulted in very early patterning defects, resulting in decreased expression of *chordin* and a concomitant increase in *vent2* expression. In contrast, there was no change in expression of *cerberus*. Although we identified very early molecular changes, we were surprised by the restricted phenotypes observed at later stages in Staufen2 morpholino injected tadpoles. After identifying that Staufen2 was required for proper development of anterior endodermal organs but not for head or other tissues, we expected to find molecular changes only after gastrulation, during neurula stages. At the moment, we cannot explain these discrepancies, but it remains possible that chordin is expressed for a short period and that this is sufficient for maintaining its function during gastrulation. Identification of Staufen2 mRNA targets should help resolve this issue.

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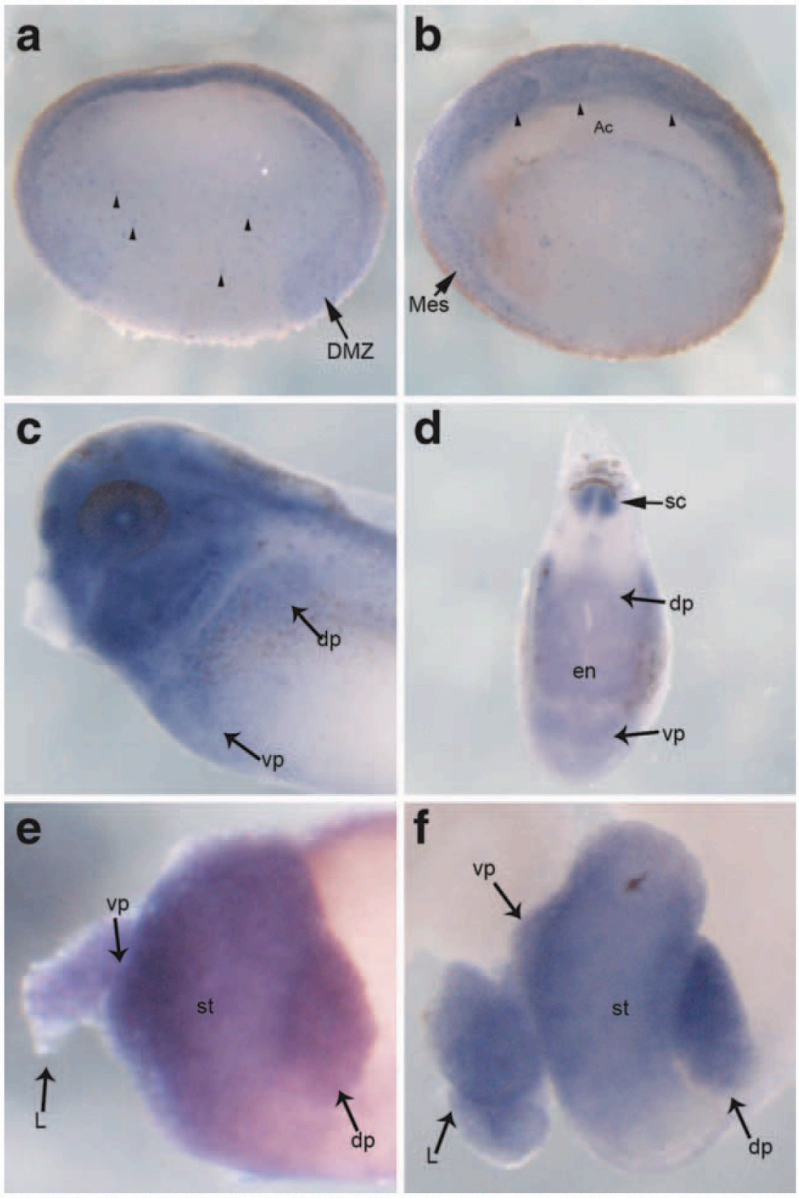

Figure 1. *Staufen2* expression in the endoderm and isolated gut tissue. A: Transverse section through embryo at NF10 showing punctate expression of *Staufen 2* throughout the mesendodermal precursor cells (arrowheads) as well as in the dorsal marginal zone (DMZ). **B:** Transverse section through embryo at NF15 showing *Staufen2* expression within the archenteron roof (Ac) endoderm (arrowheads). Expression is also detected in the mesoderm (Mes). **C:** NF35 embryos showing*Staufen2* expression throughout the head, in the spinal cord and in the dorsal (dp) and ventral (vp) pancreatic buds. **D:** Sagittal section through *Staufen2* expressing region of NF35 embryo showing broad expression in the endoderm (en) and in the spinal cord (sc). **E,F:**Whole guts from NF40 and NF44 tadpole, expression was found throughout the pancreas, liver and stomach, becoming slightly stronger at later stages. St, stomach; L, liver; dp, dorsal pancreas; vp, ventral pancreas.

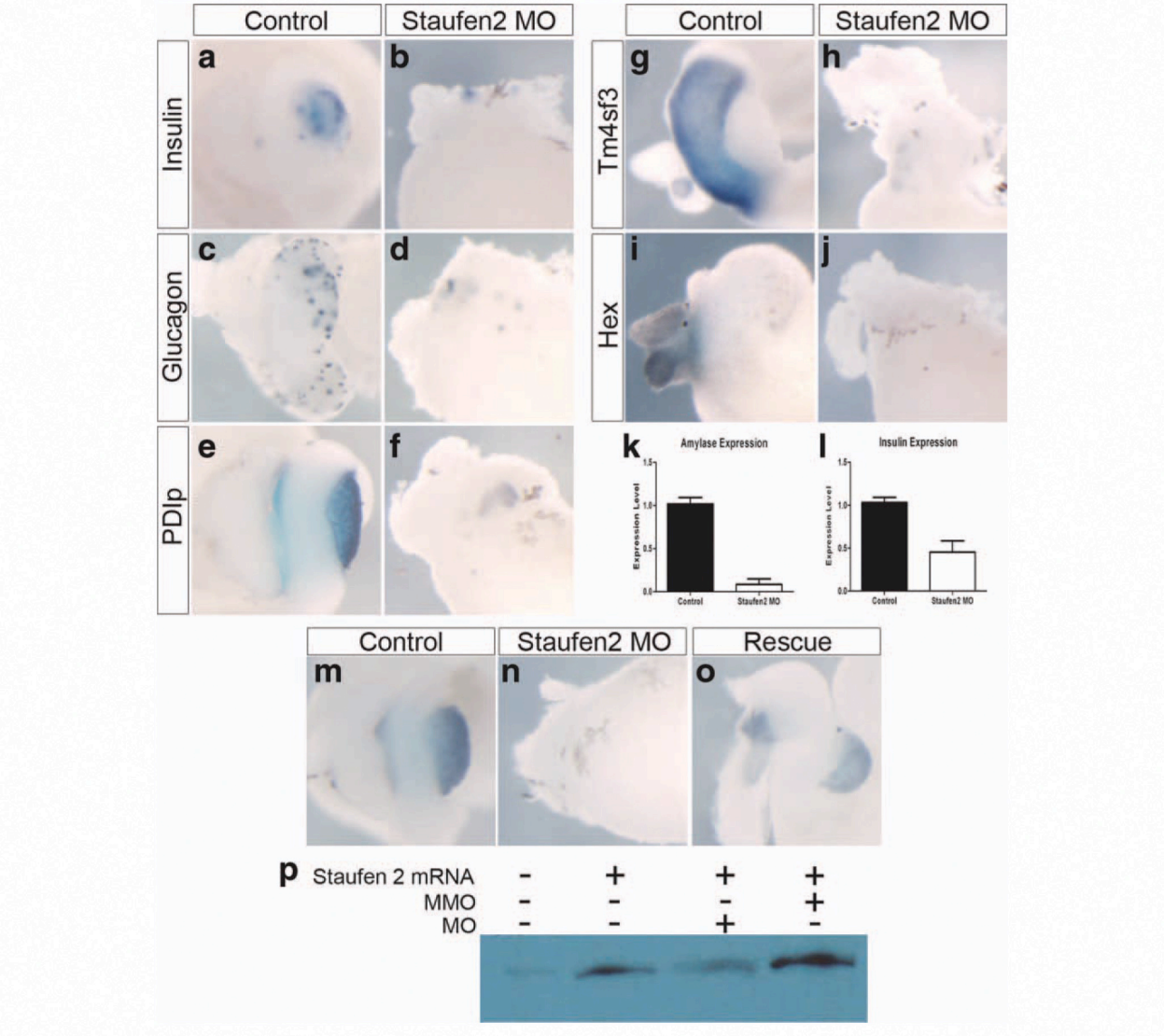

Figure 2. Staufen2 is required for anterior endodermal organ formation. A,B: *Insulin* expression at NF44 is almost completely abolished (75%, n=16). **C,D:** *Glucagon* expression at NF44 is almost entirely lost in the stomach and pancreas (67%, n=6). **E,F:** Expression of *PDIp* is almost completely lost in Staufen2 knockdown animals at NF44 (75%, n=12). **G,H:** *tm4sf3* gene expression is lost at NF44 (78%, n=9). **I,J:** Expression of *hex* is lost at NF44 (100%, n=11). **K,L:** Real-time PCR analysis of endocrine and exocrine markers in Staufen2 knockdown embryos for amylase in control and Staufen2 knockdown whole guts at NF44 and insulin expression in whole embryos at NF35. Each bar is an average of 8 individual whole tadpoles. Amylase expression is almost lost, and insulin expression is decreased by more than 2-fold. **M–P:** Flag-Staufen2 mRNA rescues the morpholino knockdown phenotype. **M:** Control NF45 whole gut stained for ptf1a mRNA showing expression in the pancreas. **N:** Whole gut from embryo injected with 40 ng of Staufen2 morpholino. *Ptf1a* expression is almost

completely lost in injected embryos (n=20). **O:** Isolated whole gut stained for *ptf1a* expression from embryo injected with 40 ng of Staufen2 morpholino and 2000 pg of flag-Staufen2 mRNA. *Ptf1a* expression was restored in 31/45 embryos. **P:** In vitro transcription translation of staufen2 mRNA with either the morpholino (MO) or the mismatch morpholino (MM).

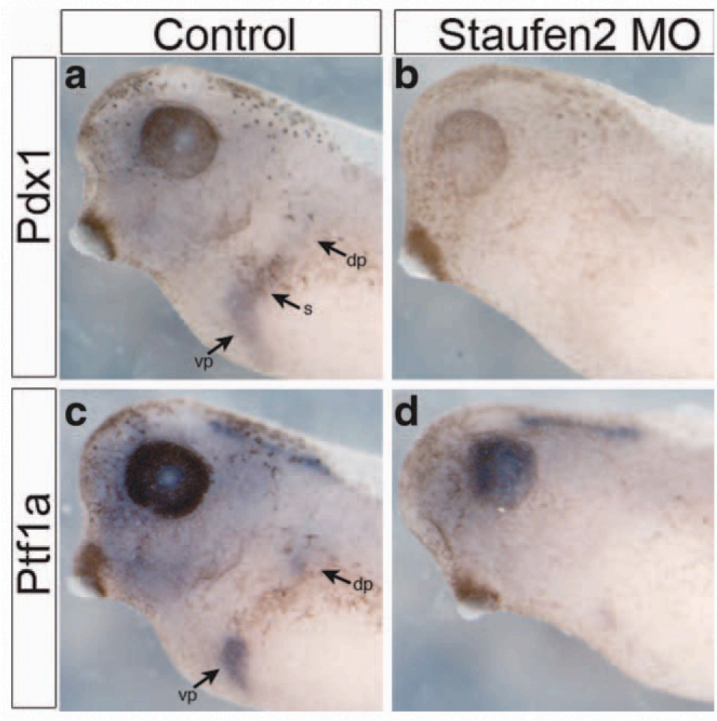

Figure 3. Staufen2 is required for the patterning of the developing anterior endoderm. **A,B:** Pdx1 expression by whole mount in situ hybridization in control and Staufen2-MO-injected embryos at NF35, expression of pdx1 is lost in the developing stomach and pancreatic anlagen (83%, n=12).. **C,D:** NF35 embryo, expression of ptf1a is almost completely lost in the developing dorsal and ventral pancreatic bud (80%, n=10).

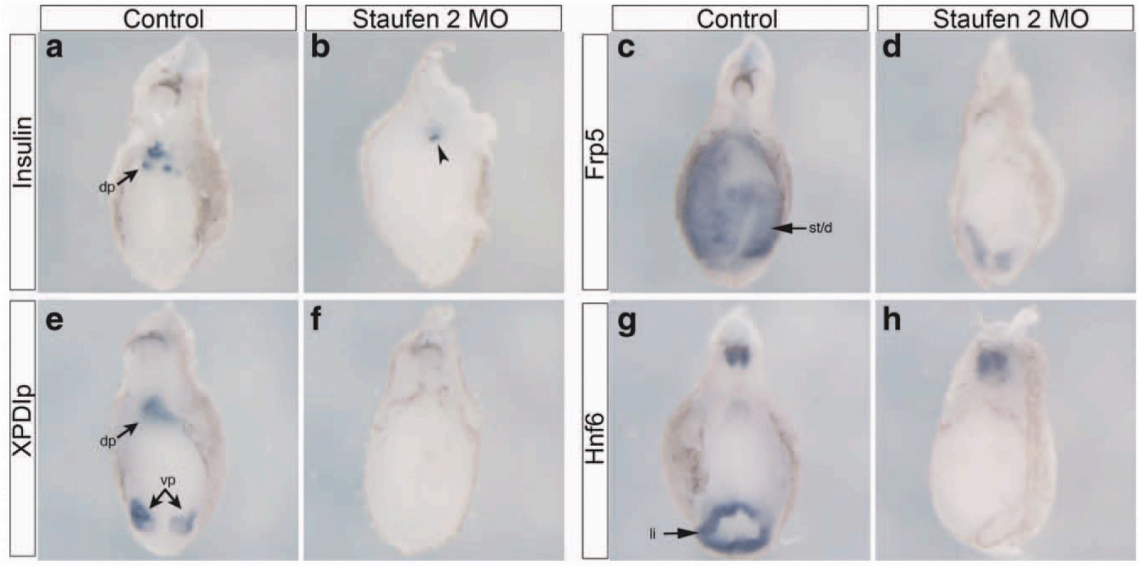

Figure 4. Patterning of anterior endoderm is perturbed in Staufen2 morpholino injected embryos. Sagittal sections through the anterior endoderm of NF35 control and Staufen2 morpholino injected embryos. **A,B:** *Insulin* expression is almost completely abolished (93%, n=14). Arrowhead in B points to the small remaining insulin expression in the dorsal pancreas. **C,D:** Expression of *PDIp* is lost in the future dorsal and ventral anlagen (100%, n=6). **E,F:** Expression of *frp5* is almost completely lost (100%, n = 7). G,H: Expression of *hnf6* is lost (63%, n=8). dp, dorsal pancreas; vp, ventral pancreas; st/du, stomach/duodenum; li, liver

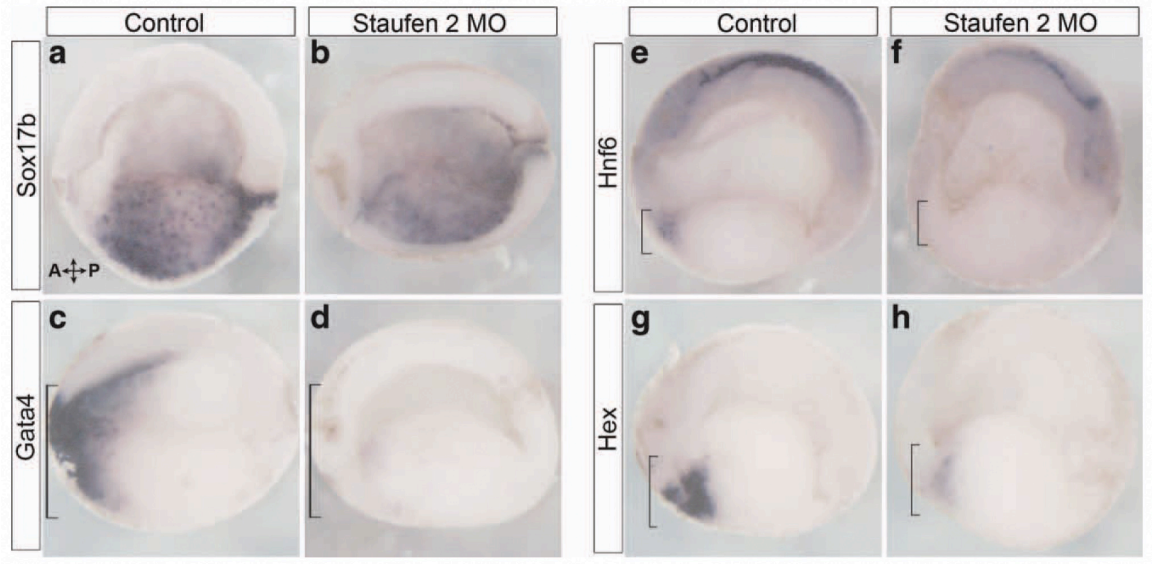

Figure 5. Staufen2 is required for the earliest stages of endoderm regionalization. Transverse section through the embryo at NF18. **A,B:** *Sox17β* expression by whole mount *in situ* hybridization in control and Staufen2-MO-injected embryos, expression of *sox17β* remains unchanged throughout the endoderm (80%, n=20). **C,D:** Expression of *gata4* is lost in the anterior endoderm (82%, n=17). **E,F:** Expression of *hnf6* is lost in the anterior endoderm (67%, n=9). **G,H:** Expression of *hex* is almost completely lost (72%, n=18). Brackets identify anterior endoderm.

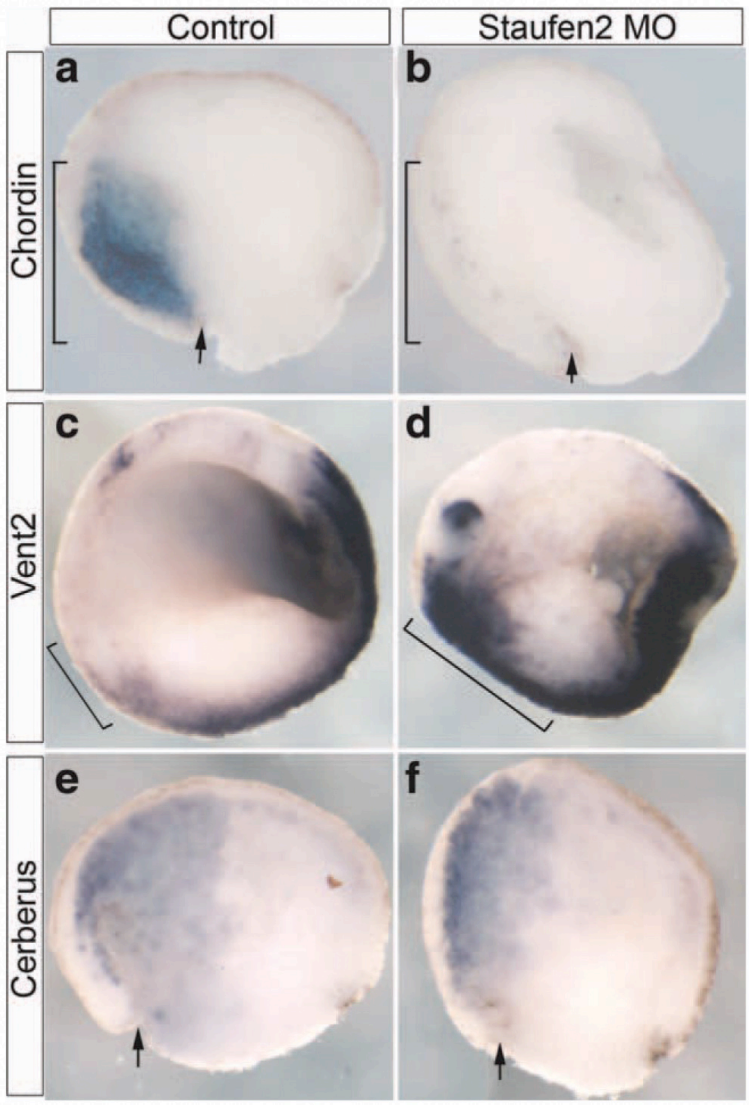

Figure 6. Staufen2 regulates early endoderm patterning through the BMP pathway. **A,B:** *Chd* expression by whole mount *in situ* hybridization in control and Staufen2-MO-injected embryos at NF10 transverse section, expression of *chd* is lost in the dorsal half of the embryo (80%, n=10). **C,D:** *Vent2* expression is significantly upregulated in the anterior half of the embryo at NF18, transverse section (75%, n=12) . **E,F:** Expression of *cerberus* is unaffected (100%, n=10). Arrows indicate dorsal lip.

Chapter 3: Staufen2 Regulation of the Wnt Signaling Pathway is Necessary for Patterning of the Endoderm

ABSTRACT

Patterning and specification of the three germ layers (ectoderm, mesoderm and endoderm) is initiated at gastrulation. The mesoderm and endoderm develop in close proximity and share similar inducing signals, leading to the assumption that they are specified and patterned together. I recently identified a novel role for Stau2, a double-stranded RNA binding protein, in patterning along the anterior-posterior axis of the endoderm. In this study, I showed that Stau2 function is required at the onset of gastrulation to maintain expression of Wnt/β-catenin signaling pathway effector proteins. Using RIP-seq of Stau2-bound mRNA targets at the onset of gastrulation, I identified many novel Stau2 targets including *gsk3β*, a known Wnt signaling inhibitor. Finally, I demonstrated that dsRBD1, dsRBD4 and dsRBD5 appear to be required for proper endoderm patterning. This study revealed that the function of Stau2 is required for the patterning of only the endoderm, providing evidence that the endoderm and mesoderm are patterned separately beginning at the onset of gastrulation.

INTRODUCTION

The vertebrate endoderm contributes to the epithelial lining of the respiratory and digestive tract and the organs associated with these systems. The regulatory network that controls patterning of the early endoderm into organ lineages is poorly understood. To date, most studies have focused on elucidating the molecular hierarchy required to establish ectoderm and mesoderm development, while the understanding of endoderm development has not been a priority (Zorn and Wells 2007). A deeper comprehension of this process will be critical for progress in understanding congenital endodermal organ defects.

In *Xenopus*, the fundamental principal governing patterning of all three germ layers is the Spemann-Mangold organizer. Named for the scientists that performed the experiments that led to its discovery, Spemann and Mangold demonstrated that transplantation of the dorsal blastopore lip of the gastrula stage newt embryo onto the ventral side of a host embryo resulted in the formation of a secondary axis. The transplanted tissue acted as an organizer, engaging neighboring host cells to form tissues of the secondary axis (Spemann and Mangold 1924). It was almost 65 years later before the first molecular signals produced by the organizer were identified, and how the organizer elicits its activity is still not fully understood. The organizer is formed in the dorsal margin of the embryo by synergism between maternal Wnt11 (mWnt11) and Nodal signaling, and is required for proper gastrulation, and axial patterning of the embryo (Watabe et al. 1995; Harland and Gerhart 1997; Reid et al. 2012). Activation of the canonical mWnt11/β-

Catenin pathway directly stimulates the transcription of target genes such as the homeodomain (HD) factors Siamois (Sia) and Twin (Twn) and the secreted factor Nodal-related-3 (Xnr3) (Lemaire et al. 1995; Fan and Sokol 1997; Laurent et al. 1997; McKendry et al. 1997). Nodal-related signals are activated in a dorsal-ventral gradient in the future endoderm and mesoderm by the combination of the β-Catenin signal and other maternal factors (Klein and Melton 1996). Nodal ligands are members of the TGFβ family of secreted growth factors, and activation of the Nodal pathway leads to the phosphorylation of Smad2 which translocates into the nucleus and complexes with transcription factors FoxH1 or Mixer to stimulate transcription of genes required for endodermal and mesodermal fates (Schneider et al. 1996; Wessely et al. 2001; Zorn and Wells 2009). The organizer patterns all three germ layers by promoting dorsal-anterior fates through activation of transcription factors including Goosecoid (Gsc), Otx2, and Lim1, as well as through inhibition of ventral-posterior fates by secreting a number of Wnt and BMP antagonists, such as Chordin, Noggin, Crescent and Cerberus. The secreted antagonists act by inhibiting BMP4 and zygotic Wnt8 (zWnt8) ligands expressed in the ventral marginal zone. BMP4 and zWnt8 promote ventral–posterior fates and restrict dorsal–anterior fates, in part by inducing the expression of the HD transcriptional repressors Vent1 and Vent2, which inhibit organizer gene expression (Klein and Melton 1996; Harland and Gerhart 1997). The differential patterning of mesoderm and endoderm seems to rely on different doses and/or the duration Wnt and Nodal signaling, but how this is regulated for proper formation of the germ layers is not clear.

Efforts to understand the complex molecular hierarchy that regulates patterning and specification of the germ layers have mainly focused on understanding the gene regulatory networks of numerous organizer-specific factors and secreted proteins (Loose and Patient 2004; Sinner et al. 2006; McLin et al. 2007; Pan et al. 2007; Zorn and Wells 2009; Rankin et al. 2011). Our understanding of how the same signals from the Spemann organizer can elicit different responses resulting in the patterning of the three germ layers have generally come from studies concentrated on ectoderm and mesoderm formation, while endoderm development has been passively included with the latter. Prior to gastrulation, the putative endoderm and mesoderm territories overlap and the equatorial cells express both endoderm and mesoderm genes. This transient cell population is collectively referred to as mesendoderm (Zorn and Wells 2009). Since the mesoderm and endoderm develop in close proximity and share similar inducing signals, many studies assumed the mesendoderm only becomes mutually exclusive in late gastrula embryos, therefore suggesting the mesoderm and endoderm are specified and patterned together. However, data suggest that specification of the germ layers occurs gradually during development. The endoderm is committed to an endodermal fate at early gastrula stages (Heasman et al. 1984), while the mesoderm, which is induced by signals from endodermal cells, is only fully committed to its fate at the end of gastrulation (Kato and Gurdon 1993). Restricted expression of genes such as *sox17* and *hex* within the endoderm prior to gastrulation provide evidence that the mesoderm and endoderm are patterned separately. Yet, it remains unclear how the endoderm is being specified separately from the mesoderm during gastrulation. To date, many studies have focused on how transcription factors regulate specification of the endoderm development. Increasing

evidence suggest a role for post-transcriptional control during differentiation and specification of the endoderm (Mayer and Fishman 2003; Spagnoli and Brivanlou 2006; Horb and Horb 2010; Bilogan and Horb 2012). However, little is known about the role post-transcriptional regulation plays in embryonic development and a better understanding may help define how the same early transcription factors can regulate the specification of three different germ layers.

RNA binding proteins (RBPs) play a vital role during embryogenesis in the regulation of gene expression through translational control, subcellular localization or mRNA stability (Kuersten and Goodwin 2003). Staufen (Stau)-related proteins are one family of double-stranded RNA-binding proteins (dsRBPs) that have been shown to play crucial roles during development. Stau was identified in *Drosophila* for its role in oocyte patterning by localizing *bicoid* and *oskar* mRNA to anterior and posterior poles, respectively (St Johnston et al. 1989; St Johnston et al. 1991; Ferrandon et al. 1994; St Johnston 1995). In vertebrates, there are two distinct Stau genes, Stau1 and Stau2. Three main functions of Staufen proteins have been characterized; mRNA localization, translational regulation and mRNA decay, while the latter has only been illustrated in mammalian cells. In *Drosophila,* Stau participates in embryonic neuroblast differentiation by mediating asymmetric segregation of *prospero* mRNA, this function is conserved in mammals where Stau regulates asymmetric localization of *prox1* in neural stem cells (Broadus et al. 1998; Schuldt et al. 1998; Shen et al. 1998; Vessey et al. 2012). In mature neurons, Stau proteins form messenger ribonucleoprotein particle (mRNP) granules and transport mRNA along microtubules in neuronal dendrites (Köhrmann et al. 1999; Tang et al.

2001; Mallardo et al. 2003; Kanai et al. 2004). In *Xenopus*, maternally encoded Stau1 and Stau2 proteins localize to the vegetal pole and regulate proper vegetal mRNA localization within the oocyte (Allison et al. 2004; Yoon and Mowry 2004). In translational regulation Stau has been shown to be involved in repression of Oskar translation during *Drosophila* oogenesis (Micklem et al. 2000). Stau-mediated mRNA decay (SMD) recruits nonsense-mediated mRNA decay (NMD) factors to Stau1 or Stau2 bound transcripts and leads to mRNA decay (Kim et al. 2005). In cell lines, SMD has been implicated in the differentiation of both myotubes and adipocytes, as well as involved in motility of human HaCat keratinocytes (Gong et al. 2009; Park et al. 2013). The mechanism of Stau function in each of these contexts is poorly understood and deeper insight into the Stau RNA and protein interactants will help decipher the complex roles Stau proteins play in development.

Previously, I reported that in *X. laevis*, *stau2* was broadly expressed within the developing endoderm beginning at gastrulation and became localized to the anterior endoderm at tailbud stages (Bilogan and Horb 2012). I demonstrated that its function was required for the proper formation of the stomach, liver and pancreas, highlighting a new biological function for Stau2. I determined Stau2 function was required for proper anterior-posterior (A-P) patterning of the endoderm during gastrulation. I showed that Stau2 knockdown led to a loss of *chordin* expression, however how Stau2 was regulating the BMP signaling pathway is still not fully understood (Bilogan and Horb 2012). I have identified a phenotype that is initiated during gastrulation whereby only endoderm

patterning is affected. Yet, the mRNA targets of Stau2 and the exact time-point at which Stau2 is required for endoderm patterning remain unknown.

To define in more detail how Stau2 function may control endoderm patterning, I determined the stage at which loss of Stau2 affects patterning of the endoderm to be NF10, the onset of gastrulation. I studied the role of Stau2 at NF10 to determine how it may regulate the complex gene network activated by the Spemann-Mangold organizer at this stage. My results demonstrate Stau2 function is required to maintain expression of Wnt/β-catenin signaling effector proteins at the onset of gastrulation, with Stau2 knockdown resulting in reduced organizer gene expression. Using RNA immunoprecipitation and RNA sequencing (RIP-seq) on Stau2 bound mRNA; I identified many novel potential Stau2 targets including *gsk3β*, a known Wnt signaling inhibitor. Finally I demonstrated that dsRBD1, dsRBD4 and dsRBD5 appeared to be required for proper endoderm patterning. Our results suggest that Stau2 constitutes a novel element in the regulation of the Wnt signaling pathway during gastrulation. Most surprising was that Stau2 function appeared to be dispensable for mesoderm patterning, providing evidence that the endoderm and mesoderm are exclusively patterned beginning at the onset of gastrulation.

METHODS

RIP-seq Analysis

All mRNA for microinjection was created using the Ambion mMessage mMachine kit. To confirm targeting, experimental mRNAs were injected along with 400pg *gfp* mRNA and targeting was verified by observing appropriate fluorescence. For functional analysis I selected only samples for which the entire anterior endoderm was targeted. 2000pg of *flag-Stau2* and *gfp* mRNAs were injected into the dorsal-vegetal blastomeres at the eight-cell stage. Whole embryos were collected at NF10 (15 embryos/set). As a control 400pg *flag-brunoL1* and *gfp* mRNA-injected embryos were also collected at NF10. Immunoprecipitation assays were preformed according to the method described before (Horb and Horb 2010). Protein-bound M2 anti-Flag beads (Sigma) were divided into two pools. One half was used for Western blot analysis to confirm the immunoprecipitation of the Flag-bound proteins using anti-Flag Ab (1:2000, Sigma). The other half was added to Trizol (Invitrogen) to isolate total bound RNA for deep sequencing.

Extracted RNA was treated with DNAse I to remove any contaminating DNA from the samples. ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre) was used to prepare RNA-seq libraries according to the manufacturer's protocol except fragmentation of RNA was only performed for 2.5 minutes. cDNA libraries were size selected for a distribution of 350-600bp using a Pippin Prep (Sage Science).

Assessment of RNA integrity and DNA sizing and quantification of the cDNA library was done with 2100 Bioanalyzer (Agilent) using the Agilent RNA 6000 Pico Kit and the Agilent High Sensitivity DNA Kit respectively. RNA sequencing was performed by the

Keck Facility at the Josephine Bay Paul Center (Marine Biology Laboratories, Woods Hole, MA) using the Illumina HiSeq 1000 sequencer in a 100bp paired end run.

Quality assessment of raw sequence data was performed using FastQC (Babraham Bioinformatics), the data was then trimmed using Trimmomatic (Lohse et al. 2012). Trimmed reads were mapped to an *X. laevis* wild-caught reference library (Horb, unpublished) using CLC Genomics Workbench (CLC bio). Candidate genes were selected as reads that mapped to the reference library specifically from the Stau2 IP RNA library, contigs that mapped to reads sequenced from the BrunoL1 IP RNA library and the Stau2 IP RNA library were discarded as non-specific mRNA targets.

RT-PCR

For RIP-seq validation, RT-PCR was performed on Flag-Stau2 bound RNA, Flag-BrunoL1 bound RNA or non-injected control whole embryos and normalized to *endodermin (edd)*.

For *gsk3β* expression in Stau2 morphant embryos, RT-PCR was performed on whole embryos injected with 40ng Stau2 morpholino into the dorsal-vegetal blastomeres at the eight-cell stage or non-injected controls and normalized to *edd*. PCR conditions using *Taq* DNA polymerase (NEB) included 3 min at 95°C preincubation, followed by 32 amplification cycles comprising 95°C for 20 sec, 55°C for 30 sec, and 68°C for 30 sec, and one cycle at 68°C for 5 min. The following primers were used:

Gsk3β (U22890) Fwd 5′-TGAAGTGTACCTGAACTTGG-3′

Gsk3β (U22890) Rev 5′-TGGATGTAGGCTAAACTGCG-3′

Smurf2 (NM_001088813) Fwd 5′-GTGCGCTAAAAACCTGGTGA-3′

Smurf2 (NM_001088813) Rev 5′-ATTGCATTGGACAGAAGCCG-3′

Dlgap4 (BC072288) Fwd 5’-ACAAAGAGGCCGTGGTATTC-3’

Dlgap4 (BC072288) Rev 5’-CCTCCTGCACACTTTCTTCA-3’

Lman2l (BC129540) Fwd 5’-ACGAAGAGAAGCAGCATGAG-3’

Lman2l (BC129540) Rev 5’-CCGGGAACATCTAGGCAATC-3’

Rpn1 (BC044106) Fwd 5’-GCTTCGCTACAGTCCAAACT-3’

Rpn1 (BC044106) Rev 5’-CGAGTGCTGCTTCTCATTCT-3’

Rmi Fwd 5’-AAAGGACCCTAGTCAGCAAAG-3’

Rmi Rev 5’-ATGGCTACGAACGCAACA-3’

BC157473 Fwd 5’-TGCATCAGGCTCCGAAATAG-3’

BC157473 Rev 5’-TTGCTGGAATGTTCCCTCTG-3’

Hace1 (BC077993) Fwd 5’-CAGCCAAACAGCAACTCTTC-3’

Hace1 (BC077993) Rev 5’-CTGCAGGTTCTTTGCGTATTC-3’

Pcdh1 (NM_001136169) Fwd 5’-CGACAATGCACCCGTCTTTA-3’

Pcdh1 (NM_001136169) Rev 5’-CGCTGTTCCCGATCCAATAC-3’

LIN28A (NM_001087449) Fwd 5’-CAGCCAATCACTGAGGAACA-3’

LIN28A (NM_001087449) Rev 5’-ATACGACCCACTTAGCAACAC-3’

Xbra (NM_001085559) Fwd 5’-TCCCTCTGCTCTCCTACTAATC-3’

Xbra (NM_001085559) Rev 5’-ATCTGTAAACCAGGCCAGTG-3’

HIRA (NM_0010875640) Fwd 5’-TGCTTCAGTCCCAGTCTTTC-3’

HIRA (NM_0010875640) Rev 5’-CAACCAGTGCCTGTATTCCT-3’

GLIS2 (NM_001088623) Fwd 5’-TGGCACCCAGCTTATCATTC-3’

GLIS2 (NM_001088623) Rev 5'-CCTTAGGGCGCTTCTCATTT-3'

Vent1 (NM_001088235) Fwd 5'-CTCCCAGCCCAATGAGAAAT-3'

Vent1 (NM_001088235) Rev 5'-TGTGCTGCTGGTCTTGTATC-3'

Xolloid (NM_001090908) Fwd 5'-GCATCGTGGTCCATGAGTTA-3'

Xolloid (NM_001090908) Fwd 5'-AGAATGTGTTCCGGGCATAG-3'

LMO4B (NM_001086236) Fwd 5'-TAACCGGATCTCCGAGTCTAC-3'

LMO4B (NM_001086236) Rev 5'-GTAACAGGACGTGCCAATCT-3'

Probes and Whole Mount *In Situ* Hybridizations

All genes were cloned using cDNA from wild-type NF10 embryos with the following primers:

Gsk3β Fwd 5'-GGAGTTGATCTTTGGAGCCA-3' and Gsk3β Rev 5'-ATC CTCGATACATGTGGCCA-3' designed based on BC108581. FoxH1 Fwd 5'-CCATGCCACCCAACAAGAGT-3' and FoxH1 Rev 5'-CTAGAGGCAGACCAGTGGGA-3' designed based on BC160705. PCR products were cloned into pCRII (Invitrogen) and confirmed by sequencing. The probe for Ptf1a-pCRscript was prepared as previously described (Jarikji et al. 2007). XSiamois-pBSKII (Not1, T7); Otx2-pBSK (Not1, T7); Gooscoid-pBSK (EcoR1, T3); Xnr3-pGEM5Z (EcoR1, T7); Vent1-pKS (Sal1, T7); Vent2-pKS (Apa1, T7) plasmids were all kindly gifted by A. Zorn.

Whole mount *in situ* hybridizations were performed as described using BM purple (Horb et al. 2003).

The Stau2 antisense morpholino oligonucleotides was designed by and purchased from Gene Tools, LLC and previously described (Bilogan and Horb 2012).

Embryo Manipulation

LiCl treatment was carried out in 0.3 M LiCl in 0.1X MMR for 10 min starting at the 32-cell stage.

mRNA

To clone Flag-Stau2, the Stau2 ORF was amplified from Stau2-pCS2+ using a forward primer with an engineered Not1 site:
5'-ATGGCACACATTGCGGCCGCGCCACCATCATCT-3' and T7 as a reverse primer. PCR product was ligated into pCRII, then cut with Not1 and cloned into Flag-CS107 cut with Not1. For mRNA synthesis, linearize with KpnI and synthesize with Sp6. The Flag-brunoL1 construct was cloned as previously described (Horb and Horb 2010). All fusions were confirmed by sequencing. All mRNAs injected into *Xenopus* embryos were made using Ambion mMessage mMachine kit.

The various RNA-binding domain mutant clones were construct by inverse PCR Phusion polymerase (NEB). The primers were designed to flank the RBD of Stau2, which have been previously described (Allison et al. 2004). Flag-stau2-CS107 was used as a template for the PCR. The following primers were used:
ΔRBD1 Fwd 5'-AGCTAGGCACACCGATATCCACAT-3'
ΔRBD1 Rev 5'-TTGGGGTTTGTCATGTTGTCATGG-3'

ΔRBD2 Fwd 5'-AGGAATGAAGATATCCCCGAGCGA-3'

ΔRBD2 Rev 5'-GTTCATGTGGATATCGGTGTGCCT-3'

ΔRBD3 Fwd 5'-ACCTCTACCTGTGATTGAAAAGCC-3'

ΔRBD3 Rev 5'-GCATCTTTATCTTCTTCAGGTCCC-3'

ΔRBD4 Fwd 5'-TCTTCCCCTGTTGCTGAGAAG-3'

ΔRBD4 Rev 5'-TTGTCCATATTCTGGGGAAGC-3'

ΔRBD5 Fwd 5'-ATGGATGGTTCTCTCATGGCTGAA-3'

ΔRBD5 Rev 5'-GGAACTATGGCACATGAGGTT-3'

All constructs were sequenced completely to confirm no mutations were present.

Protein

Analysis of endogenous Gsk3β protein in Stau2-MO injected embryos was done as follows: 40ng of Stau2-MO was injected into the both cells of a 2 cell stage embryos and at NF10 protein extract was isolated from groups of 5 embryos (control and Stau2-MO). The housekeeping protein α-actin was used to normalize total amount of protein between the control and Stau2-MO embryos using polyclonal α-α-actin antibody (1:16,000; Sigma). Endogenous Gsk3β protein levels were determined using the polyclonal α-Gsk3β antibody (1:200; sc-9166 Santa Cruz).

RESULTS

Stau2 Function is Required for the Maintenance of the Wnt Signaling Pathway

I previously performed a Stau2 loss-of-function analysis in which I determined that Stau2 knockdown led to hypoplasia of anterior endodermal organs. This phenotype appeared to be caused in part by the requirement of Stau2 function for the expression of *chordin* during gastrulation (Bilogan and Horb 2012). Previous studies have shown that Chordin knockdown produced a moderately ventralized phenotype, and that Chordin was an essential component downstream of the Wnt/β-catenin and Nodal signaling pathways (Oelgeschlager et al. 2003). I showed that knockdown of Stau2 led to a complete loss of *chordin* expression at NF11/12, however our results did not produce a ventralized phenotype as I only observed defects within the endoderm patterning (Oelgeschlager et al. 2003; Bilogan and Horb 2012). Since *chordin* expression was known to be required for axial patterning in all three germ layers, yet I only observed an endoderm specific phenotype, it was next important to determine exactly when loss of Stau2 phenotype was initiated. I examined a number of molecular markers of the Wnt/β-catenin and Nodal signaling pathways in late blastula stage embryos (NF9) and two hours later at the onset of gastrulation (NF10). Expression of Nodal effectors, *foxh1* and *mixer* were not affected by loss of Stau2 at NF9 or NF10, demonstrating that Stau2 does not control Nodal signaling (Fig. 1a-h). For the Wnt/β-catenin effectors *sia, twn* and *xnr3,* I found normal expression at NF9 in Stau2-MO injected embryos (Fig. 1i, j, m, n, data not shown); however, at NF10 expression of these markers was lost in the dorsal-anterior region of the embryo (Fig. 1k, l, o, p, data not shown). These results suggest that Stau2 function is

not required for the initial expression and function of Wnt/β-catenin target genes, but is required to maintain their expression in the anterior endoderm at NF10.

Wnt/β-catenin and Nodal signaling have been shown to be essential for the expression of several organizer genes, including *gsc, hex, otx2* and *lim1* (Rankin et al. 2011; Reid et al. 2012). To determine whether loss of maintenance of *sia*, *twn* and *xnr3* expression in Stau2 knockdown embryos affected organizer gene expression, I examined the expression of *otx2* and *gsc* at NF9 and N10. *Otx2* expression was not affected by loss of Stau2 function in late blastula embryos (Fig. 1q, r), however expression of *otx2* was reduced in the organizer at NF10 (Fig. 1s, t). Similar results were observed with *gsc* expression in Stau2 morphants, with its expression only reduced at NF10 and not at NF9 (Fig. 1u-x). These results correlate with previous studies that demonstrated transcription of *gsc* was dependent on the combined integration of Wnt/β-catenin and Nodal signaling (Reid et al. 2012). Taken together, these results suggest that Stau2 is required for the maintenance of Wnt/β-catenin signaling effectors leading to a reduction in organizer gene expression, residual expression is likely maintained by Nodal signaling.

Since Stau2 appeared to regulate Wnt/β-catenin signaling at the onset of gastrulation, I further evaluated the effect of Stau2 knockdown on a third signaling pathway working during gastrulation, namely the BMP signaling pathway which I previously showed was affected by loss of Stau2 (Bilogan and Horb 2012). Interestingly when I analyzed *chordin* expression in NF9 and NF10 Stau2 knockdown embryos expression was maintained, a

result that is seemingly different from the observed loss of Wnt/β-catenin signaling effector expression I previously noted (Fig. 1). Most strikingly, while the expression of *chordin* was not lost, the expression domain was reduced and *chordin* was no longer expressed in the deep endoderm cells, which are normally fated to become the anterior endoderm (Fig. 2a, b, data not shown). Since Chordin functions to antagonize BMP4 signaling in the dorsal half of the embryo, I tested whether this reduction in *chordin* expression would alleviate repression of BMP4 by analyzing the expression of *vent1/2*. In Stau2 knockdown embryos the expression domain of *vent1/2* was expanded dorsally (Fig. 2c, d), likely because of the loss of *chordin* expression.

To determine whether *chordin* expression in the deep endoderm cells was required for proper patterning of the dorsal-anterior/ventral-posterior axis of the endoderm, I tested whether the loss of anterior endodermal organ phenotype caused by Stau2-MO could be rescued by overexpression of *chordin* mRNA. Co-injection of Stau2-MO plus *chordin* mRNA into the dorsal-anterior endoderm effectively restored anterior endoderm organ morphology, similar to the Stau2-MO plus *stau2* mRNA rescue (Fig. 2e-h) (Bilogan and Horb 2012). These data indicate that Stau2 function is required for *chordin* expression in cells fated to become anterior endoderm, without it ventral-posterior fates expand into the dorsal-anterior endoderm. Knockdown of Stau2 leads to a complete loss of expression of Wnt/β-catenin effectors *sia, twn* and *xnr3*, while downstream targets *otx2* and *gsc* expression is reduced, but still present. *Chordin* expression was shown to be regulated by Wnt/β-catenin signaling, however while Stau2 knockdown results in a complete loss of Wnt/β-catenin effector expression, *chordin* is only lost in the deep endoderm cells. It is

likely that expression of a specific combination of genes within the embryo is required to pattern the anterior endoderm and regulate expression of *chordin.*

Identification of Stau2-Associated mRNAs

I have identified a novel biological function for Stau2 in endoderm patterning during gastrulation, however the mechanism by which of Stau2 regulates expression of *chordin* and Wnt/β-catenin signaling but not Nodal signaling is still unknown. As Stau2 likely has multiple mRNA targets during gastrulation, our gene expression analysis could not reveal the full extent of Stau2 function. To determine the mechanism of Stau2 in patterning, I set out to identify gastrula stage mRNA targets of Stau2. *Flag-stau2* mRNA was injected into the two dorsal-vegetal blastomeres of the eight-cell stage embryo and embryos were harvested at NF10, which was the stage when I first observed gene expression changes in the Stau2 knockdown embryos (Fig. 3a). I immunoprecipitated Flag-Stau2 protein from whole embryos using α-Flag coated beads (Stau2 IP), extracted the Stau2 bound mRNA using Trizol and prepared this RNA for high-throughput sequencing; this was done from three independent biological replicates (Fig. 3a). For comparison, I used RNA extracted from an immunoprecipitation of another RNA binding protein, BrunoL1 that is not expected to interact with the same mRNA targets as Stau2 (Fig. 3a). Following RIP-seq, the libraries were mapped to an *X. laevis* reference library prepared by *de novo* assembly from wild-caught embryos (Horb lab, unpublished data).

To identify Stau2-specific mRNA transcripts I compared the reference contigs mapped by Stau2 IP reads to BrunoL1 IP reads. I then eliminated any overlapping reads between the two that mapped to the same contig; with this strict cut-off I may have discarded genuine Stau2 targets, but I increased confidence that those I identified were genuine. To validate the mRNA targets identified in the Stau2 IP library I performed RT-PCR on 10 of the most enriched targets from the Stau2 IP (Fig. 3b). cDNA made from RNA isolated from Stau2 IP or BrunoL1 IP was used as a template for RT-PCR, as a control I used cDNA made from whole embryo RNA that was isolated at the same time (Fig. 3b). I also used *ef1a* as a control for the BrunoL1 IP, as it was previously shown to be a BrunoL1 target (Horb and Horb 2010). *Ef1a* was present in the BrunoL1 IP and not the Stau2 IP samples (Fig. 3b) Overall, I confirmed and validated 10 of the top targets that were revealed by the RIP-seq as putative targets in the Stau2 IP, but not in the BrunoL1 IP. These data suggest that the identified transcripts are likely to be genuine targets of Stau2, however I did identify exactly how Stau2 regulates these targets.

To provide insight into the different targets regulated by Stau2, I used DAVID (Database for Annotation, Visualization and Integrated Discovery) to identify functional relationships from our list of Stau2 associated mRNA. DAVID offers functional annotation tools to identify Gene Ontology (GO) terms from lists of genes (Huang et al. 2009a; Huang et al. 2009b). Using this program I identified a variety of biological processes from our target list, including functions involved in the regulation of transcription and RNA metabolism to cell adhesion and GTPase signal transduction (Fig. 3c). To validate the mRNA targets identified in the most abundant GO term, regulation

of transcription, I preform RT-PCR on the targets identified in this GO cluster (Fig. 3b). Each transcript was detected only in the NF10 whole embryo and in the Stau2 IP sample (Fig. 3b). Our results were consistent with previously characterized functions of human Stau2 associated mRNAs identified by RIP-Chip from HEK293T cells (Furic et al. 2008). Similar GO terms associated between human and *X. laevis* Stau2 targets included regulation of transcription, macromolecular metabolism and phosphorylation (Furic et al. 2008). These results suggest that I have likely identified genuine Stau2 mRNA targets and that Stau2 in *X. laevis* appears to associate with mRNA carrying out similar functions to those identified in humans, suggesting Stau2 function may be conserved across vertebrates.

Our initial analysis identified Wnt/β-catenin signaling effectors and various organizer factors as being reduced in Stau2 morphants. RIP-seq of Stau2 IP RNA did not identify any of these Wnt/β-catenin signaling transcription factors are targets, however I did identify another member of the canonical Wnt signaling pathway, glycogen synthase kinase 3β (Gsk3β) as a potential Stau2 target. Gsk3β is a serine-threonine kinase that phosphorylates β-catenin protein and triggers its degradation (Dominguez and Green, 2000, Aberle et al., 1997, Pai et al., 1997, Yost et al., 1997). I validated *gsk3β* as a genuine target of Stau2 by RT-PCR (Fig 3b). Since I observed a reduction of Wnt/β-catenin signaling gene expression in Stau2 morphants, and Gsk3β acts as an inhibitor of the Wnt/β-catenin signaling pathway, I hypothesized that loss of Stau2 led to an increase in Gsk3β. To test whether Stau2 may be regulating Gsk3β by stimulating decay of the

mRNA transcript, I analyzed the expression level of *gsk3β* mRNA by *in situ* hybridization and RT-PCR in control and Stau2-MO samples. By *in situ* analysis, *gsk3β* expression was unaffected by loss of Stau2 at NF9 and NF10 (Fig. 4 a-d), these results were confirmed by RT-PCR (Fig. 4e). Next, I tested whether Stau2 may be regulating translation of Gsk3β by examining expression of Gsk3β protein in control and Stau2 knockdown embryos. In wild-type lysates, a double band at ~42kDa was detected via western analysis (Fig. 4i). In Stau2-MO injected embryo lysates, Gsk3β expression levels were the same as in the control. In conclusion, these results demonstrated that Stau2 does not appear to be involved in mRNA decay or translational regulation of Gsk3β (Fig. 4i). Further experiments will be required to determine how Stau2 is regulating Gsk3β activity, whether through mRNA localization or perhaps splicing.

Since I did not observe a difference in transcript or protein levels of Gsk3β in Stau2 morphant embryos, I wanted to confirm Stau2 was indeed regulating Gsk3β function. To do so I examined whether knockdown of Stau2 was able to rescue lithium chloride (LiCl) treatment of embryos. LiCl is a known inhibitor of Gsk3β activity and treatment of embryos at the 32-cell stage with LiCl radically dorsalized embryos due to induction of organizer specific genes throughout the entire marginal zone (Fig. 4f, g) (Kao and Elinson 1988; Klein and Melton 1996; Schneider et al. 1996; Oelgeschlager et al. 2003). I hypothesized that Stau2 may play a role in the inhibition of Gsk3β activity in the dorsal endoderm, allowing Wnt/β-catenin signaling effectors to be expressed. To test whether knockdown of Stau2 could counteract the inhibitory effect of LiCl, I injected Stau2-MO

into the two ventral-vegetal blastomeres of an eight-cell embryo prior to LiCl treatment. Knockdown of Stau2 in the ventral half of the embryo along with LiCl treatment resulted in a partial rescue of axis formation (Fig. 4h). These results suggested that Stau2 does indeed regulate the activity of Gsk3β in a negative manner, however how Stau2 targets *gsk3β* mRNA and affects the activity at the protein level will require further investigation.

Not All Stau2 RBDs are the Same

To further define the mechanism of Stau2 action, I sought to determine which region of Stau2 was required for its function. Stau2 contains five dsRBDs, and I wanted to determine which dsRBDs might be required for proper patterning of the endoderm. Stau2 mutants were constructed by the removal of a single dsRBD and the Stau2-MO binding site of these clones was replaced by the Flag epitope (ΔRBD-Stau2). *ΔRBD-stau2* mRNA was injected alone or co-injected along with Stau2-MO into the two dorsal-vegetal blastomeres of an eight-cell stage embryo. Similar to the effects seen with overexpression of full length *stau2* mRNA, injection of *ΔRBD-stau2* mRNA alone did not produce a phenotype (Fig. 5a,c,e,g,i). The ability of a given *ΔRBD-stau2* mRNA to rescue the Stau-MO phenotype differed for each of the RBDs. *ΔRBD2-stau2* mRNA injected Stau2 morphants showed the greatest rescue of morphology and pancreatic gene expression (Fig. 5e,f). *ΔRBD4-stau2* mRNA injected Stau2 morphants were partially able to rescue pancreatic gene expression, however the morphology of the anterior gut remained abnormal (Fig. 5g.h). Similar results were observed in *ΔRBD5-stau2* mRNA injected

Stau2 morphants (Fig. 5i,j). *ΔRBD1-stau2* mRNA injected Stau2 morphants resembled Stau2-MO injected embryos, and did not rescue morphology or gene expression in the anterior endoderm (Fig. 5c,d). I was not able to conclusively test the function of dsRBD3 in anterior endoderm development. These results suggested that individual dsRBDs within the Stau2 protein have different functions, and that dsRBD2 does not appear to be required for endoderm patterning, since it produced the greatest rescue of the Stau2-MO phenotype.

Next, I wanted to determine which dsRBDs were required for binding *gsk3β* mRNA as well as other putative target mRNAs. *ΔRBD-stau2* mRNA was injected into eight-cell embryos and at NF10 the ΔRBD-Stau2-mRNA complex was immunoprecipitated. Total RNA was then extracted from the immunoprecipitate and RT-PCR was used to determine whether *gsk3β* mRNA was associated with the ΔRBD mutant Stau2. When *ΔRBD2-stau2* or *ΔRBD3-stau2* mRNA were injected, *gsk3β* mRNA co-immunoprecipitated with the mutant Stau2 proteins (Fig. 5k). However, when *ΔRBD1-stau2*, *ΔRBD4-stau2* or *ΔRBD5-stau2* mRNA was injected, *gsk3β* was not detected. Expression of the general endoderm marker, *edd*, served as our control and was not detected in the immunoprecipitated fractions (Fig. 5k). These results show that dsRBD2 and dsRBD3 were not required to complex with *gsk3β* mRNA, suggesting that Stau2 requires dsRBD1, dsRBD4 and dsRBD5 to effectively form a complex with *gsk3β* mRNA. It may be possible that multiple RBDs may bind a single target mRNA, which may be the case for dsRBD1, dsRBD4 and dsRBD5 with *gsk3β*. Knockdown of Stau2 produces a complex phenotype

that was not fully rescued by co-injection of *chordin* mRNA, suggesting that Stau2 may have additional mRNA targets besides *gsk3β* during gastrulation. This was also demonstrated through the identification of numerous potential Stau2-associated targets by the RIP-seq. To test which dsRBDs were required to complex with potential targets I performed RT-PCR on Stau2 associated targets identified by the RIP-seq using ΔRBD-Stau2 IP RNA. Most of the Stau2-targets tested still complexed with ΔRBD2 and ΔRBD3 mutants, while only the ΔRBD3 mutant lost the ability to bind of *lin28a* (Fig. 5k). These results suggest that dsRBD2 and often dsRBD3 are not required to associate with a variety of mRNA targets. Since certain transcripts appear to be unaffected by the loss of any given dsRBD, it is likely these targets are being pulled down via protein-protein interactions. Alternatively, more than one dsRBD may be required for specific binding. This is the first report to show differential binding of the dsRBD to potential targets, future studies will be required to determine whether binding to different domains determines how a transcript is regulated.

DISCUSSION

Patterning and establishment of the germ layers is dependent on the formation of the Spemann-Mangold organizer. Elevated Wnt/β-catenin and Nodal signaling is integrated within the cells on the dorsal side of the blastula embryo to generate organizer gene transcription in spatio-temporal manner (Klein and Melton 1996). In the work presented here, I demonstrated that the function of Stau2 is required at the onset of gastrulation to maintain Wnt/β-catenin signaling effector proteins and organizer gene transcription, but was not required for Nodal signal inputs. Using RIP-Seq I identified numerous potential Stau2 mRNA targets including an inhibitor of Wnt signaling, *gsk3β*. I showed that while knockdown of Stau2 did not affect *gsk3β* transcript or protein levels, loss of Stau2 was able to partially rescue the Gsk3β inhibitory effect of LiCl in the embryo. Finally, I showed that overexpression of only *ΔRBD2-stau2* mRNA could rescue the Stau2-MO phenotype (ΔRBD3 was not tested), and that Stau2 mutants lacking dsRBD2 or dsRBD3 could complex with *gsk3β* mRNA at gastrulation. I propose that at the onset of gastrulation Stau2 is required to regulate the activity of Wnt signaling, this may be accomplished through an interaction between *gsk3β* transcript and dsRBD1, dsRBD4, and dsRBD5 of the Stau2 protein.

In this study, I showed that Stau2 function is required to maintain Wnt/β-catenin signaling at the onset of gastrulation since knockdown of Stau2 led to loss of expression of the Wnt/β-catenin effectors *sia, twn* and *xnr3* within the organizer. Sia and Twn have been identified as regulators of multiple organizer genes, including Gsc, Chordin and Hex

(Harland and Gerhart 1997; Bae et al. 2011; Rankin et al. 2011). In previous studies, expression of a dominant-repressive form of Sia (Eng-Sia) or knockdown of both Sia and Twn together resulted in a loss of organizer gene expression and disrupted axis formation (Fan and Sokol 1997; Kessler 1997; Ishibashi et al. 2008; Bae et al. 2011). While I showed knockdown of Stau2 resulted in loss of both *sia* and *twn*, I observed a milder phenotype of loss of anterior endoderm formation, with no observed affect on mesoderm development (Bilogan and Horb 2012). Though Stau2 was ubiquitously expressed within the early embryo; I injected the two dorsal-vegetal blastomeres of the eight-cell stage embryo, which specifically targeted the anterior endoderm and part of the mesoderm. This explained the specific loss of anterior endoderm organ later, however the loss of *sia* and *twn* at gastrulation suggested a more severe axial defect should have been observed. Both spatial and temporal expression of *sia* and *twn* needs to be taken into account when comparing experiments. In the Sia and Twn knockdown studies, the morpholino or *eng-sia* constructs were injected into the two dorsal blastomeres at the 4-cell stage embryo, targeting the entire dorsal side of the embryo (Fan and Sokol 1997; Kessler 1997; Bae et al. 2011). It is possible that Sia and Twn may activate transcription of downstream targets in a temporal or concentration dependent manner. I showed that in Stau2 morphants, *sia*, *twn* and organizer-specific targets were initially expressed in late blastula embryos (NF9), however this expression was not maintained after the onset of gastrulation (NF10). It may be possible that early Sia and Twn were required to establish the germ layers and pattern future ectoderm and mesoderm, while later Sia and Twn pattern endoderm. In the Stau2 morphants, short expression of *sia* and *twn* may have been enough to induce anterior

fates in the ectoderm/mesoderm, while maintained expression of *sia* and *twn* was required to induce anterior endoderm fates.

Gsk3β is a Potential Novel Stau2 Target

I identified Gsk3β as a potential Stau2 target from our RIP-seq experiment. As a known inhibitor of the Wnt/β-catenin signaling pathway, I hypothesized that Stau2 function may be required to restrict the expression of *gsk3β* since loss of Stau2 resulted in a loss of expression of Wnt/β-catenin effectors, *sia, twn* and *xnr3*. I report here that Stau2 did not appear to target the *gsk3β* transcript for mRNA decay, nor did Stau2 appear to affect its translation. I was not able to test whether Stau2 was required for proper mRNA localization of *gsk3β*, however this could explain why transcript and protein levels of Gsk3β were unchanged. Nevertheless, I validated it as a Stau2 mRNA target and Stau2 appeared to be required upstream of Sia since injection of *sia* mRNA can partially rescue the Stau2 knockdown phenotype (data not shown). The ability of Stau2-MO to partially rescue the inhibitory effects of LiCl on Gsk3β also provided evidence that Stau2 was functioning to restrict the active protein levels of Gsk3β.

High-Throughput Analysis of Stau2 Targets

Prior studies have identified mRNA targets of Stau in E13-14 mouse cortex, in differentiated neurons in rat brain, in immortal cultured cells lines and in adult *C. elegans* (Furic et al. 2008; Maher-Laporte and Desgroseillers 2010; Kusek et al. 2012; LeGendre

et al. 2013). Our study along with most of the others has rendered an extensive and heterogeneous list of potential Stau targets. One explanation for the abundance of identified targets is that *Xenopus* Stau2 has five unique dsRBDs and potential protein interaction domains, allowing it to interact with several transcripts, either directly or indirectly through interactions with other proteins (Allison et al. 2004; Kusek et al. 2012). My analysis likely identified direct as well as indirect RNA targets since the IP conditions did not require direct Stau2-mRNA interactions. Our RIP-Seq results clustered into similar GO terms as Stau targets identified in previous high-throughput studies using vertebrate model systems, while it seemed comparably less similar to the recent targets identified in *C. elegans* (Furic et al. 2008; Maher-Laporte and Desgroseillers 2010; Kusek et al. 2012; LeGendre et al. 2013). I did identify various biologically validated Stau mRNA targets including *prox1*, the mammalian homolog of *prospero*, which appears to be a conserved Stau target (Broadus et al. 1998; Vessey et al. 2012). I also identified a few members of the ADP-ribosylation factor (Arf) gene family including *arf1*, which has been described as an SMD target of Stau1 in mammalian cultured cells. While others have not detected *arf1* in high-throughput identification of Stau-associated mRNAs in human HEK293T cells (Furic et al. 2008), both our study and Furic et al., 2008 identified another member of the ARF gene family, *arf3* (Furic et al. 2008). Differences in identified Stau2 mRNA targets between studies likely exist since the samples collected for analysis came from different developmental stages and different cell types. Stau as well as other RBPs have been shown to serve different functions in a context-dependent manner (Wessely and De Robertis 2000; Tran et al. 2007). I am the first to report findings on Stau2 targets during early embryogenesis and the first to use

RNA-seq to identify Stau2 IP targets. Through this technique I appear to have identified conserved as well as new Stau2 targets. Interestingly many of the validated novel Stau2 targets enriched in the GO term regulation of transcription have previously identified roles in patterning. Xolloid, Vent1 and LMO4b are involved in ventral-posterior patterning of the embryo and Xbra is a well-known early mesodermal marker. Studies of Lin28a and Smurf2 suggest a role in germ layer specification and boundary formation. Some of these genes can also regulate or be regulated by the Wnt signaling pathway (Smurf2, Glis2, HIRA). I believe that this approach has provided an overview of the Stau2-enriched set of transcripts in the developing endoderm at the onset of gastrulation.

Stau proteins contain multiple dsRBDs, each of which is unique. Most Stau homologs contain five dsRBDs, while human Stau1 protein only contain four, lacking dsRBD1. Our understanding of which dsRBDs are required to bind mRNA is minimal. Our analysis of mutant Stau2 protein function *in vivo* allowed for a better understanding of the RNA specificity for a given dsRBD. I found that the deletion of dsRBD1, dsRBD4 or dsRBD5 prevented binding of *gsk3β* transcript, while dsRBD2 and dsRBD3 did not. In line with this result, only *ΔRBD2-stau2* mRNA could rescue the Stau2-MO phenotype (ΔRBD3 was not tested), suggesting dsRBD1, dsRBD4 and dsRBD5 are required to bind *gsk3β* mRNA directly or interact with other proteins that bind *gsk3β* mRNA. In *Xenopus* oocytes, expression of Stau lacking dsRBB1 and dsRBD5 blocked vegetal RNA localization (Yoon and Mowry 2004). In *Drosophila* Stau, dsRBD1, dsRBD3, and dsRBD4 bound dsRNA *in vitro*, whereas dsRBD2 and dsRBD5 did not (Micklem et al. 2000). While in mammalian cells, dsRBD3 and dsRBD4 constitute the main active

dsRNA-binding sites (Park et al. 2013). The only other report studying the affinity of full-length Stau to bind dsRNA found that at least two domains of Stau1 in *C. elegans* were required to bind dsRNA with high affinity, however they used randomly generated RNA sequences that formed a 23-bp stem in electrophoretic mobility shift assays (LeGendre et al. 2013). Our results suggest a conserved function of dsRBD4 between *Xenopus*, *Drosophila* and humans. Further studies are required to understand how *X. laevis* Stau2 associates with different RNA targets, either directly or indirectly via protein partners and how it regulates the fate of its different targets.

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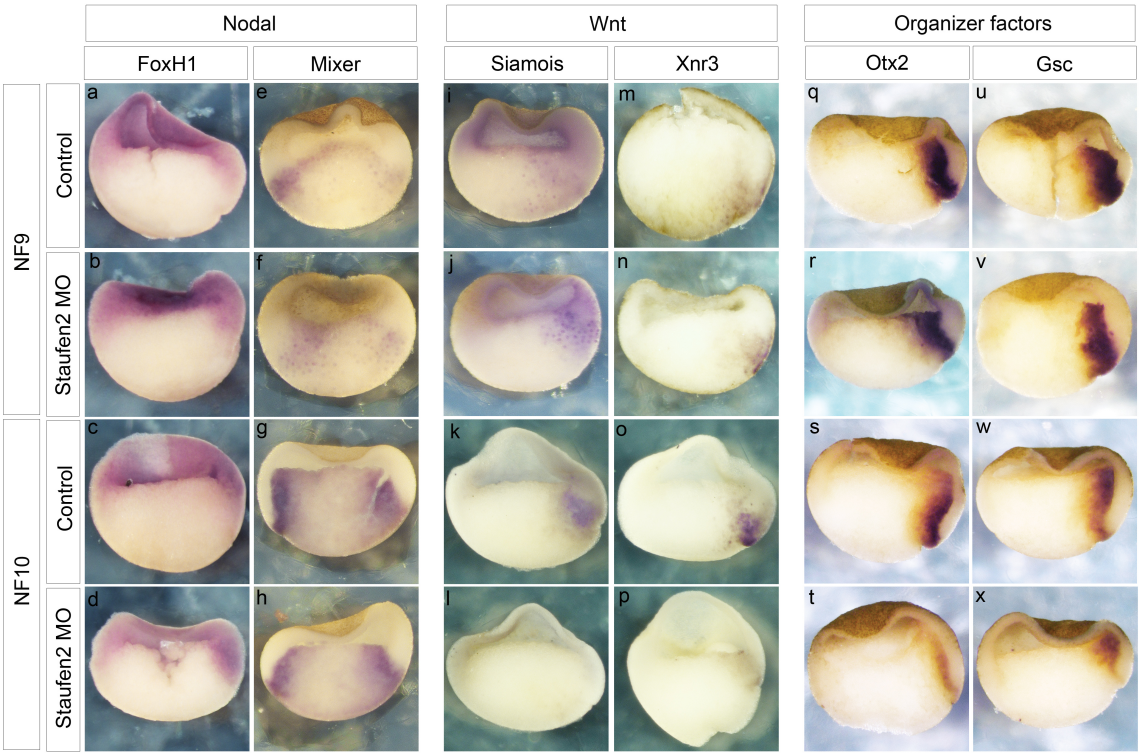


Figure 1. Staufen2 regulates early endoderm patterning through the Wnt signaling pathway. Whole mount *in situ* hybridization in control and Staufen2-MO-injected embryos transverse section. **A,B:** Expression of *foxh1* is unchanged in the Staufen2 knockdown embryos at NF9 (100%, n=8). **C,D:** At NF10, expression of *foxh1* remains unchanged (100%, n=9). **E,F:** Expression of *mixer* is unchanged in the Staufen2 knockdown embryos at NF9 (100%, n=11). **G,H:** At NF10, expression of *mixer* remains unchanged (100%, n=9). **I,J:** Prior to gastrulation, expression of *siamois* is unchanged in the Staufen2 knockdown embryos (100%, n=15). **K,L:** At gastrulation, expression of *siamois* is lost in the dorsal half of the embryo (85%, n=20). **M,N:** Prior to gastrulation, expression of *xnr3* is unchanged (100%, n=10). **O,P:** At gastrulation, expression of *xnr3* is almost completely lost in the dorsal half of the embryo (78%, n=9). **Q,R:** At NF9, expression of *otx2* is unchanged in the Staufen2 knockdown embryos (100%, n=10). **S,T:** At gastrulation, expression of *otx2* is reduced in the dorsal half of the embryo (71%, n=14). **U,V:** In the late blastula stage embryo, expression of *gsc* is unchanged (100%, n=12). **W,X:** At gastrulation, expression of *gsc* is reduced in the dorsal half of the embryo (83%, n=12).

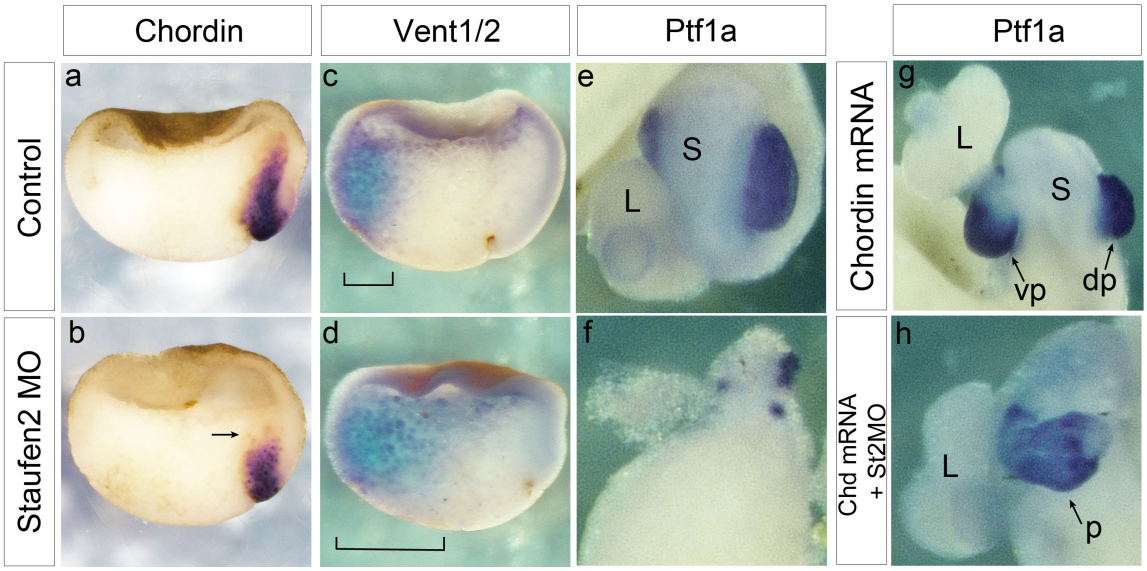
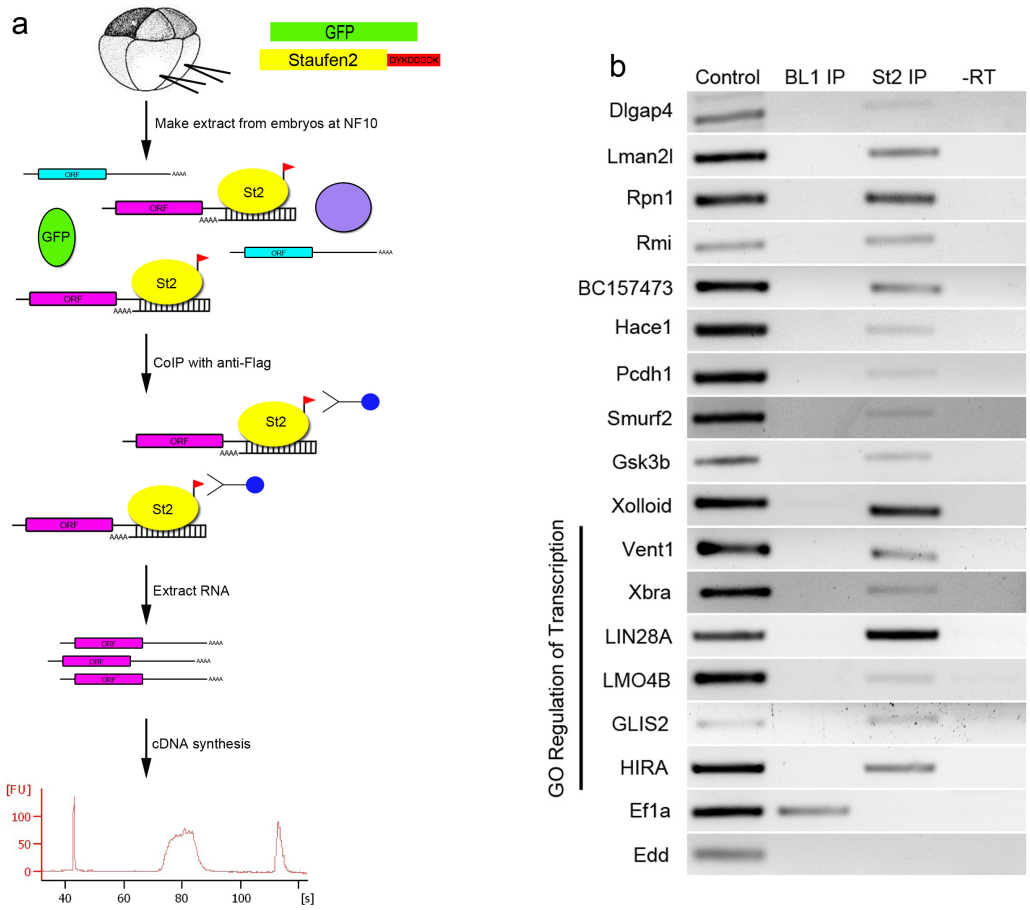


Figure 2. Staufen2 function is required upstream of Chordin. A-D: Whole mount *in situ* hybridization in control and Staufen2-MO-injected embryos transverse section at the onset of gastrulation (NF10). **A,B:** Expression of *chordin* is lost in the deep endoderm cells in the Staufen2 knockdown embryos, arrow (67%, n=15). **C,D:** At NF10, expression of *vent1/2* is expanded dorsally, bracket (100%, n=8). **E-H:** Whole mount *in situ* hybridization for *ptf1a* on isolated guts at NF46. **E:** Expression of *ptf1a* is restricted to the dorsal and ventral pancreatic buds. **F:** Expression of *ptf1a* and formation of the anterior endodermal organs is lost. **G:** Overexpression of 200pg *chordin* mRNA has no effect on anterior endoderm organ formation (95%, n=20). **H:** Co-injection of 200pg *chordin* mRNA and Staufen2 MO can rescue *ptf1a* expression and partially rescue organ formation (64%, n=14). S, stomach; L, liver; dp, dorsal pancreas; vp, ventral pancreas; p, pancreas.



c

ID	Term	Count	P-Value
GO:0006355	Regulation of transcription	413	5.20E-07
GO:0051252	Regulation of RNA metabolic process	285	5.00E-04
GO:0006468	Protein amino acid phosphorylation	142	6.40E-02
GO:0006259	DNA metabolic process	84	1.30E-02
GO:0007155	Cell adhesion	50	7.10E-04
GO:0006974	Response to DNA damage stimulus	49	7.50E-03
GO:0034660	ncRNA metabolic process	48	1.60E-03
GO:0051056	Regulation of small GTPase mediated signal transduction	41	4.50E-04

Figure 3. RIP-seq identifies endogenous Staufen2 mRNAs. A: Scheme for identification of Staufen2 associated mRNAs. α-flag M2 beads were used to immunoprecipitate Staufen2-Flag from injected NF10 embryo. RNA was isolated from the immunoprecipitations, cDNA was synthesized and amplified, and sequence by the Illumina HiSeq 1000. RT-PCR validation of RIP-Seq results. **B:** total RNA was isolated from the immunoprecipitate (IP), reverse transcribed, and PCR-amplified using gene-specific primers. *Edd* served as a loading control. *Ef1a* is a transcript that did not appear on our list of Staufen2-associated mRNAs but is known to associate with BrunoL1. Control, non-injected control; BL1 IP, *brunoL1-flag* mRNA-injected IP; St2 IP, *stau2-flag* mRNA-injected IP; -RT, control reaction without reverse transcriptase. **C:** List of major Gene Ontology terms associated with Staufen2 targets. Count, refers to the number of Staufen2 targets within each group.

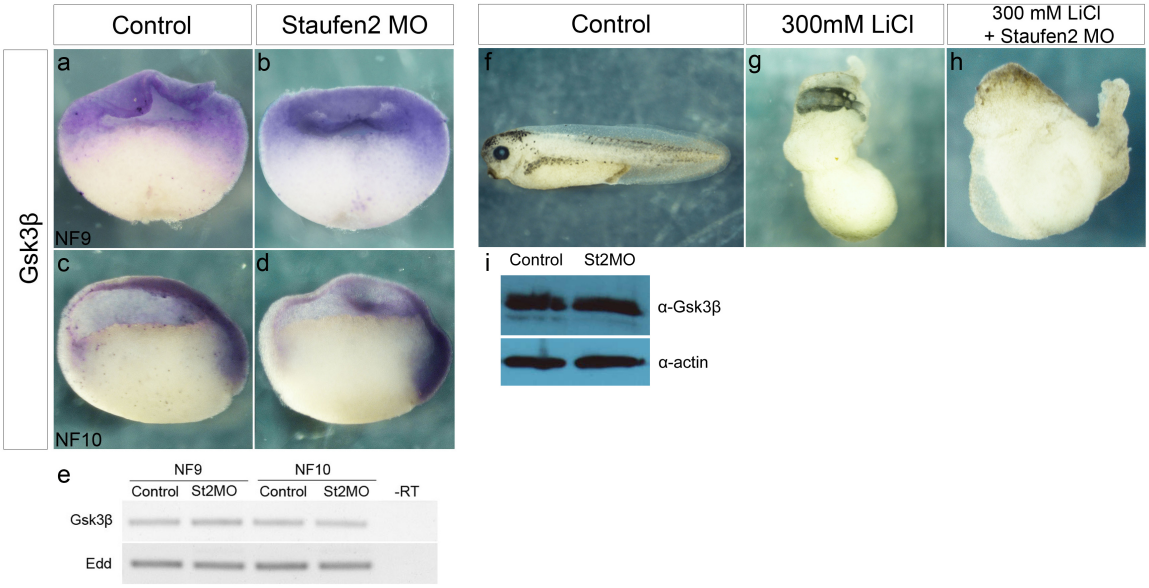


Figure 4. Staufen2 function is required to regulate Gsk3β function during gastrulation. A-D: Whole mount *in situ* hybridization in control and Staufen2-MO-injected embryos transverse section. **A,B:** Expression of *gsk3β* is unchanged in the Stau2 knockdown embryos at NF9 (100%, n=8). **C,D:** At NF10, expression of *gsk3β* remains unchanged (100%, n=8). **E:** RT-PCR for *gsk3β* at NF9 and NF10, expression is unchanged in control and Staufen2 knockdown embryos. **F:** Control tadpole at NF39 **G:** NF39 tadpole exposed to 300 mM LiCl for 10 minutes at 64-cell stage **H:** NF39 tadpole exposed to 300 mM LiCl for 10 minutes at 64-cell stage and injected Staufen2 MO into the ventral vegetal cells at 8–cell stage can partially rescue the dorsalizing affect of inhibiting Gsk3β activity. **I:** Levels of endogenous Gsk3β protein in control and Stau2-MO injected embryos, demonstrating that there was no changed Gsk3β protein levels in Stau2 knockdown embryos.

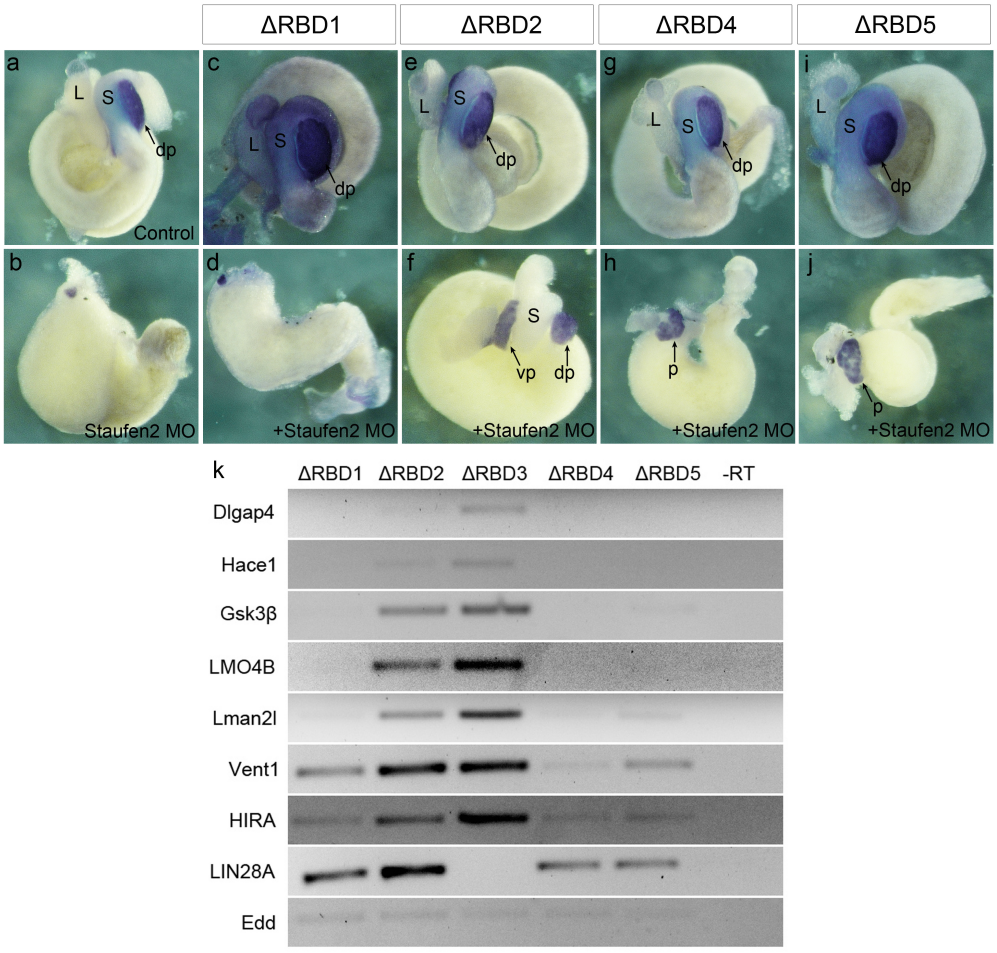


Figure 5. Staufen2 dsRBDs 1, 4 and 5 are required for proper anterior endoderm organ formation. Whole mount *in situ* hybridization in control and Staufen2-MO-injected embryos for *ptf1a* on NF46 isolated whole guts. **A:** Expression of *ptf1a* is restricted to the dorsal and ventral pancreatic buds. **B:** Expression of *ptf1a* and formation of the anterior endodermal organs is lost. **C,D:** Co-injection of ΔRBD1-Staufen2 mRNA with Staufen2 MO does not rescue *ptf1a* expression or anterior endoderm morphology (100%, n=13). **E,F:** Co-injection of ΔRBD2-Staufen2 mRNA with Staufen2 MO can rescue *ptf1a* expression and anterior endoderm morphology (67%, n=18). **G,H:** Co-injection of ΔRBD4-Staufen2 mRNA with Staufen2 MO partially rescues *ptf1a* expression and anterior endoderm morphology (75%, n=12). **I,J:** Co-injection of ΔRBD5-Staufen2 mRNA with Staufen2 MO partially rescues *ptf1a* expression and anterior endoderm morphology (57%, n=28). **K:** RT-PCR for ΔRBD-Staufen2 associated mRNA. Total RNA was isolated from immunoprecipitate of ΔRBD-Staufen2-Flag at NF10; reverse transcribed, and PCR-amplified using gene-specific primers. Gsk3β transcript was only associated with ΔRBD2-Staufen2-Flag and ΔRBD3-Staufen2-Flag. *Edd* served as a loading control. -RT, control reaction without reverse transcriptase S, stomach; L, liver; dp, dorsal pancreas; vp, ventral pancreas; p, pancreas.

Chapter 4: Microarray Analysis of Xenopus Endoderm Expressing Ptf1a

Bilogan CK and Horb ME. 2012. *Genesis* 50:853-70

ABSTRACT

Pancreas specific transcription factor 1a (Ptf1a), a bHLH transcription factor, has two temporally distinct functions during pancreas development; initially it is required for early specification of the entire pancreas, while later it is required for proper differentiation and maintenance of only acinar cells. The importance of Ptf1a function was revealed by the fact that loss of Ptf1a leads to pancreas agenesis in humans. While Ptf1a is one of the most important pancreatic transcription factors, little is known about the differences between the regulatory networks it controls during initial specification of the pancreas as opposed to acinar cell development, and to date no comprehensive analysis of its downstream targets has been published. In this paper, we use *Xenopus* embryos to identify putative downstream targets of Ptf1a. We isolated anterior endoderm tissue overexpressing *Ptf1a* at two early stages, NF32 and NF36, and compared their gene expression profiles using microarrays. Our results revealed that Ptf1a regulates genes with a wide variety of functions, providing insight into the complexity of the regulatory network required for pancreas specification.

INTRODUCTION

The pancreas is an endodermal organ comprised of exocrine and endocrine cells. Exocrine tissue includes acinar cells that secrete digestive enzymes and ductal cells that transport the enzymes to the duodenum. Endocrine tissue is composed of five cells types, α, β, δ, ε and PP, which secrete glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide hormones, respectively, into the bloodstream.

The embryological origin of the pancreas is conserved from mammals to amphibians, where the pancreas develops from separate dorsal and ventral buds (Kelly and Melton 2000; Gittes 2009; Pearl et al. 2009). In *Xenopus laevis*, the dorsal bud arises first at NF35/36 just below the notochord at the level of the pronepheros. The ventral pancreas derives from two anlagen at the junction of the liver bud and developing duodenum at NF37/38. Morphological movements of the gastrointestinal tract during NF39 and 40 lead to the repositioning of the pancreatic buds driving their fusion and the formation of a single discrete organ (Kelly and Melton 2000; Jarikji et al. 2009; Pearl et al. 2009). Prior to the emergence of the dorsal pancreatic bud, punctate expression of the beta cell differentiation marker *insulin* is detectable by in situ hybridization as early as NF32; subsequent expression within the ventral pancreas does not occur until NF47 (Kelly and Melton 2000; Horb and Slack 2002). Unlike *insulin*, the expression of the other two endocrine markers, *glucagon* and *somatostatin,* are not restricted to the pancreas. These two genes are initially expressed in a punctate pattern within the stomach and duodenum at NF41, and are only detected in the pancreas beginning at NF44/45. The rapidity of

pancreas development in *Xenopus*, coupled with its embryological and molecular benefits, makes it a useful system for functional studies on pancreas development (Blitz et al. 2006; Harland and Grainger 2011; Pearl et al. 2012).

In the last few years our lab has utilized these benefits to characterize the function of several new genes in *Xenopus* pancreas development. First, in studying liver to pancreas transdifferentiation we found that Ptf1a and Pdx1 were sufficient to convert liver to pancreas in *Xenopus* transgenics (Horb et al. 2003; Jarikji et al. 2007). Second, we isolated individual dorsal and ventral pancreatic buds prior to fusion and used microarrays to compare their gene expression profiles. From this we characterized the function of three different genes, Insm1 (zinc finger transcription factor), Tm4sf3 (tetraspanin) and Brunol1 (RNA binding protein) and showed that they were involved in endocrine cell differentiation, fusion of the pancreatic buds and proliferation of endodermal progenitor cells, respectively (Horb et al. 2009; Jarikji et al. 2009; Horb and Horb 2010). Third, we showed that Staufen2, an RNA binding protein, played an essential role in early patterning of the anterior endoderm (Bilogan and Horb 2012). Fourth, we performed an exhaustive functional analysis of Rfx6, a neonatal diabetes candidate gene, and through microarray analysis identified new target genes involved in development of several anterior endodermal organs (Pearl et al. 2011). Fifth, we recently described a novel protocol to maximize production of pancreatic beta cells over alpha cells by controlled overexpression of the pancreatic endocrine transcription factor Ngn3 in naïve endoderm; using this gain-of-function phenotype we isolated endoderm shortly after Ngn3 expression and identified novel targets that function downstream of Ngn3

(Oropez and Horb 2012). These studies demonstrate the benefits of using *Xenopus* to study pancreas development, namely the ability to perform both gain- and loss-of-function studies in naïve endoderm and the ability to make transgenics rapidly for tissue-specific overexpression studies. In this paper we describe a combined gain-of-function approach with microarray analysis to identify new target genes of a pancreatic transcription factor (Ptf1a) in *Xenopus* endoderm.

The basic helix-loop-helix (bHLH) protein Ptf1a (pancreas specific transcription factor 1a) is one of the first endodermal transcription factors restricted to early pancreatic progenitors before overt morphogenesis of the dorsal and ventral buds (Kawaguchi et al., 2002). Ptf1a has different stage-specific functions that are crucial for proper pancreas organogenesis. Initially, it is required to specify undifferentiated foregut endoderm into a pancreatic fate (Kawaguchi et al. 2002). Later, its expression becomes restricted to the cells at the tip of the pancreas where it functions in the initiation and maintenance of acinar cells (Krapp et al. 1996; Masui et al. 2007). The different functions are largely dependent on protein binding partners of Ptf1a, specifically which RBPJ paralog is bound by Ptf1a. RBPJ, the vertebrate Suppressor of Hairless [Su(H)], is found in the early PTF1 complex (PTF-J) and is required for the epithelial growth and development of the dorsal and ventral pancreatic buds. The PTF1-J complex binds to the Rbpjl promoter, activates its expression, and is eventually replaced by the RBPJL in the PTF1 complex (PTF1-L) during acinar cell differentiation (Masui et al., 2007). The PTF1-L complex then activates the promoters of genes encoding the secretory digestive enzymes (Beres et al., 2006). In humans, loss-of-function gene mutations in PTF1A lead to pancreatic and cerebellar

agenesis (Sellick et al. 2004). Similarly, loss-of-function studies in mice and zebrafish revealed that functional Ptf1a is essential for exocrine cell development and the development of a subset of endocrine cells (Krapp et al. 1998; Kawaguchi et al. 2002; Lin et al. 2004; Zecchin et al. 2004).

In *Xenopus*, we found Ptf1a to be both necessary and sufficient for development of the pancreas. Morpholino knockdown of Ptf1a resulted in a complete loss of exocrine cells and loss of a subset of endocrine cells, while overexpression of Ptf1a was found sufficient to promote ectopic development of pancreatic cells (Afelik et al. 2006; Jarikji et al. 2007). Overexpression of *ptf1a* mRNA in naïve endoderm was sufficient to respecify early stomach and duodenal cells into both acinar and endocrine pancreas cells, while a superactive version was only capable of promoting an acinar cell fate. Similarly, we showed that transgenic overexpression of Ptf1a at later stages (after the foregut organs had formed) was able to promote ectopic endocrine and exocrine cell fates within the stomach and duodenum. Though we know Ptf1a can specify both endocrine and exocrine cell fates, the transcriptional hierarchy downstream of Ptf1a is still unknown.

In an attempt to elucidate the gene regulatory network activated by Ptf1a in early pancreas development we set out to identify gene expression changes that occurred upon overexpression of Ptf1a at the onset of pancreas development. Taking advantage of the Ptf1a overexpression phenotype, we compared control endodermal tissue to Ptf1a overexpression tissue at two early time-points, NF32 and NF36. This led to the

identification of many genes with altered expression levels; the genes identified had varied functions revealing the diverse roles of Ptf1a in early pancreas development. We describe the expression of seven candidate genes with functions ranging from transcription, vesicle fusion and cell adhesion. In addition, we identified novel genes expressed within the developing pancreatic endoderm.

METHODS

Microarray Analysis

All mRNA for microinjection was created using the Ambion mMessage mMachine kit. To confirm targeting, experimental mRNAs were injected along with 400pg *gfp* mRNA and targeting verified by observing appropriate fluorescence. For functional analysis we selected only samples for which the entire anterior endoderm was targeted. 800pg *ptf1a* and *gfp* mRNAs were injected into the dorsal vegetal blastomeres at the eight-cell stage. Anterior endoderm explants were collected at NF32 and NF36. RNA extraction of the sample sets (15 explants/set) was performed using TRIzol (Invitrogen) and purified using the RNeasy Micro Kit (Qiagen). RNA analysis, cDNA preparation and hybridization were performed by Genome Québec (McGill University, Montréal).

Microarray results were analyzed using Affymetrix Expression Console and normalized using the Probe Logarithmic Intensity ERror estimation (PLIER) algorithm. Differential gene expression was analyzed using consecutive sampling with bin size of 25 (Novak et al. 2002; Guilbault et al. 2006; Novak et al. 2006a; Novak et al. 2006b). Representative standard deviations in each bin were calculated using non-linear regression to determine the boundaries of probability intervals. Candidate genes were selected as genes that lay beyond the probability interval of 0.9. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO series accession number GSE34193.

RT-PCR

RT-PCR was performed on isolated anterior endoderm explants from *ptf1a* expressing and non-expressing embryos and normalized to EF1-α. PCR conditions using *Taq* DNA polymerase (Invitrogen) included 1 min at 94°C preincubation, followed by 26, 28 or 30 amplification cycles (except Ef1-α which only had 20, 22 or 24 cycles) comprising 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and one cycle at 72°C for 10 min. The following primers were used:

Stxbp1 Fwd 5'-TCTGCATCGGGTATCCGTTTCCTT-3' and

Stxbp1 Rev 5'-ATAAAGGTCGGGAGTGGTCGTTGT-3'

TA-2 Fwd 5'-ACAGTTATACCCAGCACAGCCCTT-3' and

TA-2 Rev 5'-ACTGAACCACAGAGCTGACACAGT-3'

Crbp2 Fwd 5'-CCGTGGGCGTTGAATTCGATGAAA-3' and

Crbp2 Fwd 5'-TTCCAGCCACGGTTGTTCTTCTCT-3'

C25H Fwd 5'-ACCTTTGCACTGGCCACTCAGTAT-3' and

C25H Fwd 5'-AAATGGTACCAGCTTGTGGGAGGA-3'

Igfbp1 Fwd 5'-TTACGGCCTAGTGAAGCAGCAGAT-3' and

Igfbp1 Rev 5'-TTCTGCGCTCAAGCATCTTTCGTG-3'

Irf1 Fwd 5'- GCACAGGCCCTTAATGTGATCCAA-3' and

Irf1 Rev 5'- GCAAACGAGTGGCAAACTGGTC-3'

Hapln3 Fwd 5'- GATGGCTGGTTGAGACTCATGTCT-3' and

Hapln3 Rev 5'- AGAAAGCTCACTCACAAGGGACG-3'

Hey1 Fwd 5'- CAGTAAAGCTGTGCATGGAAGGG-3' and

Hey1 Rev 5'- GTTGTATAGTCCGGGTTCATGTGC-3'

Sestrin1 Fwd 5'- CCTCCCCCGGATATAGATGT-3' and

Sestrin1 Rev 5'- CCTGCACTCGAAAAGTAGGC-3'

Syndecan-4 Fwd 5'- GCTAAGCCCAAAACATTGGA-3' and

Syndecan-4 Rev 5'- AAGGAAACTGCAACGACCAG-3'

Probes and Whole Mount *In Situ* Hybridizations

All genes were cloned using cDNA from wild-type NF35 embryos with the following primers:

Hapln3 Fwd 5'-GACCCAATTGTTTTGGGGCTACCT-3' and Hapln3 Rev 5'-GGGAGCGCAGTGAGTGCAGG-3' designed based on BC046259. Hey1 Fwd 5'-TCCTCCGCTGCTCTCCTCCA-3' and Hey1 Rev 5'-GGGTGATGTCGCACCCAAGCA-3' designed based on BC084410. Igfbp1 Fwd 5'-CTGGATCCCCTGAAAACAGA-3' and Igfbp1 Rev 5'-GGACCTGGGATTTTCTGGAT-3' designed based on BC060008. Irf1 Fwd 5'-TGCCATTGCCTGACAGCACA-3' and Irf1 Rev 5'-GGGCCTGTGCAAACCGATGC-3' designed based on BC059984. Syndecan-4 Fwd 5'-TCCTGCTGCTTTTAGCGCTGGTT-3' and Syndecan-4 Rev 5'-ACCAGGCCAACCAACGTGCC-3' designed based on DQ116029. Syntaxin 1B Fwd 5'-CAGAGCATCTCTGAGCATCT-3' and Syntaxin 1B Rev 5'-GATTCTTTCAGAGTCCCAGG-3' designed based on BC084156. Syntaxin binding protein 1 ordered from XDB (http://xenopus.nibb.ac.jp/) contig number xl103a06. PCR products were cloned into pCRII (Invitrogen) and confirmed by sequencing. Whole

mount *in situ* hybridizations were performed as described using BM purple (Horb et al. 2003).

RESULTS

Microarray Analysis Endodermal Tissue Overexpressing Ptf1a

We previously showed that overexpression of Pf1a was sufficient to convert stomach and duodenum to pancreas (Jarikji et al. 2007). On the basis of this phenotype, we sought to identify downstream targets of Ptf1a by isolating endodermal tissue overexpressing Ptf1a at two early time-points and comparing the gene expression changes using microarrays. The first microarray was performed at NF32, which is shortly after endogenous Ptf1a expression begins (named MA32); the second microarray was performed at NF36 (MA36), which is 8 hours after the first time-point.

We injected *ptf1a* and *gfp* mRNAs together or *gfp* mRNA alone into the two dorso-vegetal blastomeres of eight-cell embryos, targeting the anterior endoderm. Forty (NF32) to forty-eight (NF36) hours later the anterior endoderm was dissected out and total RNA isolated. Fifteen endoderm explants were pooled for each RNA preparation, and both control and experimental samples were collected from the same clutch of embryos. This was done in triplicate at NF32 and in quadruplicate at NF36, each replicate coming from sibling embryos (Fig. 1a). The NF36 microarray was performed first using the Affymetrix 3' Xenopus laevis Genome 1.0 GeneChip, and the NF32 microarray second using the Affymetrix 3' Xenopus laevis Genome 2.0 GeneChip (the NF36 microarray was performed prior to the release of the 2.0 GeneChip). Primary analysis was done using the Affymetrix algorithm PLIER and globally normalized to 100% of the mean value. Multiple t-tests were used, including a Cyber-T test, a probability interval of 0.9 and 0.95 was set and only genes with a minimal fold change of 2.0 were identified as candidate

genes. The analysis yielded 296 probe sets upregulated at NF32 and 846 probe sets upregulated at NF36.

In agreement with our previous data that showed Ptf1a was sufficient to promote ectopic acinar cell development (Jarikji et al. 2007), we found upregulated expression of numerous exocrine genes in endodermal tissue overexpressing Ptf1a at both time-points. Carboxypeptidase A (CPA) was upregulated 4-fold in both MA32 and MA36 (Table 1) and Carboxypeptidase B1 (CPB1) was upregulated 6-fold in MA36 (Table 3). Endocrine genes were also found upregulated, including Pax6, NeuroD, Carboxypeptidase E (CPE) and Somatostatin. Pax6 and CPE were found upregulated 2.7-fold and 2.46-fold in MA32 (Table 2). NeuroD and somatostatin were found upregulated 5-fold and 4.33-fold in MA36 (Fig. 1b,c, Table 3). In conjunction with our previous results that showed loss of stomach and duodenal tissue, we found down-regulated expression of several stomach and duodenal genes, such as villin-1 and intestinal fatty acid binding protein (IFABP) in endoderm overexpressing Ptf1a (data not shown, see GEO data GSE34193). In summary, we found the changes in gene expression identified in the microarrays were consistent with our earlier data, suggesting genes identified in the microarray are involved in promoting the ectopic pancreas phenotype.

Identification of Temporally Regulated Genes

To compare differential gene up-regulation in the two different time-points, we separated the genes based on whether they were upregulated in both MA32 and MA36 or only in

one time-point. Using MA32 as the base we identified 297 genes to be upregulated greater than 2-fold in both MA32 and MA36 (Table 1). At NF32, we identified 143 genes to be upregulated only at this stage, while at NF36 there were 702 upregulated genes (Tables 2 & 3). Because of the extended amount of time between onset of endogenous Ptf1a expression and sample collection, we believe those genes upregulated at the later stage represent secondary or tertiary targets of Ptf1a and not direct targets.

To provide insight into the different genes regulating pancreas specification downstream of Ptf1a at NF32 versus NF36, we used DAVID (Database for Annotation, Visualization and Integrated Discovery) to map the upregulated genes from both microarrays to enriched GO Biological Process categories (Huang et al. 2009a; Huang et al. 2009b). Gene annotation enrichment analysis revealed the top three functions at MA32 were regulation of transcription, regulation of RNA metabolic process and biological adhesion. At this stage there appears to be enrichment of genes that may function in cell fate specification and in cell biological processes, such as adhesion for tissue patterning. In comparison, at MA36 the top three functions were ion transport, nitrogen compound biosynthetic process and purine nucleotide metabolic process. As expected, genes at this later stage appear to be enriched in functions indicative of differentiated cells in comparison to naïve cells undergoing specification.

Temporally regulated genes were also mapped using SwissProt to determine enriched functions. The top 3 enhanced keywords at MA32 were developmental proteins, calcium

and intermediate filaments. At MA36, the top 3 enhanced functional similarities were transmembrane related proteins, calcium and intermediate filaments. The enrichment of developmental proteins at MA32 suggested Ptf1a was regulating the expression of genes required to define the pancreatic fate at this early stage. At MA36, the most enriched protein family was transmembrane related proteins, which included many proteins with functions in signal transduction pathways (e.g., guanylyl cyclase 1, EPH receptor B1, Syndecan-4). The difference between the enriched protein families across the time-points correlates with our analysis that upregulated genes at NF32 are functioning in cell fate specification, while at NF36 the cells are more differentiated and these putative secondary or tertiary Ptf1a target genes are involved in maintaining proper cell function.

Analysis of the 1142 probe sets upregulated in both microarrays showed the most enriched GO Biological Process category was transcriptional regulation. Of the 45 transcription factors that were upregulated, we identified known pancreatic transcription factors that function downstream of Ptf1a (NeuroD1, Pax6, Sox9), which have been shown to be expressed in the pancreas at these two stages. We also identified many genes with known functions in pancreas development that have yet to be identified as Ptf1a targets (JunB, IRF1, Hey1). However, the majority of upregulated genes have unknown functions in pancreas development (Sox3, Hairy2, FoxC1), though related family members have been shown to play a role in pancreas development. The identification of both known and unknown pancreatic genes suggested that these new transcription factors might be part of the initial Ptf1a gene regulatory network. However, further functional

analysis is required to validate and understand the roles of these potential pancreatic transcription factors.

Genes Upregulated in Ptf1a Microarray are Expressed During Early Pancreas Specification

To validate genes involved in early pancreatic specification from the upregulated probe sets, we chose to examine the spatial and temporal expression of 94 genes by whole-mount *in situ* hybridization (ISH). We confirmed 50 of these genes to be expressed in anterior endoderm, but we focused our attention on the 32 that showed specific expression within the mature pancreas (between NF40 and NF46). Of the 32 genes expressed in the mature pancreas, 9 genes showed expression within the anterior endoderm at the level of the developing pancreas between NF32-36. The 9 endodermally expressed genes were: syntaxin binding protein 1 (Stxbp1), putative transmembrane protein TA-2, cholesterol 25-hydroxylase (C25H), Insulin-like growth factor binding protein 1 (Igfbp1), interferon regulatory factor 1 (Irf1), hyaluronan and proteoglycan link protein 3 (Hapln3), hairy/enhancer-of-split related with YRPW motif 1 (Hey1), sestrin1, syndecan-4.

According to the microarray analysis six of the genes, TA-2, C25H, Irf1, Hapln3, Hey1 and Syndecan-4, were found significantly upregulated at both NF32 and NF36; Igfbp1 and Sestrin1 were only upregulated at NF36, whereas Stxbp1 was only upregulated at NF32. Consistent with the microarray data, we confirmed relative expression for seven of

these genes by RT-PCR (Fig. 2). However, we found expression of C25H and TA-2 to be reduced in the Ptf1a samples at MA36 (Fig. 2). As a control, we examined expression changes for one of the genes found decreased in both MA32 and MA36, cellular retinol binding protein 2 (Crbp2). In agreement with the microarray data, we found it to be decreased in Ptf1a overexpressing endoderm (Fig. 2). These results suggested that genes identified in the analysis were targets of Ptf1a, and based on the confirmation of the RT-PCR and their known functions and putative roles in pancreas development, we examined seven genes in more detail, Hey1, Irf1, Stxbp1, Syntaxin 1B, Igfbp1, Hapln3 and Syndecan-4.

Hey1

Hey1 is a bHLH transcriptional repressor protein that was shown to interact with Ptf1a and is expressed in both endocrine and exocrine cells of the human pancreas (Ghosh and Leach 2006; Johansson et al. 2008). In *Xenopus* embryos, we first detected *hey1* expression at NF32 along the periphery of the endoderm (Fig. 3a,b). At later stages expression became restricted to the stomach and pancreas (Fig. 3c). To confirm the RT-PCR data we compared the spatial expression of *hey1* in control and *ptf1a*-injected embryos. At NF32, expression of *hey1* in *ptf1a*-injected embryos was stronger particularly in the ventral and dorsal region of the endoderm where the pancreatic buds will arise (Fig. 6a,b). At NF36, expression of *hey1* remained much stronger in the endoderm in comparison to the controls (Fig. 6c,d). Expression of *hey1* in the developing endoderm and up-regulation in the region of the prospective pancreatic buds suggests it is

a downstream target of Ptf1a *in vivo* and likely plays a role in pancreatic cell specification.

Irf1

Irf1 was originally identified as a regulator of interferon (IFN)-β and is a key factor in the transcriptional regulation of the IFN response, but it has also been studied for its role in cell growth regulation (Fujita et al. 1988; Kröger et al. 2002). In the domain of the developing pancreas in *Xenopus* at NF32, *irf1* was expressed along the ventrolateral endoderm; this expression expanded laterally by NF36 (Fig. 3d,e). Within the mature gut, the expression was dispersed throughout the duodenum and liver (Fig. 3f). The developing anterior endoderm not only gives rise to the pancreas, but also the liver, stomach and intestine. Since overexpression of Ptf1a is sufficient to convert stomach and duodenum to pancreas it is possible that Ptf1a may activate genes, such as Irf1, that inhibit proper stomach and duodenum development.

Stxbp1

In our microarray, we also identified several proteins involved in vesicle fusion. As examples, we focused our analysis on Stxbp1 and Syntaxin 1B, as they were both upregulated over two-fold. Stxbp1 was originally identified for it role in exocytosis through yeast genetics as Sec1 (Ferro-Novick and Jahn 1994). Homologs have been identified in many species from *Caenorhabditis elegans* (UNC-18) to mammals (Munc-18) (Hosono et al. 1992; Hata et al. 1993). Stxbp1 is known to function in neuronal cells

as well as in pancreatic islets and insulin-producing cells (Jacobsson et al. 1994). In *Xenopus* embryos, *stxbp1* was expressed within the dorsal mesendoderm border at NF32 (Fig. 4a), by NF36 it was expressed in a punctate fashion within the dorsal endoderm at the level of the developing pancreatic bud (Fig. 4b). In the mature gut, *stxbp1* expression was punctate throughout the stomach, duodenum and pancreas (Fig. 4c,d). These results indicate that *stxbp1* was expressed in a punctate pattern in the developing dorsal pancreatic bud, similar to the expression of *insulin* in the dorsal anlagen prior to pancreas morphogenesis.

Syntaxin 1B

Syntaxin 1B is a member of the t-SNARE family, these proteins are located in the plasma membrane and play a fundamental role in vesicle docking and fusion (Ferro-Novick and Jahn 1994). Stxbp1 can directly bind Syntaxin, this interaction has been shown to result in negative regulation of the insulin secretory machinery in insulin-secreting HIT-T15 cells (Hata et al. 1993; Zhang et al. 2000). In *Xenopus* embryos *syntaxin 1B* was not expressed within the developing pancreatic buds (Fig. 4e,f), but it was uniformly expressed through the mature stomach, liver and pancreas (Fig. 4g,h). At both NF32 and 36 in *ptf1a*-injected embryos, *syntaxin 1B* expression was ectopically expressed in the dorsal and ventral endoderm (Fig. 6e-h). At this early stage *insulin* mRNA is only beginning to be expressed, and it is unclear what role these genes, which are implicated in insulin-release, might play at such an early stage in development.

Igfbp1

Insulin-like growth factor-binding proteins (IGFBPs) are secreted proteins that bind to Insulin-like growth factors (IGFs) and sequester them from binding to the IGF receptor, which plays a crucial role developmental growth and metabolism (Hwa et al. 1999; Duan 2002). In confirmation of the microarray, we found *igfbp1* to be expressed within the developing endoderm at all stages examined. At NF32 it was expressed in the ventral endoderm and dorsolateral mesoderm (Fig. 5a). Eight hours later at NF36 expression within the endoderm increased, and was now found to extend into the dorsal endoderm (Fig. 5b). In isolated guts at NF45, expression was strongest in the liver, but was also found diffuse throughout the posterior part of the gut (Fig. 5c). In *ptf1a* overexpressed embryos expression was expanded in the ventral endoderm at NF32 (Fig. 6m,n). Similarly at NF36 *igfbp1* expression was stronger in the ventral endoderm in comparison to the control and the punctate pattern was more abundant (Fig. 6o,p). Concentrated *igfbp1* expression in the ventral endoderm was amplified in *ptf1a*-injected embryos, and based on these observations we consider that it may play a role specific for only ventral pancreas development.

Hapln3

Hyaluronan and proteoglycan-binding link protein 3 (Hapln3) is a member of the link-protein family HAPLN, in *Xenopus* Hapln3 was identified as an essential extracellular matrix protein that stabilizes hyaluronan matrix formation during cardiogenesis (Ito et al. 2008). Our analysis of *hapln3* expression showed it to be highly expressed within the most ventral endoderm at NF32 (Fig. 5d). At later stages, *hapln3* expression was barely

detectable in the endoderm (Fig. 5e,f). In embryos overexpressing *ptf1a* mRNA, *hapln3* was expressed in broader and stronger pattern in the ventral endoderm at NF32 (Fig. 6i,j). At NF36, ectopic expression of *hapln3* was observed in the ventral endoderm in *ptf1a*-injected embryos (Fig. 6k,l). These results indicate that *hapln3* is expressed in the ventral endoderm prior to morphogenesis of the pancreatic buds, and this ventral expression was upregulated in embryos overexpressing *ptf1a* mRNA in the developing anterior endoderm.

Syndecan-4

Lastly, we focused on Syndecan-4, which as a member of the Syndecan family is a heparan sulfate bearing transmembrane protein involved in cell adhesion and linkage to the cytoskeleton (Whiteford et al. 2008). At NF32 faint *syndecan-4* expression was detected in the ventral endoderm (Fig. 5g). By NF36, expression within the most ventral portion of the endoderm was increased substantially (Fig. 5h). In NF44 guts *syndecan-4* was most strongly expressed within the stomach, but expression was also detected in the liver (Fig. 5i). These observations indicate that *syndecan-4* is expressed in the ventral endoderm during stages when the ventral pancreatic cells are being specified.

DISCUSSION

Numerous studies have addressed the transcription factor hierarchy involved in pancreas development; none have examined in detail the Ptf1a transcriptional network. Ptf1a is a particularly interesting gene in this cascade, as it is one of the earliest transcription factors to have restricted expression within the developing pancreatic buds. Ptf1a plays two roles, early in initial pancreas specification and later in maintenance of mature pancreatic exocrine cells. While a greater understanding of its function in exocrine cell maintenance has been achieved, much less is known about the genes activated downstream of Ptf1a during initial stages of pancreas development. In this article, we have used mRNA overexpression and microarray analysis to identify early downstream targets of Ptf1a that may play a role in initial specification of the pancreas. From this study, we identified numerous genes that were not previous known to function in pancreas development.

Through microanalysis of Ptf1a overexpression over two time-points we have identified over 800 genes that are temporally regulated within the anterior endoderm during the initial stages of pancreas development, and have validated a subset of these which show expression within the anterior endoderm. Many of these have no prior known function in pancreas development. A total of 45 transcription factors were identified in our analysis, and while the majority of these have no known role in pancreas development, several established regulators of pancreas development were observed (NeuroD1, Atf3, Sox9). This suggests the other upregulated transcription factors in our data sets are likely to also play a role in pancreas development.

Using gene annotation enrichment analysis we have identified differences between the two time-points. At NF32 the most enhanced gene sets are involved in transcriptional regulation, biological adhesion and have known functions during development. This suggests that Ptf1a directly activates genes involved specification of the pancreatic cells and developing tissue. Genes upregulated at NF36 are likely secondary or tertiary targets of Ptf1a, and at this stage the pancreas has formed into buds that will soon fuse to form a discrete organ. From this data set, we identified many genes enriched in transmembrane function and ion transport.

Although Ptf1a has been shown to be essential for development of a subset of endocrine cells, little is know about how it specifies these cells. To begin to define the Ptf1a endocrine regulatory circuit we sought to identify overlap between the Ptf1a and endocrine Ngn3 regulatory network, which we recently published (Oropez and Horb 2012). This analysis identified a list of 14 genes potentially involved in endocrine cell development (data not shown). Five of the identified genes have previous known pancreatic function (CPE, Hey1, Hes5, Ptgs2, NeuroD4). The identification of all these genes in both the Ngn3 and Ptf1a microarrays strongly suggest they function downstream of these key transcription factors in early pancreas specification, but exactly what role they might play in early pancreas specification downstream of Ptf1a is not known. However, this overlap provides a starting point for further evaluation of these genes to define the overlap between the Ptf1a and Ngn3 pathways.

Endodermally Expressed Candidate Genes

The specification of pancreatic cell lineages involves sequential activation of several classes of transcription factors, in this study we identified two transcription factors expressed within the developing endoderm and upregulated by Ptf1a: Irf1 and Hey-1. Irf1 has been studied for its involvement in immune response, including islet inflammation, but has other known roles in tumor suppression, apoptosis and cell growth regulation (Fujita et al. 1988; Kröger et al. 2002). *Irf1* was upregulated 3-fold at NF32 and 7-fold at NF36, and given its previous known functions it is possible the role of Irf1 downstream of Ptf1a may be to inhibit growth of naïve duodenal cells into a mature organ.

Early in pancreas development the Notch signaling pathway regulates cell fate differentiation. Ultimately it activates target genes, including the Hairy Enhancer of Split (HES) family of bHLH transcriptional repressors, which maintain cells in an undifferentiated state. Later in development Notch has been shown to delay the onset of the exocrine lineage; effector proteins of Notch, including Hes1, Hey1 and Hey2 are capable of interacting with Ptf1a and preventing the PTF1 complex from binding to target DNA (Esni et al. 2004; Ghosh and Leach 2006). It is likely that Hey1 is interacting with Ptf1a to maintain pancreas progenitor cells in an undifferentiated state leading to an increase in cell growth and pancreatic cells.

We also identified genes with known functions in mature pancreatic cells, such as Stxbp1 and Igfbp1, but their role in pancreas development has not yet been studied. In mice there are three Munc18 (the mammalian stxbp1 homolog) isoforms: Munc18a, Munc18b and Munc18c, all of which are expressed in islet β-cells and function in insulin release (Spurlin et al. 2004; Tomas et al. 2008; Oh and Thurmond 2009). These cytosolic proteins are also capable of binding to Syntaxin with high affinity, and this interaction is known to have both inhibitory and positive roles in vesicle transport (Lehtonen et al. 1999). Not only is binding partner Syntaxin 1B upregulated in the microarrays, but so are many other components of the SNARE complex, such as SNAP-25, Synaptotagmin-like 1, Synaptotagmin 4, Synaptophysin A and B and VAMP-2. At this early stage in pancreas development at which our microarrays are performed insulin is not yet being released, suggesting that Stxbp1 and components of the SNARE complex may have other functions early during pancreas development, such as generation of polarization of specific cells leading to a differentiated cell fate (Lehtonen et al. 1999).

Igfbp1 has been shown to function independently of IGF, it can inhibit the mitogenic activity of epidermal growth factor (EGF) when bound to certain integrins and it also enhances migration of smooth muscle cells, extravillous trophoblast cells and Chinese hamster ovary cells (Jones et al. 1993; Gockerman et al. 1995; Chakraborty et al. 2002; Cavaillé et al. 2006). Recently Igfbp1 has been shown to activate kinases and small monomeric GTPases to affect cell migratory behavior of oligodendrocytes (Chesik et al., 2010). It may be possible that Igfbp1 has IGF-independent effects during pancreas development, involving the migration of precursor cells into defined anlagen.

Finally, we identified two genes with novel expression patterns within the early pancreatic endoderm, Hapln3 and Syndecan-4. Hapln3 is an extracellular matrix protein that stabilizes hyaluronan matrix formation. Hyaluronan is shown to play roles cell-cell adhesion, migration, proliferation and differentiation (Comper and Laurent 1978; Shimabukuro et al. 2005). Recently, Hapln3 has been shown to be necessary for cardiogenesis through its role in hyaluronan matrix formation around the developing heart in *X. laevis* (Ito et al. 2008). Has2, the catalytic enzyme which synthesizes hyaluronan, was shown to be expressed in the same cardiac region as Hapln3 (Ito et al. 2008), but was also increased in procine neonatal pancreas cells stimulated with EGF, where it was suggested it may play a role in the proliferation and migration of pancreatic duct cells (Jeon et al. 2004). It is possible that Hapln3 up-regulation in Ptf1a injected embryos may aid in hyaluronan matrix formation of the ectopic hyaluronan synthesized by Has2 leading to proliferation of Ptf1a cells resulting in ectopic pancreas.

Our results are the first to show expression of *hapln3* and *syndecan-4* at such an early stage in the developing anterior ventral endoderm. To our knowledge, this is also the first time that *hey1, syntaxin 1B, hapln3 and igfbp1* have been shown to be ectopically expressed in the developing endoderm in response to *ptf1a* mRNA overexpression *in vivo.* The fact that we found all of these genes to be upregulated only in the ventral endoderm at the level of the developing ventral pancreatic buds suggests that Ptf1a

function is more pronounced in this region of the endoderm than in more central, dorsal or posterior areas.

While all these genes were identified as upregulated in the early endoderm in response to Ptf1a overexpression, many do not have restricted expression within the pancreas and have been previously identified in other cell types. This is not uncommon; several key pancreatic genes are expressed in multiple tissues, such as Pdx1 which is also expressed in the duodenum, or Ngn3, NeuroD and Ptf1a, which are also expressed in neural tissue (Miller et al. 1994; Lee et al. 1995; Sommer et al. 1996; Sellick et al. 2004). The ability of these genes to be involved in cell fate specification in multiple tissues rely on networks of other tissue-specific genes, it is possible that the genes we have identified in our microarray may function in pancreatic specification when expressed with their pancreatic-specific cofactors.

In conclusion, we are the first to report results from a microarray analysis of Ptf1a. We have found that Ptf1a modulates the expression of genes with a range of functions, from transcription to cell adhesion. Ptf1a is a key transcription factor necessary for pancreas development, and genes identified through this microarray are likely to play critical roles downstream of Ptf1a in defining cells along a particular pancreatic lineage.

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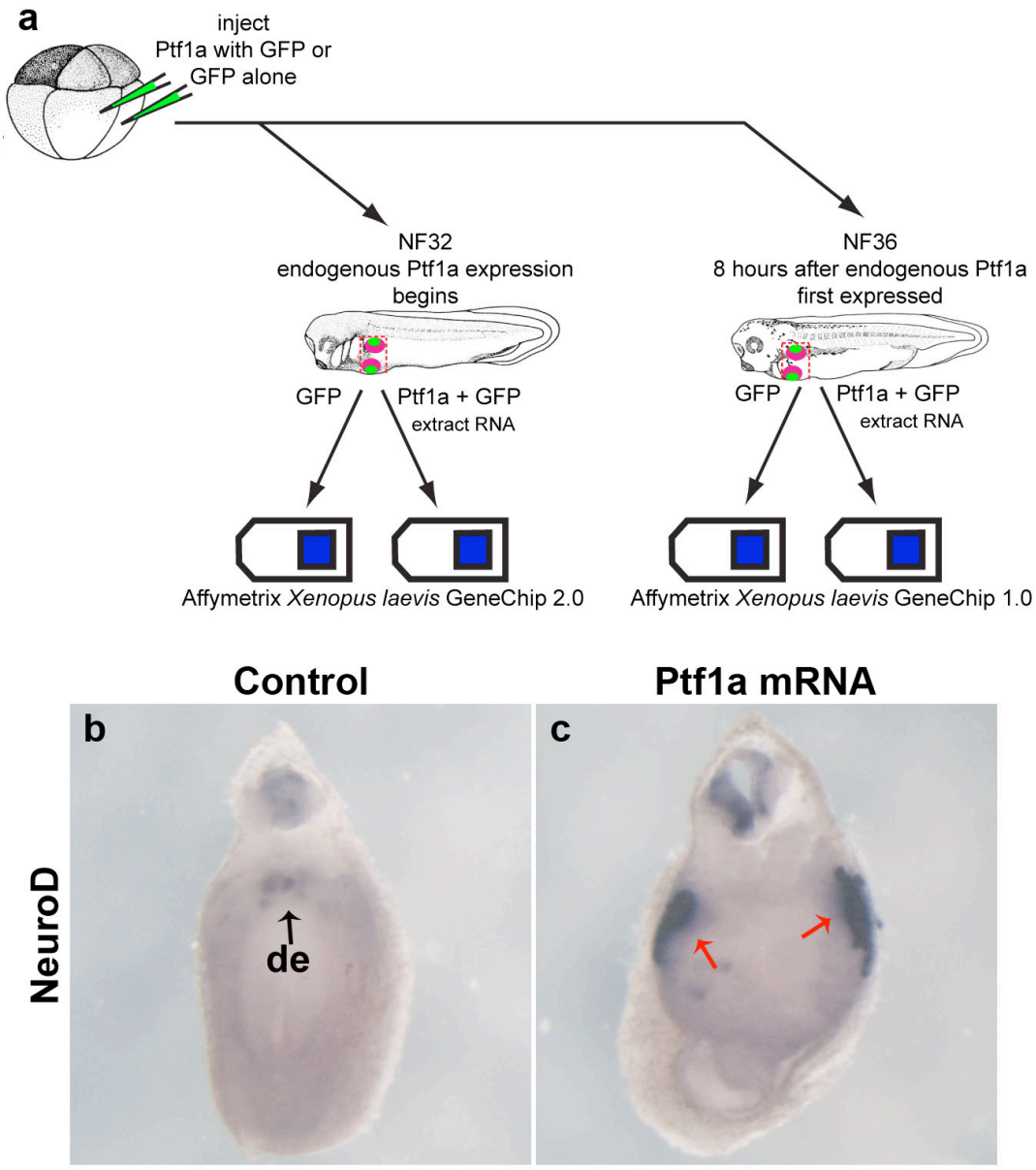

Figure 1. Microarray experiment schematic and ISH verification. A: Schematic diagram of microarray experiment. Eight-cell stage embryos were injected with either 800pg of Ptf1a mRNA or 400pg of GFP mRNA. Samples were collected at NF32 and NF36; RNA was extracted and hybridized to Affymetrix Xenopus laevis GeneChip 2.0 and Affymetrix Xenopus laevis GeneChip 1.0, respectively. **B**: NeuroD expression in gfp-injected control embryos at NF32, punctate expression in the dorsal endoderm (black arrow). **C**: NeuroD expression in Ptf1a-injected embryos at NF32, increased expression in the dorsal endoderm (red arrows). de, dorsal endoderm.

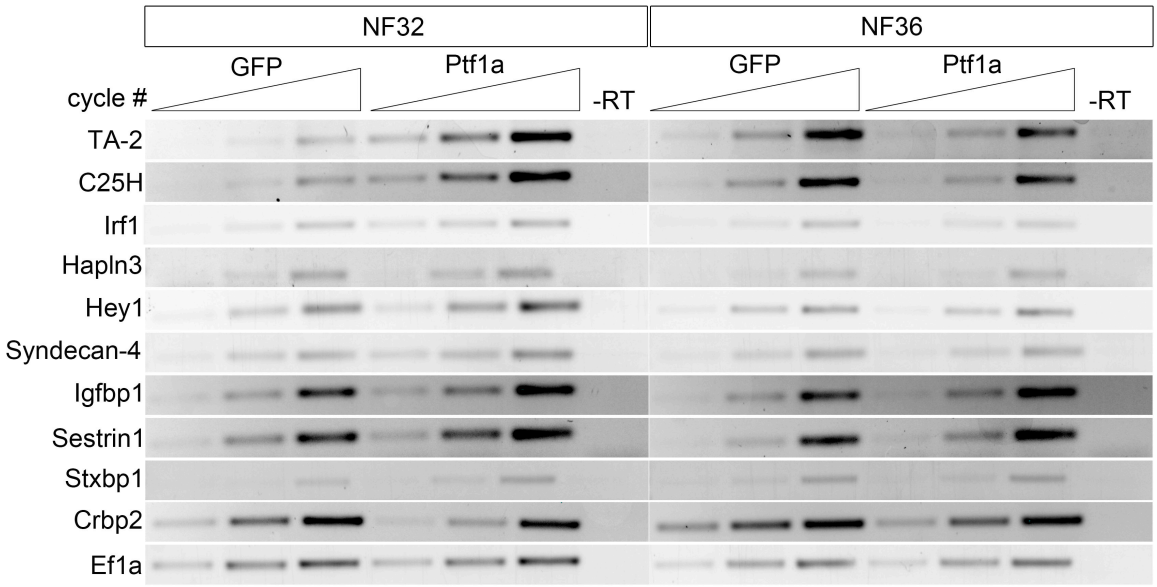

Figure 2. RT-PCR verification of selected differentially expressed genes from the microarray analyses. GFP and Ptf1a sample data provided from three different cycle time-points. GFP, *gfp* mRNA-injected control; Ptf1a, *ptf1a* mRNA-injected; -RT, control reaction without reverse transcriptase.

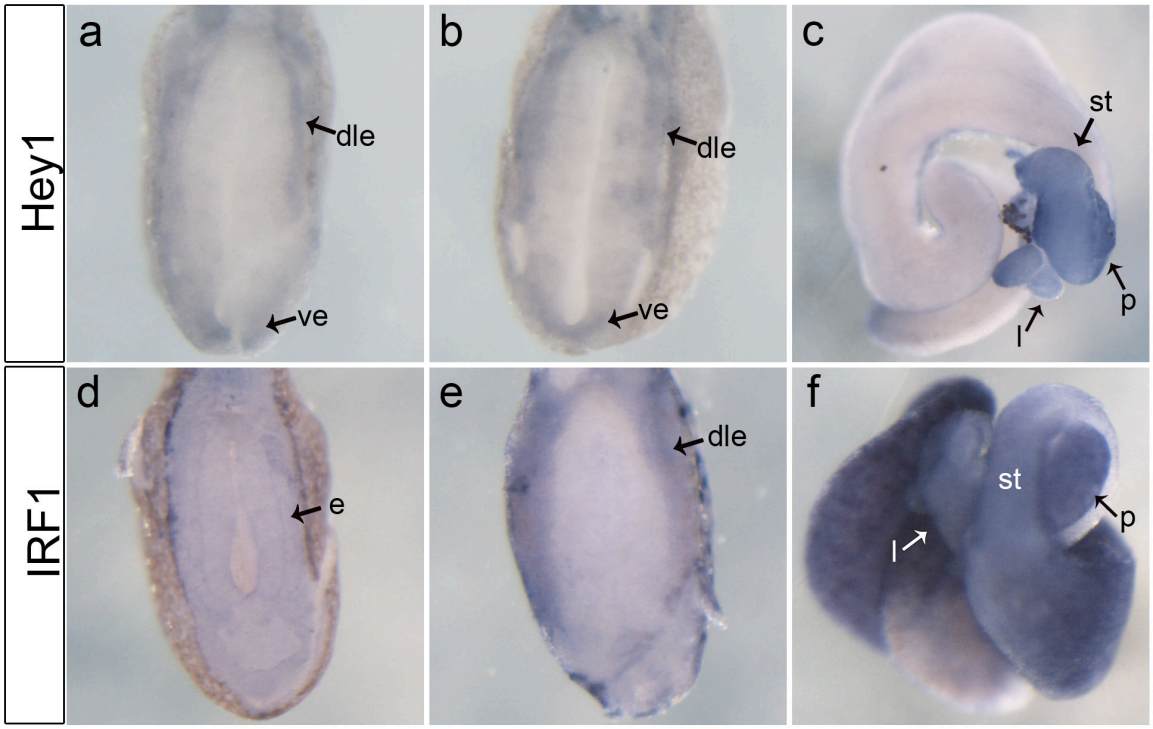

Figure 3. Expression pattern of transcription factors Hey1 and Irf1 in the developing endoderm. **A:** Transverse section through anterior endoderm at NF32 showing expression of *hey1* restricted to the outer periphery of the endoderm. **B:** Expression of *hey1* is more diffuse within the endoderm of the NF36 embryo, transverse section. **C:** NF44 whole gut showing expression of *hey1* throughout the stomach, liver and pancreas. **D:** Transverse section through anterior endoderm at NF32 showing expression of *irf1* throughout the entire endoderm. **E:** Transverse section at NF36 shows restricted expression of *irf1* along the dorsolateral endoderm. **F:** NF44 whole gut showing expression of *irf1* throughout the stomach, liver, pancreas and in the intestine. dle, dorsolateral endoderm; ve, ventral endoderm; e, endoderm; l, liver; st, stomach; p, pancreas.

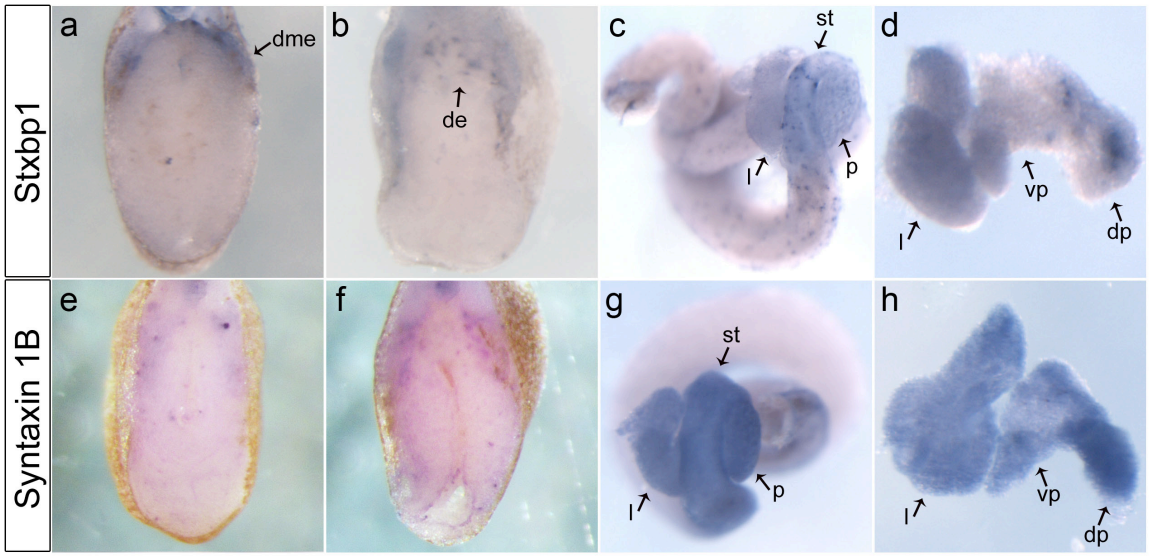

Figure 4. Expression of SNARE complex proteins in the developing endoderm. A: *Stxbp1* expressed along the dorsal mesendoderm periphery in the transverse section at NF32. **B:** Transverse section at NF36 shows punctate expression of *stxbp1* restricted to the dorsal endoderm. **C:** Whole gut at NF44, *stxbp1* has a punctate expression throughout the gut. **D:** NF42 liver and pancreas showing *stxbp1* punctate expression in the dorsal pancreas and more diffuse expression in the liver. **E:** *syntaxin 1B* has minimal punctate expression within the endoderm at NF32. **F:** At NF36 there is an increase in the punctate expression of *syntaxin 1B* in the endoderm, transverse section. **G:** NF44 whole gut showing expression of *syntaxin 1B* in the stomach, liver and duodenum. **H:** NF42 liver and pancreas with expression throughout the liver and pancreas. dme, dorsal mesendoderm; de, dorsal endoderm; l, liver; st, stomach; p, pancreas; dp, dorsal pancreas; vp, ventral pancreas.

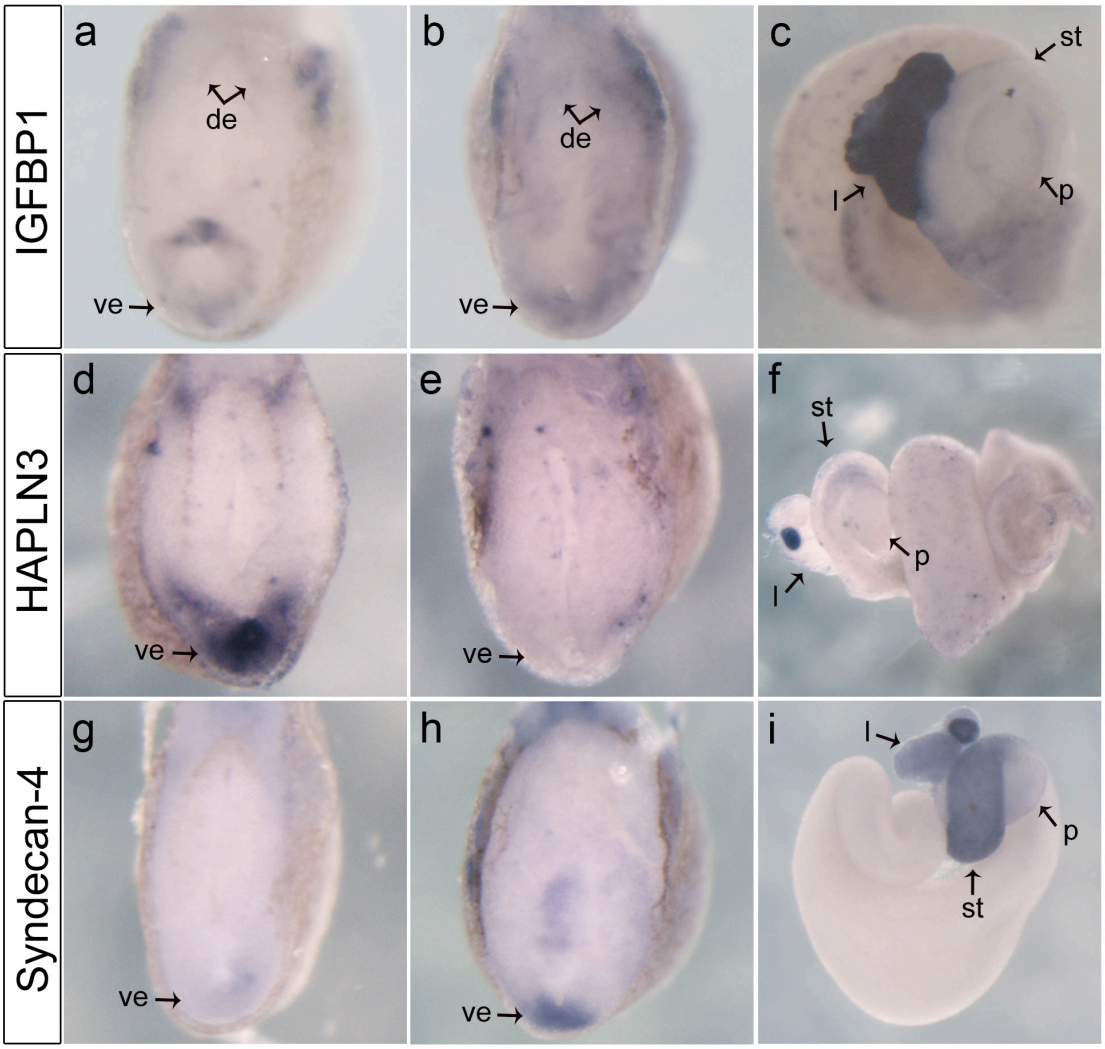

Figure 5. Expression of cell shape/adhesion genes in the developing endoderm. A: Transverse section through anterior endoderm at NF32 showing expression of *igfbp1* restricted to the dorsal and ventral endoderm. **B:** At NF36 *igfbp1* is expressed most highly in the dorsal and ventral endoderm, but diffusely throughout the rest of the endoderm, transverse section. **C:** NF45 whole gut showing *igfbp1* strongly expressed in the liver, and lighter in the duodenum. **D:** *Hapln3* is expressed in the ventral endoderm in the transverse section at NF32. **E:** Transverse section at NF36, *hapln3* expression is lost in the ventral endoderm, but is punctate through the endoderm. **F:** NF45 whole gut showing strong expression of *hapln3* in the gall bladder, and punctate expression through the pancreas and duodenum **G:** *Syndecan-4* is only expressed in the most ventral region of the endoderm shown in the transverse section of NF32. **H:** Transverse section at NF36 is showing expression of *syndecan-4* only in the most ventral part of the endoderm, but stronger than at NF32. **I:** NF44 whole gut shows expression of *syndecan-4* in the stomach and liver, and slightly in the pancreas. de, dorsal endoderm; ve, ventral endoderm; l, liver; st, stomach; p, pancreas.

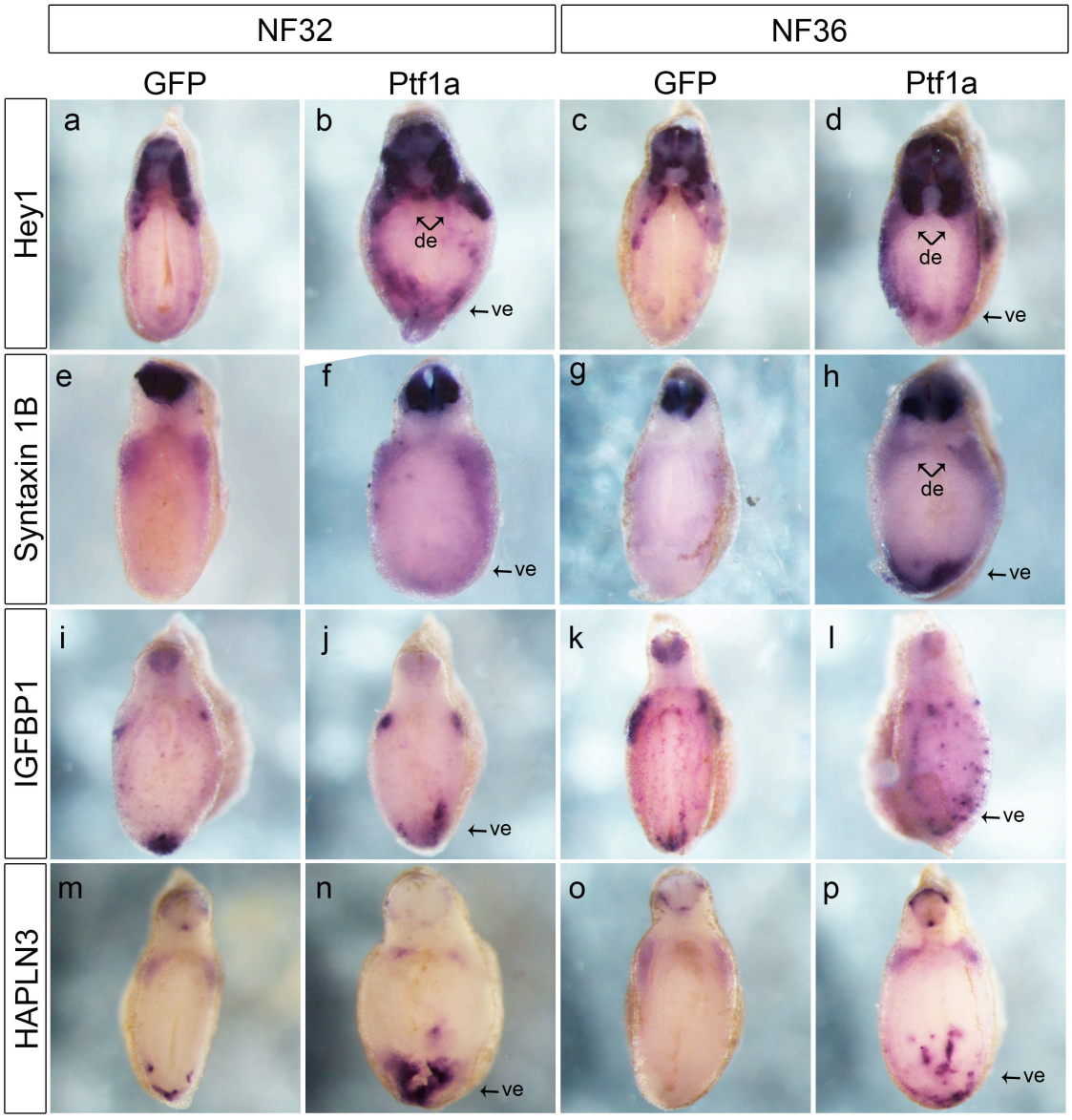

Figure 6. Ptf1a overexpression promotes ectopic expression of candidate genes in the developing endoderm. All images are transverse section through the anterior endoderm. **A,B:** Stronger expression and expanded domain of *hey1* in the anterior endoderm of a *ptf1a* mRNA injected embryo at NF32 (7/10). **C,D:** At NF36, expression of *hey1* is stronger along the periphery of the endoderm (8/11). **E,F:** At NF32, *syntaxin 1B* expression is stronger in the dorsal endoderm and expands ectopically towards the ventral endoderm (7/10). **G,H:** Expression of *syntaxin 1B* is much stronger in the dorsal and ventral endoderm of *ptf1a*-injected embryos at NF36 (6/10). **I,J:** *igfbp1* expression is expanded in the ventral endoderm but unchanged in the dorsal endoderm of *ptf1a*-injected embryos at NF32 (9/9). **K,L:** At NF36 *ptf1a*-injected embryos the punctate expression of *igfbp1* is stronger and more abundant (8/11). **M,N:** Expression domain of

hapln3 in the ventral endoderm is stronger and broader in *ptf1a*-injected embryos at NF32 (6/10). **O.P:** At NF36, expression of *hapln3* is ectopically expressed in the ventral endoderm but unaffected in the dorsal endoderm (5/10). de, dorsal endoderm; ve, ventral endoderm.

Table 1. Genes upregulated Ptf1a microarray at NF32 and NF36.
Genes found upregulated in both MA32 and MA36 relative to control samples. Genes are listed in descending order based on relative values in MA32. Unigene ID numbers that did not represent a known gene and duplicated genes were removed from this table. $p < 0.05$.

		Fold Change	
Gene Title	**Unigene ID**	**MA32**	**MA36**
Matrix metalloproteinase 1	Xl.26520	5.98	10.39
Pancreas-specific transcription factor 1a	Xl.29862	5.81	7.74
Immunoresponsive gene 1	Xl.53505	5.70	10.06
Interleukin-1-beta	Xl.415	5.58	9.37
Peripherin	Xl.16232	5.51	5.26
Transmembrane protein TA-2-like	Xl.736	5.50	9.44
Apolipoprotein O-like	Xl.24724	5.30	4.97
ELAV-like protein 4	Xl.1036	5.27	4.22
Soluble toll-like receptor 5	Xl.53947	5.23	8.52
Cholesterol 25-hydroxylase	Xl.18444	5.13	8.40
Matrix metalloproteinase 18	Xl.17465	4.98	9.10
Fast skeletal troponin C beta	Xl.24572	4.94	4.74
Stathmin-like 2	Xl.11	4.90	5.08
Troponin T type 3 (skeletal, fast)	Xl.3362	4.89	4.96
Dihydropyrimidinase-like 3	Xl.5161	4.76	3.03
Similar to Parvalbumin (calcium binding protein)	Xl.9653	4.41	4.13
Olfactomedin 4	Xl.17389	4.40	4.86
Tumor necrosis factor, alpha-induced protein 3	Xl.1952	4.40	6.15
Neuronal pentraxin I	XlAffx.54	4.37	5.99
Oncomodulin	Xl.48817	4.31	5.77
Synaptotagmin 4	Xl.34868	4.06	2.95
Tumor necrosis factor receptor superfamily, member 12A	Xl.34690	3.96	4.82
Carboxypeptidase A1	Xl.73839	3.96	3.85
Jun B proto-oncogene	Xl.52744	3.96	5.46
Actinin, alpha 3	Xl.76514	3.95	3.11
TNFAIP3 interacting protein 2	Xl.47119	3.84	6.47
Amphiphysin	Xl.14350	3.82	2.87
Platelet-activating factor receptor	Xl.4290	3.81	6.27
Early growth response protein 1-A	Xl.637	3.79	5.44
Internexin neuronal intermediate filament protein, alpha	Xl.13474	3.74	3.41
Syntaxin 1B	Xl.49106	3.69	3.29
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Xl.78163	3.58	6.09
Phosphoglycerate mutase 2	Xl.12227	3.55	4.81
Proto-oncogene c-Fos	Xl.10283	3.48	4.39
Interferon regulatory factor 1	Xl.1419	3.45	7.13
Suppressor of cytokine signaling 3	Xl.26512	3.45	5.73
Tumor necrosis factor receptor superfamily, member 11b	Xl.47506	3.44	4.37
Pleiotrophin	Xl.900	3.34	4.41
Early B-cell factor 2	Xl.547	3.19	4.15
Olfactomedin 1	Xl.8515	3.16	2.40

Matrix metalloproteinase 9	Xl.25126	3.13	4.96
Mab-21-like 1	Xl.29719	3.13	2.58
NUAK family, SNF1-like kinase, 2	Xl.8315	3.03	3.12
Growth arrest and DNA-damage-inducible, gamma	Xl.12125	2.98	4.01
TRAF interacting protein TANK	Xl.53193	2.98	3.47
Pleckstrin homology-like domain family A member 2	Xl.57018	2.82	2.89
Hes5.2-a	Xl.586	2.76	3.06
Thrombospondin 3	Xl.198	2.61	4.37
Keratin	Xl.76286	2.61	5.84
Hyaluronan and proteoglycan link protein 3 (Hapln3)	Xl.22996	2.52	5.35
TRAF-interacting protein with FHA domain-containing protein A	Xl.49742	2.51	3.57
TNFAIP3 interacting protein 1	Xl.15972	2.48	3.73
Sox3	Xl.22	2.48	2.59
Claudin 5	Xl.14214	2.47	4.47
Rho-related GTP-binding protein Rho6 precursor	Xl.7206	2.43	4.11
Hairy/enhancer-of-split related with YRPW motif 1 (Hey1)	Xl.469	2.43	4.68
Adenosine deaminase	Xl.80690	2.42	4.65
Tribbles homolog 1	Xl.75411	2.41	3.26
B-cell translocation gene 5	Xl.51810	2.20	3.96
Serpine1	Xl.25094	2.18	3.80
B-cell translocation protein 2	Xl.48530	2.13	3.57
FXYD domain containing ion transport regulator 6	Xl.18084	2.12	3.43
Bone morphogenetic protein 2 A	Xl.1138	2.04	3.18
ATPase, Na+/K+ transporting, alpha 2 polypeptide	Xl.10662	2.03	3.98

Table 2. Genes upregulated in Ptf1a microarray at NF32.
Genes have been sorted based on their fold change in MA32 relative to control samples. Only those with greater than 2-fold upregulation are listed. Unigene ID numbers that did not represent a known gene and duplicated genes were removed from this table. $p < 0.05$.

Gene Title	Unigene ID	Fold Change
Matrix metallopeptidase 8	Xl.49512	5.21
Fatty acid binding protein 7, brain	Xl.48742	5.00
Thrombospondin 4	Xl.9871	4.92
Tubulin, beta 3	Xl.57483	4.75
Similar to oncomodulin	Xl.3153	4.70
Fast troponin I	Xl.2142	4.62
Myosin light chain, phosphorylatable, fast skeletal muscle	Xl.6263	4.60
Oncomodulin	Xl.14726	4.48
Calcium ATPase at 60A	Xl.11405	4.46
Fast skeletal troponin C beta	Xl.1032	4.34
SNAP25a	Xl.8686	4.32
Slow troponin I	Xl.11074	4.30
Neuritin 1-A	Xl.11951	4.04
Low molecular weight neuronal intermediate filament	Xl.992	3.99
Alpha-actinin-2	Xl.34825	3.95
Growth associated protein 43	Xl.9994	3.90
Similar to Parvalbumin (calcium binding protein)	Xl.25492	3.87
Neuritin-B precursor	Xl.34198	3.85
Neuro-oncological ventral antigen 1	Xl.47571	3.81
Solute carrier family 1	Xl.20736	3.80
Troponin I type 2 (skeletal, fast)	Xl.18396	3.78
Myosin binding protein H	Xl.6142	3.77
Guanine nucleotide binding protein (G protein), gamma 3	Xl.24553	3.67
Tropomyosin 2 a isoform 1	Xl.21893	3.58
Solute carrier family 1	Xl.20736	3.58
Muscle-related coiled-coil protein	Xl.57797	3.50
Phosphoglycerate mutase 2	Xl.84002	3.36
Enolase 1 alpha	Xl.66912	3.34
Calcium-dependent secretion activator 1	Xl.26179	3.34
Growth associated protein 43	Xl.1133	3.33
Enolase 3, beta muscle	Xl.19056	3.32
Beta-synuclein	Xl.14705	3.26
Sox10	Xl.1588	3.24
Actin, alpha skeletal muscle	Xl.70068	3.22
Synuclein, gamma (breast cancer-specific protein 1)	Xl.11348	3.22
Middle molecular weight neurofilament protein NF-M(1)	Xl.173	3.19
Aspartyl beta-hydroxylase	Xl.23399	3.10
RUN and FYVE domain containing 3	Xl.8516	3.01
Acetylcholine receptor subunit alpha-1-A	Xl.1118	3.00
Desmin	Xl.2665	2.99
Syntaxin binding protein 1	Xl.26111	2.96
Jun oncogene	Xl.541	2.93

Dihydropyrimidinase-like 4	Xl.77348	2.90
Jun proto-oncogene	Xl.542	2.90
Similar to calsequestrin 2 (cardiac muscle)	Xl.15700	2.87
Protein tweety homolog 1-A	Xl.11254	2.85
Collapsin response mediator protein 1	Xl.50837	2.83
Chondromodulin-I precursor	Xl.21971	2.79
Synaptophysin	Xl.230	2.79
Transcription factor Sp8	Xl.49163	2.77
Neural cell adhesion molecule L1	Xl.21565	2.76
Tubulin	Xl.8919	2.76
Protocadherin 8	Xl.72042	2.75
CUGBP Elav-like family member 3-B	Xl.12160	2.73
CUGBP Elav-like family member 3-A	Xl.986	2.73
Pax6	Xl.647	2.69
Synaptophysin	Xl.232	2.66
Dihydropyrimidinase-like 2	Xl.3001	2.65
Kinesin family member 5A	Xl.18417	2.64
Middle molecular weight neurofilament protein NF-M(2)	Xl.174	2.61
VENT homeobox 1	Xl.1420	2.57
MICAL-like 2	Xl.34034	2.56
Synaptotagmin 4	Xl.48319	2.55
Neuritin 1-A	Xl.11951	2.53
Tubulin beta-2 chain	Xl.121	2.50
Diacylglycerol kinase iota	Xl.16337	2.49
Carboxypeptidase E	Xl.8483	2.46
Cell adhesion molecule 3 precursor	Xl.23549	2.45
Neuronal pentraxin-2 precursor	Xl.11864	2.45
Reticulon-1-B	Xl.8555	2.45
Actin, alpha skeletal muscle 3	Xl.24656	2.43
Lysyl oxidase homolog 3 precursor	Xl.13606	2.42
Similar to myozenin 1	Xl.21921	2.41
Trimeric intracellular cation channel type A	Xl.45053	2.39
Seizure protein 6 homolog precursor	Xl.51942	2.36
Guanine nucleotide-binding protein G(O) alpha subunit 1	Xl.20976	2.36
Fox-1 homolog C	Xl.57891	2.35
Coiled-coil domain containing 28B	Xl.9739	2.35
Zinc finger protein ZIC 1	Xl.1796	2.33
RAB33A, member RAS oncogene family	Xl.47462	2.33
Enah/Vasp-like	Xl.12544	2.32
Wnt-8	Xl.49	2.29
Stathmin-like 3	Xl.21810	2.29
Hes-4-A	Xl.25977	2.20
Sodium/potassium-transporting ATPase subunit beta-1-interacting protein 4	Xl.55497	2.20
Zinc finger protein 36, C3H type, homolog	Xl.24023	2.17
Calcium channel, voltage-dependent, beta 3 subunit	Xl.53876	2.09
Hes5.1	Xl.48575	2.03

Table 3. Genes upregulated in Ptf1a microarray at NF36.
Genes have been sorted based on their fold change in MA36 relative to control samples. Only those with greater than 4-fold upregulation are listed. Unigene ID numbers that did not represent a known gene and duplicated genes were removed from this table. $p < 0.05$.

Gene Title	Unigene ID	Fold Change
Phosphoenolpyruvate carboxykinase 1, cytosolic	Xl.12459	8.92
Glucose-6-phosphatase, catalytic	Xl.12866	7.44
Ornithine decarboxylase-2	Xl.8949	7.23
Phosphoenolpyruvate carboxykinase 1 (soluble)	Xl.18730	7.03
Similar to glucose-6-phosphatase	Xl.5165	6.95
Uroplakin III	Xl.25311	6.65
Insulin-like growth factor binding protein 1	Xl.34669	6.61
Na+/K+ -ATPase beta 2 subunit	Xl.53667	6.57
Hydrogen/potassium-exchanging ATPase 12A b	Xl.2659	6.46
Uncoupling protein 3 (mitochondrial, proton carrier)	Xl.49433	6.46
Arginase, non-hepatic 1	Xl.892	6.43
Carboxypeptidase B1	Xl.26001	6.38
Similar to deoxyribonuclease I-like 3	Xl.6017	6.26
CCAAT-enhancer binding protein delta	Xl.29876	6.24
Serine/threonine-protein kinase Sgk1-A	Xl.7842	6.22
Anterior gradient 2	Xl.25847	6.21
Paired box protein Pax-9	Xl.21602	6.06
Uroplakin 1B	Xl.8143	5.93
Tripartite motif-containing 29	Xl.9051	5.85
Keratin, type I cytoskeletal 47 kDa	Xl.66586	5.82
Cornifelin homolog B	Xl.23712	5.82
Matrix metalloproteinase 13	Xl.9058	5.73
Caveolin-3	Xl.53974	5.69
Peroxisomal membrane protein 2, 22kDa	Xl.83319	5.66
Pyruvate dehydrogenase kinase 2	Xl.1717	5.55
ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B1	Xl.15867	5.54
Claudin 4	Xl.53796	5.54
Purine nucleoside phosphorylase	Xl.57569	5.53
Arrestin domain containing 2	Xl.19508	5.52
Otogelin	Xl.847	5.50
V-type proton ATPase subunit G 3	Xl.34612	5.43
Serpin peptidase inhibitor, clade E, member 2	Xl.12045	5.31
Glutathione S-transferase P 1	Xl.54920	5.27
L-lactate dehydrogenase C chain	Xl.4591	5.25
Glucose-6-phosphatase, catalytic, 2	Xl.47697	5.21
Guanylyl cyclase-1	Xl.906	5.17
Prostaglandin-endoperoxide synthase	Xl.78035	5.09
Serine dehydratase	Xl.72557	5.05
Phospholipid scramblase 1	Xl.60828	5.04
Neurogenic differentiation 1(NeuroD)	Xl.26304	5.02
Carbonic anhydrase XII	Xl.10362	4.97
Amiloride-sensitive sodium channel subunit beta-2	Xl.1175	4.97

Keratin 15	Xl.57085	4.97
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7	Xl.8104	4.95
Claudin 1	XL.16310	4.94
Sox9	Xl.1690	4.92
Tetraspanin 1	Xl.39094	4.92
X-epilectin	Xl.6048	4.91
ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	Xl.8939	4.88
Sciellen	Xl.15658	4.88
Calpain 9	Xl.47406	4.87
Pendrin-like anion exchanger	Xl.10887	4.78
Lectin, galactoside-binding, soluble, 3	Xl.1779	4.76
Collagen, type II, alpha 1 (col2a1)	Xl.606	4.74
ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A, isoform 1	Xl.54891	4.72
Collagen, type I, alpha 1	Xl.47042	4.65
Collagen, type I, alpha 2	Xl.49126	4.65
Sestrin-1	Xl.12255	4.58
Collagen, type II, alpha 1 (col2a1)	Xl.606	4.57
Alkaline phosphatase	Xl.1299	4.57
Collagen, type VI, alpha 1 (col6a1)	Xl.9863	4.55
Cleavage stimulation factor, 3' pre-RNA, subunit 3, 77kDa, b	Xl.24776	4.53
T-box transcription factor TBX1	Xl.51448	4.50
Forkhead box protein E1	Xl.50069	4.48
Versican	Xl.70991	4.46
Villin-like	Xl.15156	4.45
Osteoglycin	Xl.21905	4.45
Synaptotagmin-like 2	Xl.52466	4.38
Fibroblast growth factor binding protein 1	Xl.48872	4.38
Augurin precursor	Xl.21897	4.37
ATPase, H+ transporting, lysosomal V0 subunit a4	Xl.2715	4.34
Somatostatin	Xl.13593	4.33
NIPA-like domain containing 4	Xl.5679	4.31
Actin, alpha 2, smooth muscle, aorta (acta2)	Xl.83891	4.30
Phosphoinositide-3-kinase interacting protein 1	Xl.77369	4.30
Distal-less homeobox 3 (dlx3-a)	Xl.838	4.30
Transcription factor AP-2 alpha (tfap2a-a)	Xl.2143	4.28
Glutamate-ammonia ligase (glutamine synthase) (glul)	Xl.47079	4.25
GDP-mannose 4, 6-dehydratase	Xl.10260	4.23
Calpain 1, (mu/I) large subunit	Xl.3776	4.23
D-dopachrome decarboxylase-A	Xl.23562	4.23
Adenylate kinase 1	Xl.25877	4.22
5-hydroxytryptamine (serotonin) receptor 3A	Xl.9068	4.22
Myosin, heavy chain 6, cardiac muscle, alpha	Xl.3161	4.22
Arrestin domain containing 3	Xl.25985	4.19
Annexin A1	Xl.2308	4.18
Macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	Xl.115	4.18

Keratin 16	Xl.15435	4.15
Sal-like 4 (sall4)	Xl.77515	4.15
Zinc finger protein 36, C3H type, homolog	Xl.26472	4.10
Distal-less homeobox 5 (dlx-5)	Xl.18401	4.09
Histidine ammonia-lyase	Xl.2256	4.08
Fucosyltransferase 6 (alpha (1,3) fucosyltransferase)	Xl.16504	4.07
Aquaporin-3	Xl.34454	4.07
Phosphatidylinositol glycan anchor biosynthesis (PIG-S)	Xl.48652	4.05
Aquaporin 1	Xl.5043	4.04
Myosin, light chain 4, alkali; atrial, embryonic	Xl.11969	4.03
Plasminogen activator, tissue	Xl.14787	4.02
Coagulation factor II (thrombin) receptor-like 2	Xl.23535	4.01

Chapter 5: Discussion and Future Perspectives

In Chapter 2, I demonstrated that *stau2*, a well-conserved dsRBP is expressed throughout the *Xenopus* embryo at gastrulation and becomes localized to neural tissue and the anterior endoderm at tailbud stages. Targeted knockdown of Stau2 function within the developing anterior endoderm and mesoderm led to the loss of stomach, liver and pancreas formation. I demonstrated that Stau2 function was required during gastrulation for proper patterning of the endoderm. Stau2 function appeared to regulate the components of the BMP signaling pathway, as expression of the BMP antagonist *chordin* was lost in gastrula stage Stau2 morphant embryos. My results suggest that Stau2 has a unique role in patterning and development of the anterior endoderm during embryogenesis.

In Chapter 3, I set out to understand in more detail how Stau2 function may be controlling endoderm patterning. I demonstrated that Stau2 function was required at the onset of gastrulation to maintain expression of Wnt/β-catenin signaling effector proteins and this lead to a reduction in organizer gene expression during gastrulation. I showed that the Stau2 knockdown loss of anterior endoderm organ function identified in Chapter 2 could be partially rescued by overexpression of *chordin* or *sia* mRNA. RIP-seq of Stau2-bound mRNA targets at the onset of gastrulation led to the identification of many novel targets including *gsk3β*, a known Wnt signaling inhibitor. I showed that while knockdown of Stau2 did not affect *gsk3β* transcript or protein levels, loss of Stau2 was able to partially rescue the Gsk3β inhibitory effect of LiCl on the embryo. Finally, I demonstrated that dsRBD1, dsRBD4 and dsRBD5 appeared to be required for proper endoderm patterning. These results suggest that Stau2 function may be essential for

endoderm patterning. As the endoderm and mesoderm germ layers develop in close proximity to each other, they are thought to be patterned together until the end of gastrulation. However, my results provide evidence that the endoderm may be patterned separately from the mesoderm, beginning at the onset of gastrulation.

In Chapter 4, I set out to gain insight into the gene regulatory network activated by Ptf1a during early pancreas development. Following a study published by the Horb lab that characterized the function of Ptf1a in *Xenopus laevis* (Jarikji et al., 2007), I wanted to identify gene expression changes that occurred upon overexpression of Ptf1a at the onset of pancreas development. I isolated anterior endoderm tissue overexpressing *ptf1a* at two early stages, NF32 and NF36, and compared their gene expression profiles to control tissue, using microarrays. This led to the identification of over 1000 genes with altered expression levels of diverse function. I identified novel genes expressed within the developing pancreatic endoderm and described the expression of seven candidate genes with functions ranging from transcription, vesicle fusion and cell adhesion. In addition, I demonstrated the altered expression of some of the genes *in vivo* in the anterior endoderm of *ptf1a* mRNA-injected embryos. These results revealed that Ptf1a regulates genes with a wide variety of functions, providing insight into the complexity of the regulatory network required for pancreas specification

In the following pages I am going to discuss the implications of my research and future directions. I will combine Chapters 2 and 3 as I consider the impact of Stau2 in endoderm development and future perspectives. I will finish with a discussion of the insight gained

from the Ptf1a microarrays presented in Chapter 4 and how I envision the subsequent path to continue to identify downstream targets of Ptf1a in *Xenopus* pancreas development.

SEPARATING ENDODERM AND MESODERM DEVELOPMENT

There are numerous diseases that affect endodermally derived cells, from congenital defects to aggressive cancers. Studying the development of endodermal organs in model systems have helped elucidate the mechanisms underlying these diseases and provide insight that can be used for the development of therapies and cures. A significant clinical application of understanding the conserved molecular network that controls endoderm development is in the production of therapeutically important endoderm cells, such as pancreatic β-cells and liver hepatocytes from embryonic stem cells or differentiated cells.

Throughout development reciprocal signaling between endoderm and mesoderm tissues plays an integral role in patterning and organogenesis. Prior to gastrulation, the vegetal cells fated to become endoderm signal to the overlying equatorial cells and induce a mesodermal fate. The existence of a common mesendoderm progenitor that can give rise to both the mesoderm and endoderm germ layer has been reported from *C. elegans* to *Xenopus*. It has been demonstrated in both zebrafish and *Xenopus* that in blastula stage embryos vegetal and marginal cells can contribute to both lineages (Tada et al., 2005; Zorn and Wells, 2007). However, the concept of a bipotential mesendoderm in mammalian development is still unknown. Studies in early mice development

demonstrate that the anterior primitive streak gives rise to anterior definitive endoderm and axial mesoderm however whether these differentiate from a common mesendoderm precursor remains elusive (Tada et al., 2005). In all vertebrates, Nodal signaling is necessary and sufficient to initiate endoderm and mesoderm development. Nodal and Wnt/β-catenin signaling play pivotal roles in the initiation of early embryonic endoderm development where they promote the expression of a complex endodermal gene regulatory network comprised by a conserved core of transcription factors (Zorn and Wells, 2009; reviewed in 2007). Activation of endodermal transcription factors along with Nodal activity has also been shown to promote endodermal fate in a feed-forward autoregulatory loop. These core endoderm factors are expressed in a spatially restricted manner prior to gastrulation when the germ layers are not yet specified, however the mesoderm and endoderm are not considered to be mutually exclusive until the end of gastrulation (Zorn and Wells, 2009; reviewed in 2007). In Chapter 3, I provided evidence that the mesoderm and endoderm are specified separately during gastrulation as the loss of Stau2 within the two germ layers only affected endodermal patterning (Fig. 1). Understanding how the mesoderm and endoderm are specified and patterned will provide insight into whether a common mesendodermal progenitor exists and if so, when the lineages do become mutually exclusive.

Gastrulation and the formation of the organizer drive the specification of the germ layers. However, how is it that a group of transcription factors and secreted proteins appear to specify vastly different germ layers? I hypothesize that it is likely that there are subtle differences in the way these organizer factors are expressed and function within all three

germ layers that have not yet be appreciated. A difficulty of studying the mesoderm and endoderm separately is that there is no clear boundary between the two tissues making it challenging to define exact spatial expression pattern of a gene. I propose there are many benefits of initiating these studies in *Xenopus*; the embryos develop *ex utero*, they are available in high quantities upon demand and their large size makes for easy micromanipulations and allows for the ability to clearly discern distinct cell populations. In fact, localization of key components of four signaling pathways, β-catenin (Wnt), MAPK (FGF), Smad1 (BMP) and Smad2 (Nodal/Vg1) have been analyzed during early *Xenopus* development to tailbud stages (Schohl and Fagotto, 2002). Schohl and Fagotto (2002) identified overlap between the expression patterns suggesting extensive interplay between pathways, as well as unexpected signal patterns (Schohl and Fagotto, 2002). Analysis of only these four signaling pathway components revealed that aspects of early development are still not well understood. Therefore, in order to understand how the organizer genes can specify all three germ layers and define boundaries between them, the first step should be a detailed study of the expression of organizer factor transcripts and proteins over a series of time points leading up to and during gastrulation. Imaging the experiments using techniques that can decipher cell morphology would also provide information about the tissue boundaries. Focusing not only on spatial expression of a given factor but also the length and concentration of a particular cells exposure to the factor may provide insight into how the same factors can differentially specify three different germ layers.

The work presented in Chapters 2 and 3 not only provide evidence that mesoderm and endoderm are specified as distinct lineages earlier than previously assumed, it also demonstrates the requirement for post-transcriptional regulation during early endoderm development (Bilogan and Horb, 2012a). Much emphasis has been focused on elucidating the transcriptional hierarchy that is activated throughout endoderm development, however many transcription factors do not appear to have expression restricted to a specific tissue. Various studies have demonstrated that these multi-tissue transcription factors can complex with different factors to elicit distinctive responses from one cell type to the next. I hypothesize that different cellular responses to the same factor in multiple lineages may be dependent on the chromatin accessibility, which may define distinct transcription factor binding across different tissues. This may be determined by formaldehyde-assisted identification of regulatory elements followed by deep sequencing (FAIRE-Seq) to identify active regulatory elements and tissue specific targets of the factors (Giresi et al., 2007).

To date, most studies have focused on how organizer genes are regulated at the transcriptional level and how they function to regulate transcription of their targets to specify the germ layers (Engleka and Kessler, 2001; Laurent et al., 1997; Rankin et al., 2011; Reid et al., 2012; Watabe et al., 1995). Evidence suggest that most organizer genes are directly activated by synergistic cooperation between the same Wnt and Nodal signaling effectors (Reid et al., 2012). Therefore another level of regulation must be required to establish the spatially and temporally distinct transcription domains of these organizer genes and their downstream targets. The cell-specific response to an organizer

transcription factor or growth factor may also be dependent on post-transcriptional or post-translational regulation. These regulations or modifications may alter an organizer proteins function or cellular location allowing it to specify one lineage over another. For example, sumoylation regulates the repressive activities of Gsc by altering its ability to bind certain promoters, including it's own and that of Xbra (Izzi et al., 2008). Determining which organizer factors are being modified may be difficult without previous indication, I hypothesize that factors that are expressed in multiple tissues at the same time are likely to be regulated or modified in different cell types, leading to cell- or tissue-specific functions. Using online resources and databases, *in silico* information could be used predict whether a particular transcript may be differentially regulated resulting in alternative splicing or transcript stability in specific cell types. Bioinformatic analysis of that particular protein would also help predict potential post-translational modifications that may alter the proteins function. Once potential post-transcriptional/translational regulations have been predicted for a given organizer factor, expression patterns of modifying proteins, such as RBPs or kinases, can be analyzed. Those of most interest would be the modifying protein with an expression domain that overlaps with the given organizer factor expression, however the expression domain of the former would be restricted to a particular tissue. This interaction and also its ability to modify the organizer factor would have to be confirmed biochemically. A deeper understanding into how organizer factors are regulated both post-transcriptionally and post-translationally may provide insight into how a small group of factors can specify vastly different germ layers.

A Concentration-Dependent Role for the Wnt Signaling Pathway in Endoderm Versus Mesoderm Specification During Gastrulation

The role of Wnt/β-catenin signaling pathway in endoderm development is conserved in both invertebrate and vertebrates; while in invertebrates, the initiation of endoderm formation does not appear to require TGFβ (Maduro and Rothman, 2002; Nakagoshi, 2005; Nishida, 2005). Studies of endoderm formation in *C. elegans*, ascidians and sea urchins have suggested that they appear to rely more significantly on the Wnt/β-catenin signaling pathway. In vertebrates however, it has been suggest that the Wnt/β-catenin pathway only plays a role in patterning of the endoderm as one of multiple contributors to early Nodal expression (reviewed in Zorn and Wells, 2007). Wnt signaling is particularly dynamic during endoderm development; first, mWnt11/β-catenin signaling promotes gastrulation and anterior endoderm development, however towards the end of gastrulation and the beginning of neurulation Wnt/β-catenin signaling has the opposite affect as it represses foregut fate in the anterior endoderm and promotes hindgut fate in the posterior endoderm. This has led to a model where at the end of gastrulation Wnt-OFF (Wnt pathway is suppressed) promotes foregut fate while Wnt-ON (Wnt pathway is activated) promotes hindgut fate (Li et al., 2008). Recently this model has proven to be too simplistic, as Wnt-antagonist Sfrp5 and receptor Fzd7 have been shown to exhibit biphasic activity; repressing Wnts at high concentrations while they are also essential at low concentrations to maintain foregut progenitors (Mii and Taira, 2009; Zhang et al., 2013). These data suggest that Wnt/β-catenin signaling has different thresholds along the A-P axis (Mii and Taira, 2009; Zhang et al., 2013). As it was previously assumed Wnt

was inhibited for foregut specification after gastrulation, it is possible that the current model for the role of Wnt/β-catenin signaling during gastrulation is too simplistic as well.

I showed that knockdown of Stau2 leads to a loss of mWnt11/β-catenin signaling pathway maintenance and this only affected endoderm patterning (Chapter 3, Fig. 1, Chapter 2). Data extrapolated from previous studies suggest that Wnt/β-catenin signaling pathway may have tissue specific functions, knockdown of Wnt11 in the dorsal blastomeres at the 4-cell stage resulted in gastrulation and heart defects (Pandur et al., 2002; Schambony and Wedlich, 2007). While Wnt11-MO targeted to the future foregut endoderm (32-cell stage injections) resulted in expanded expression of the anterior endoderm domain at the expense of the posterior foregut (Li et al., 2008). Interestingly, expression of many organizer factors have been shown to be initiated after MBT prior to the onset of gastrulation (Sasai et al., 1995; Wylie et al., 1996). Which raises the question of what is the function of these factors prior to gastrulation? Using a morpholino, translation of the target is inhibited upon injection and does not allow for temporal control of the targets inhibition. Knockdown of Stau2 in the two dorsal vegetal blastomeres at the 8-cell stage lead to a loss of *sia, twin* and *xnr3* at NF10 (the onset of gastrulation) and a reduction in expression of the organizer factors (Chapter 3, Fig.1). While data based on MO knockdown experiments suggest that organizer factors are required at the onset of gastrulation for the formation and patterning across all three germ layers, Stau2 knockdown embryos retain normal patterning of the ectoderm and mesoderm, and only anterior endoderm patterning was perturbed (Chapter 3, Fig. 1, Chapter 2). This data provides evidence that expression of organizer factors prior to

gastrulation function to pattern the ectoderm and mesoderm, suggesting axial patterning is initiated prior to gastrulation.

In Chapter 3, I proposed a possible explanation for the endoderm-specific phenotype we observed in the Stau2 knockdown embryos may be that Sia and Twn activate transcription of downstream targets in a temporal or concentration dependent manner (Fig. 1). It may be possible that early/short Sia and Twn function is required to establish the germ layers and to pattern future ectoderm and mesoderm, while later/maintained Sia and Twn functions to pattern endoderm (Fig. 1). This supports the hypothesis of a concentration or time sensitive role of Wnt/β-catenin signaling in mesoderm patterning versus endoderm patterning. To test this, a dominant inhibitor of Sia, the *eng-sia-GR* mRNA construct, can be injected at different concentrations into the dorsal vegetal cells and can then be activated at different time-points prior to and during gastrulation. Previous studies have shown that expression of *eng-sia* mRNA in the dorsal domain of the embryo leads to complete loss of axis formation and organizer gene expression (Fan and Sokol, 1997; Kessler, 1997). While temporal regulation of Sia inhibition has not yet been studied, results presented in Chapter 3 suggest that loss of *sia* expression at the onset of gastrulation leads to a loss of anterior endoderm formation. I expect early inhibition of *sia* expression would reflect previous data and result in a loss of axial patterning across all three germ layers, and that this axial defect will become less severe with later inhibition of *sia* expression. I would also expect the inhibition of *sia* expression at the onset of gastrulation to have a similar phenotype as the Stau2 morphant embryos, since the Stau2-MO anterior endoderm phenotype could be partially rescued by

overexpression of *sia* mRNA (data not shown). These experiments would provide insight into how the Wnt/β-catenin signaling pathway is able to regulate the specification of endoderm separately from its role in specification of the other germ layer lineages.

An unresolved issue with regarding endoderm-specific regulation by Stau2 is its ubiquitous expression across all three germ layers in the blastula and gastrula embryo (Chapter 2, Fig. 1). In the described experiments I am specifically targeting the developing dorsal mesoderm and endoderm (Dale and Slack, 1987). At this point, it is unknown how Stau2 may regulate patterning of the endoderm but not the mesoderm, however it is likely through accessibility to target mRNA and protein-protein interactions. Allison et al. (2004) showed that Stau proteins are phosphorylated by the MAPK pathway in *Xenopus* oocytes, however it is unknown whether phosphorylation alters Stau function. In the early *Xenopus* embryo, FGF signaling is specifically activated in the mesoderm and it specifically acts on the MAPK pathway. While MAPK pathway can be activated by several tyrosine kinases, in early *Xenopus* development it seems to be largely activated downstream of the FGF pathway (Schohl and Fagotto, 2003). I hypothesize that Stau2 may be phosphorylated by the MAPK pathway in the mesoderm during early development, this may alter its function through its ability to bind mRNA targets or form RNP complexes. Phosphorylation of RBPs such as chicken ZBP1 (VgRBP1 homolog) and mouse FMRP have been shown to alter their ability to bind mRNA and regulate translation (Hüttelmaier et al., 2005; Muddashetty et al., 2011). Ultimately this post-translational modification may define how Stau2 functions differently in the endoderm versus the mesoderm.

Gsk3β is also a Component of the Non-Canonical Wnt Signaling Pathway

The non-canonical Wnt signaling pathway has also been shown to be involved in endoderm development, in this situation Wnt ligand activates a β–catenin-independent pathway which signals through the Rho-family GTPases and Jun-N-kinases (JNK) (Li et al., 2008; Wallingford and Habas, 2005; Zhang et al., 2013). During endoderm development and gut morphogenesis the non-canonical Wnt pathway has been shown to regulate cytoskeletal dynamics, cell polarity and cell shape changes (Li et al., 2008; Zhang et al., 2013). While the mechanisms involved in the non-canonical pathway are not well understood, recent efforts have determined that both the canonical and non-canonical pathways use common intracellular components, including Disheveled (Dvl), axin and Gsk3β to activate pathway specific co-receptors and trigger different responses (Grumolato et al., 2010). There is evidence suggesting that the canonical and non-canonical branches are mutually antagonistic, however, it was recently shown in *Xenopus* that both pathways can be activated simultaneously in the same tissue (Grumolato et al., 2010; Zhang et al., 2013). In Chapter 3, I was not able to fully rescue the Stau2 knockdown phenotype by overexpression of *chordin* or *sia* mRNA (Chapter 3, Fig. 2, data not shown). There are two possible reasons for the incomplete rescue. First, Stau2 may be regulating other targets not related to the Wnt/β-catenin signaling pathway that would not be rescued by overexpression of *chordin* or *sia* mRNA. Second, Stau2 may regulate the non-canonical Wnt signaling pathway through Gsk3β as *gsk3β* was identified as a potential Stau2 mRNA target. Since Chordin and Sia are targets of the Wnt/β-catenin signaling pathway, overexpression of *chordin* or *sia* mRNA would not be able rescue the

dis-regulation of the non-canonical Wnt/JNK pathway caused by the knockdown of Stau2. It is likely that both possibilities may be occurring. To test whether knockdown of Stau2 affects the non-canonical Wnt signaling pathway, western blot analysis of the phosphorylation of JNK and RhoGTPase from the endoderm of Stau2-MO-injected embryos will be necessary. Since Stau2 appears to be required to inhibit Gsk3β activity, I hypothesize this will result in an increase in phospho (activated) receptors which can be observed by western blot of Stau2-MO embryos.

Post-Transcriptional Regulation of Gsk3β by Stau2

We identified *gsk3β* as a target of Stau2 and reported that Stau2 does not appear to target the *gsk3β* transcript for mRNA decay, nor does Stau2 appear to affect its translation (Chapter 3). The ability of the Stau2-MO to partially rescue the inhibitory effects of LiCl on Gsk3β activity provides evidence that Stau2 is functioning to restrict the active protein levels of Gsk3β. Gsk3β is a constitutively active protein and to date it appears that the regulation of Gsk3β activity is controlled mainly post-translationally, involving complex mechanisms that are each dependent on specific signaling pathways (Medina and Wandosell, 2011). In the Wnt/β-catenin signaling pathway, inhibition of Gsk3β phosphorylation of β-Catenin appears to be regulated by phosphorylated PPPSPXS motifs of the Wnt co-receptor LRP6 and by sequestration of Gsk3β inside multivesicular bodies (MVBs) reducing the cytosolic levels of Gsk3β but not the activity level (Taelman et al., 2010; Wu et al., 2009). There are a few studies that have noted that Gsk3β is located in cytosolic, nuclear and mitochondrial compartments of cells and Gsk3β pools

appear to relocate into growth cones in response to external signals (Eickholt et al., 2002; Zhou et al., 2004). Since Gsk3β is constitutively active, it would be advantageous to restrict its translation to specific regions within the cell where it is required to function or its activity can be sequestered.

I reported that knockdown of Stau2 led to a decrease in Wnt/β-catenin effector expression, which is negatively regulated by Gsk3β, suggesting that in the knockdown embryos Gsk3β activity was increased (Fig. 1). I propose a mechanism by which Stau2 regulates Gsk3β by localizing the transcript either to regions of the cell that are rich in inhibitors that sequester Gsk3β activity or where it cannot interact with β-Catenin and lead to its degradation. Transport of *gsk3β* by Stau may be required to prevent Gsk3β protein from acting ectopically before it reaches its destined location. This could be tested by confocal analysis of whether the localization of the *gsk3β* transcript using fluorescence *in situ* hybridization (FISH) or the localization of the Gsk3β protein using immufluorescence is altered in Stau2-MO embryos. While further biochemical analysis is also required to confirm the mechanism by which Stau2 may be regulating Gsk3β activity, these would be one of the first results to identify a form of post-transcriptional regulation of Gsk3β. The results presented in Chapter 3 along with future experiments suggested above could link Stau2 as an integral regulator of the Wnt signaling pathway.

STAU2 RIP-SEQ ANALYSIS

In Chapter 3, I performed RNA-seq analysis on Stau2 IP mRNA (RIP-seq) to identify Stau2 mRNA targets. This is the first time RNA-seq was used to identify Stau2 associated mRNA targets. Attempts to identify Stau targets have been preformed in other studies and in all studies, including ours, the output was a large number of potential targets that contain direct as well as indirect mRNA targets (Furic et al., 2008; Kusek et al., 2012; LeGendre et al., 2013; Maher-Laporte and DesGroseillers, 2010). Functional clustering of Stau targets from these studies and our own identify a wide variety of biological processes. While some genes and processes are conserved between studies, each study was performed at different developmental stages and within different tissues. I hypothesize that targets identified in the Stau2 RIP-seq performed in Chapter 3 may play a role in anterior endoderm specification during gastrulation. To determine whether RIP-seq targets are involved in endoderm patterning, their expression pattern should be confirmed within the developing endoderm by *in situ* hybridization. Knockdown and mRNA gain-of-function studies can be used to determine whether their function is necessary for endoderm patterning.

For the analysis in Chapter 3, RIP-seq reads were mapped to an *X. laevis* wild-caught reference library (Horb, unpublished) using CLC Genomics Workbench (CLC, Denmark). Candidate genes were selected as reads that mapped to the reference library specifically from the Stau2 IP RNA library, contigs that mapped to reads sequenced from the BrunoL1 IP RNA library and the Stau2 IP RNA library were discarded as non-

specific mRNA targets. Two approaches were used to ascertain relevant candidates to pursue, the estimation of gene expression value, reads per kilobase of exon model per million mapped reads (RPKM) and the functional clustering using DAVID. First, since I eliminated any overlapping reads between Stau2 IP RNA library and the BrunoL1 IP RNA library that mapped to the same contig, there is increased confidence that the reads identified as Stau2 targets are genuine. Therefore the reads with the highest RPKM are most likely to be the most abundant Stau2 mRNA targets at the onset of gastrulation. Second, DAVID was used to identify functional relationships from our list of Stau2 associated mRNA. This was used as a preliminary step for data exploration, the goal being to identify particular patterns in the data that would provide insight into specific signaling pathways or functions that Stau2 may be regulating. The main issue with the approach described above is lack of quantitation regarding the differential expression between the Stau2 IP and BrunoL1 IP reads. Recently, an alternate approach was used to quantify the differential expression levels between reads found in the two libraries (Robert Freeman, personal communication). In this second approach, Trinity was use to build a new transcript set by combining all of the Stau2 and BrunoL1 data (three Stau2 data sets and three BrunoL1 data sets) (Grabherr et al., 2011). Next, the original reads from each of the data sets were mapped to the new transcript reference to get quantitative data, and finally the differential expression program EBSeq was used to identify the genes and isoforms that were differentially expressed across the Stau2 and BrunoL1 data sets (Leng et al., 2013). From this analysis, 1000 transcripts were determined to be statistically significant ($P < 0.05$). Not only does this second approach provide a much more statistically sound group of potential Stau2 targets, it also provides a more

manageable number. Within this second analysis, previously identified Stau targets, SERPINE1 and Arf1, were expressed, suggesting that we have identified genuine Stau2 targets (Kim et al., 2005; 2007). To identify which transcripts to pursue from the second analysis I propose two approaches. First, review the literature of the potential targets to identify known expression patterns during gastrulation, whether they may be differentially expressed in the germ layers and if the target transcript has been shown to be transported or regulated in RNP complexes. Second, I would identify the 3'UTR sequence for the targets and identify whether they contain SINEs, as Stau has been shown to bind dsRNA formed by base-pairing between SINEs (Wang et al., 2013). After using this approach to make an informed decision to pursue certain candidate targets from the RIP-seq, I propose their expression pattern during gastrulation be confirmed by *in situ* hybridization and knockdown and mRNA gain-of-function studies can be used to determine whether their function is necessary for endoderm patterning.

LncRNAs Form Complexes with Stau2

Long noncoding RNA (lncRNA) are RNAs longer than 200 bases that do not encode mRNA, rRNA or tRNA (Lee and Kikyo, 2012). LncRNAs control many cell functions, including development, cell differentiation and cancer and lncRNA can regulate all processes of mRNA metabolism. LncRNAs can function as scaffolds to assemble RNA-protein complexes, they can also aid in the recruitment of these complexes to target genes (Lee and Kikyo, 2012). Stau proteins have been show to complex with lncRNAs and mediate a variety of functions through two different mechanisms. First in Stau-mediated

mRNA decay (SMD), Stau binding to most identified SMD targets requires an interaction between the Stau binding site (SBS) in the 3'UTR of the target and a SINE element in a lncRNA (Gong and Maquat, 2011; Wang et al., 2013). Stau can then bind the dsRNA complex formed between the target 3'UTR and lncRNA and promote decay of the target using NMD proteins (Gong and Maquat, 2011; Wang et al., 2013). Second, the lncRNA TINCR was recently identified to complex with Stau1, however this interaction did not occur through an SBS and the interaction was RNA-independent. Interestingly, this complex appeared to regulate the stabilization of differentiation mRNA targets to ensure their expression (Kretz et al., 2013). These novel findings suggest that lncRNAs may be a critical component of Stau mRNPs.

From the Stau2 RIP-seq described in Chapter 3, the second most abundant transcript identified shares 40% similarity to human TINCR lncRNA (data not shown). These exciting results suggest that the Stau-TINCR complex is conserved in *Xenopus* and may provide a mechanism for Stau-mediated regulation of endoderm development. I propose Stau2 and TINCR may form a complex within the developing endoderm and this complex may interact with certain transcripts to maintain their expression. Since Stau2 appears to regulate endoderm specification but not mesoderm development, it may be possible that within endoderm, TINCR and Stau2 interact with endoderm-specific genes and maintain their expression. As Stau2 is expressed within the developing endoderm and mesoderm, this hypothesis would be based on the assumption that TINCR is not present in the mesoderm or does not interact with Stau2 in the mesoderm. TINCR expression should be confirmed by *in situ* hybridization. Regardless of whether the TINCR-Stau2

interaction is endoderm-specific, confirmation of the complex should be performed to confirm that the TINCR and Stau2 complex in *Xenopus* and whether it previous identified functions in mRNA decay and mRNA stabilization are conserved (Kim et al., 2005; Kretz et al., 2013). Confirmation of the complex could be performed by purification of the Stau2 protein via TINCR by overexpression of TINCR_MS2bs and pFLAG-MS2 expression plasmids (Gong et al., 2012). The pFLAG-MS2 would bind the exogenous TINCR and bound components; an anti-FLAG IP could then be performed to recover the TINCR complex. The interaction with Staufen2 could be confirmed by western blot. This protocol would also allow for the identification of bound mRNA targets by either RT-PCR or RNA-seq. Future understanding of the Stau2-lncRNA complex may provide deeper insight of how Stau elicits multiple functions within a given cell.

Identifying the Stau2 Binding Site and Defining Targets

In Chapter 3, I showed that, at least in *Xenopus*, different dsRBDs appear to have the ability to bind to different mRNA targets (Chapter 3, Fig. 5). This is in contrast to work in *C. elegans* where it was suggested that each dsRBD can bind non-specifically to dsRNA (LeGendre et al., 2013). However in *Drosophila* and humans, only certain Stau dsRBDs can bind RNA (Micklem et al., 2000; Park et al., 2013). Though there have been recent advances such as the identification of a SBS in mRNA decay targets (Gong and Maquat, 2011; Kim et al., 2005; Wang et al., 2013), our understanding of how Stau proteins select mRNA targets is still lacking.

In order to identify dsRBD-specific mRNA targets I propose utilizing the catalytic deaminase domain of Adenosine deaminase acting on RNA (ADAR). ADARs are a conserved family of proteins that edit RNA by converting adenosines to inosines in dsRNA substrates (Savva et al., 2012). I hypothesize that addition of the ADAR catalytic deaminase domain to the carboxy-terminal domain of Stau2 will allow us to identify direct mRNA targets of Stau2, as the catalytic domain will edit the RNA bound by the Stau dsRBDs. In addition, we could add the catalytic deaminase domain to ΔRBD-Stau2 mutants to determine what targets a given dsRBD is bound to. These constructs would be injected into the embryo then RIP-seq would be performed to identify edited mRNA targets. This would provide insight into the role of specific dsRBDs as well as identify direct targets of Stau2. The detection of additional targets would also serve to confirm the recently identified SBS and identify other potential sequences required for Stau2 binding.

Stau2 is a multi-functional RBP that is conserved from *Drosophila* to humans. The data presented here are the first to identify a role for Stau2 in endoderm development and the first to suggest a function in the Wnt signaling pathway. Future studies of the role of Stau in vertebrate development will likely identify its invaluable functions. These studies will also provide a deeper understanding of the role of post-transcriptional regulation within the cell.

IDENTIFYING DOWNSTREAM TARGETS OF PTF1A

The bHLH transcription factor Ptf1a is required for proper development of the pancreas, retina and nervous system (Fujitani et al., 2006; Glasgow et al., 2005; Kawaguchi et al., 2002; Roux et al., 1989; Sellick et al., 2004). Within the pancreas, Ptf1a plays a biphasic role in development; early expression of Ptf1a is throughout the pancreatic multipotent progenitor cells (MPCs) and is required for the specification of foregut endodermal cells to adopt a pancreatic fate (Kawaguchi et al., 2002). Over time, Ptf1a expression becomes restricted to acinar cells and directly promotes expression of terminal exocrine differentiation genes such as Elastase and Carboxypeptidase 1 (Cockell et al., 1989; Krapp et al., 1996). While the genes targeted by Ptf1a in exocrine differentiation are well defined, it is not specifically known how Ptf1a regulates pancreatic specification.

Studies attempting to produce ectopic β-cells from within the pancreas have focused on Ptf1a since acinar cells are the most abundant tissue in the pancreas and Ptf1a is expressed in both acinar cells and MPCs (Hesselson et al., 2011; Pan et al., 2013). Acinar cells can give rise to endocrine cells under injury induced reprogramming, and this was recently shown to require Ptf1a (Pan et al., 2013). It appears that the spatio-temporal regulation of Ptf1a is tightly regulated, as suppression of Ptf1a activity can restore developmental plasticity and induce endocrine cells (Dong et al., 2008; Hesselson et al., 2011). Few studies have focused on the downstream targets activated by Ptf1a in pancreatic MPCs, however such studies would provide insight into the gene regulatory network involved in pancreatic specification. To date, a handful of direct downstream

targets have been identified. Known pancreas factors Pdx1, Nkx6.1, Onecut1 and Mnx1 are direct targets of Ptf1a, and Ptf1a promotes their expression within pancreatic progenitors (Miyatsuka et al., 2007; Thompson et al., 2012; Wiebe et al., 2007). Comparison of differentially regulated E10.5 pancreas progenitors in Ptf1a KO mice to those of controls led to the identification of novel direct targets such as BMP7, Nr5a2, RhoV and P2rx1 (Holmstrom et al., 2011; Thompson et al., 2012).

In Chapter 4, I performed a comparison of differently regulated genes in *X. laevis* embryos overexpressing *ptf1a* mRNA to those of controls (Bilogan and Horb, 2012b). In retrospect it is clear that the experimental design behind the Ptf1a microarrays had a few flaws. First, the time-points chosen where too late after the onset of endogenous Ptf1a expression to identify direct targets, NF32 is eight hours after Ptf1a is first expressed. If the experiments were to be repeated, this could easily be fixed by collecting samples within an hour of endogenous Ptf1a expression. A second flaw was the dissection of the anterior endoderm explant that also contained mesodermal and ectodermal cells. I think that this led to significant background in the microarray results, making it difficult to identify actual Ptf1a targets. I would suggest injecting *ptf1a* mRNA into transgenic embryos that expressed GFP under the control of a pancreas-specific marker or anterior endodermal marker. Dissection of the GFP-positive tissue could then be performed more precisely. Alternatively, FACS sorting could be used to isolate GFP-positive cells. A third flaw was collecting the two time-points from separate clutches of embryos, and using a different microarray chip for each time-point. While different microarray chips are no longer an issue with the advancements of RNA-seq, if possible I would suggest

collecting time-points from the same clutch for each replicate then using deep sequencing to identify targets.

Despite the flaws in the experimental design, the Ptf1a microarrays described in Chapter 4 still provide information about the molecular network activated by Ptf1a. We identified over 1000 genes over two time-points during early pancreatic specification that are likely involved in the gene regulatory network downstream of Ptf1a in pancreas development. I demonstrated that many of these targets were expressed within the developing endoderm and that their expression was increased in the endoderm by *ptf1a* mRNA overexpression. I hypothesize that targets identified in the earlier time-point are potentially novel direct targets of Ptf1a. In order to confirm that the identified genes are direct targets of Ptf1a, chromatin immunoprecipitation (ChIP) will need to be performed. Direct binding of Ptf1a to the chromatin surrounding the potential targets would provide evidence that they may be direct. Additional evidence, including mutating potential sequences within the promoter bound by Ptf1a and electrophoretic mobility shift assays (EMSA) would be required to confirm that Ptf1a directly promotes expression of novel targets identified in the microarray. A better understanding of the regulatory network involved in Ptf1a directed pancreas development would provide insight it how mature pancreatic cells could be altered to regain multipotency to produce β-cells.

CONCLUSION

The ultimate goal behind studying the progression from germ layer specification to organ formation is to generate a complete model of the molecular pathway involved in endoderm development, in multiple model species. This will require large-scale high-throughput approaches such as RNA-seq on different tissues throughout development. Following on this by detailed biochemical analysis will be required to determine how factors may function together. Ultimately, a complete understanding will allow researchers to produce endoderm *in vitro* using stem cells or by transdifferentiation from other mature cells, which would provide tremendous therapeutic promise for many diseases. A complete model for the complex regulatory network would also allow understanding of the pathology of endodermal diseases likely leading to a more streamline and predictable path to drug discovery.

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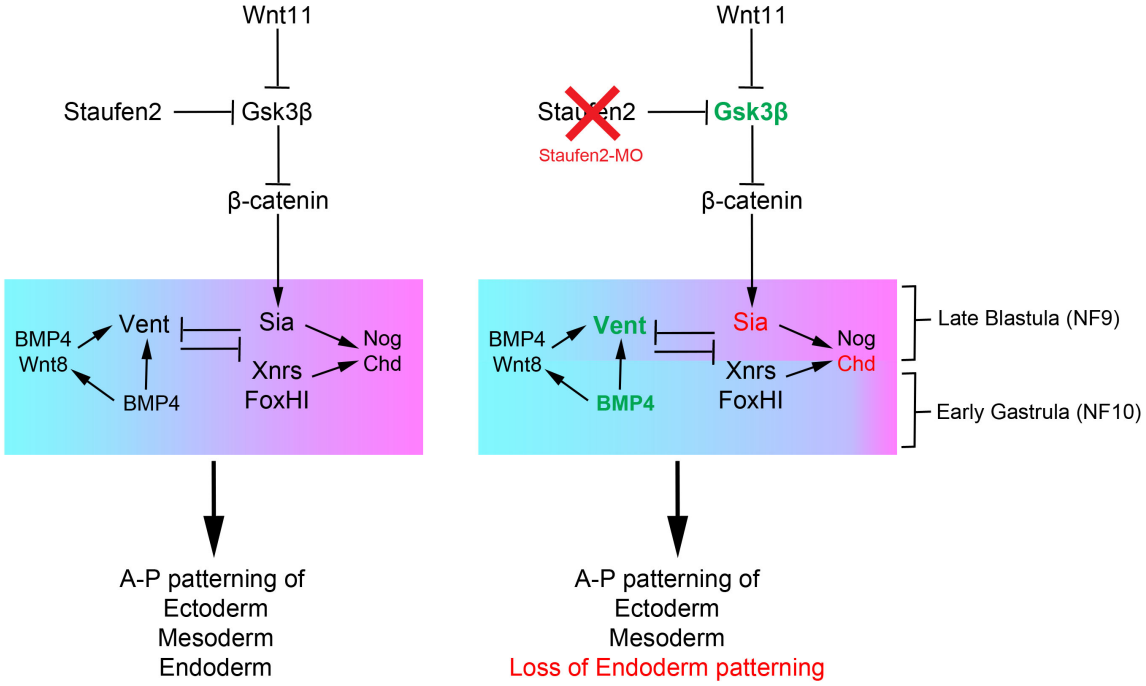

Figure 1. Model of Stau2 regulation on endoderm development. The Wnt11 signaling pathway activates Wnt effector proteins Siamois and Twin in the dorsal-anterior half of the embryo shown in purple half. The Wnt signaling pathways along with the Nodal signaling pathway activate Chordin and Noggin along with organizer transcription factors in the dorsal margin. Repression of dorsal-anterior factors by BMP, zygotic Wnt8 and Vents in the ventral-posterior half of the embryo is shown in the blue half. This leads to the proper patterning of all three germ layers. When Stau2 is knockeddown by morpholino-injection into the dorsal-vegetal cells of an eight-cell embryo the Wnt11 signaling pathway is initiated (NF9) but not maintained (NF10). Knockdown of Stau2 leads to a loss of maintenance of *siamois* expression at NF10 and of the expression domain of *chordin* within the dorsal margin of the embryo shown in red. Expression of ventral-posterior genes *bmp4* and *vent1/2* are expanded dorsally, shown in green. The loss of maintained Wnt11 signaling effector proteins leads to the loss of anterior endoderm patterning without any effect on ectoderm or mesoderm patterning.

Appendix: Xenopus Pancreas Development

Pearl, EJ, **Bilogan, CK**, Mukhi, S, Brown, DD, Horb, ME, 2009. *Dev. Dyn*. 238, 1271–1286.

I wrote the sections titled “Morphological Development” and “Pancreatic Specification (after NF30)” and I performed some of the *in situs* in Fig. 3-5.

SPECIAL ISSUE REVIEWS–A PEER REVIEWED FORUM

Xenopus Pancreas Development

Esther J. Pearl,[1–3] Cassandra K. Bilogan,[1,3,4] Sandeep Mukhi,[5] Donald D. Brown,[5] and Marko E. Horb[1–4*]

Understanding how the pancreas develops is vital to finding new treatments for a range of pancreatic diseases, including diabetes and pancreatic cancer. *Xenopus* is a relatively new model organism for the elucidation of pancreas development, and has already made contributions to the field. Recent studies have shown benefits of using *Xenopus* for understanding both early patterning and lineage specification aspects of pancreas organogenesis. This review focuses specifically on *Xenopus* pancreas development, and covers events from the end of gastrulation, when regional specification of the endoderm is occurring, right through metamorphosis, when the mature pancreas is fully formed. We have attempted to cover pancreas development in *Xenopus* comprehensively enough to assist newcomers to the field and also to enable those studying pancreas development in other model organisms to better place the results from *Xenopus* research into the context of the field in general and their studies specifically. *Developmental Dynamics 238:1271–1286, 2009.* © 2009 Wiley-Liss, Inc.

Key words: Xenopus; pancreas; RA; Wnt; TGFβ; Pdx1; Ptf1a; metamorphosis; thyroid hormone

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INTRODUCTION

The pancreas is an important organ in the digestive system. It is located next to the stomach and is composed of two types of cells, endocrine and exocrine, which secrete hormones, such as insulin and glucagon, and digestive enzymes, such as trypsin, respectively (Fig. 1). In *Xenopus*, the endocrine cells are arranged in clusters in the mature pancreas, and the exocrine cells in a floral pattern (Fig. 1D). The main disease associated with a malfunctioning pancreas is diabetes, a disease whereby sufferers are unable to store sugar from their bloodstream due to defects in either insulin production or recognition. Other diseases affecting the pancreas include exocrine pancreatic insufficiency, where the digestive enzymes are not made and the patient cannot properly digest food; pancreatitis, the inflammation of the pancreas, which can be caused by many conditions including cystic fibrosis and alcoholism; and pancreatic cancer, one of the most lethal cancers with a 5-year survival rate below 5% (Hezel et al., 2006; Nair et al., 2007; Maitra and Hruban, 2008).

By understanding the way the pancreas forms and what genes are involved in its development, it is envisioned that new drug targets for the treatment of these diseases will be discovered or that techniques to produce *insulin*-expressing cells de novo will be developed. Most of the research in pancreas development thus far has been performed in more traditional model organisms, such as mouse and chick (Kume, 2005; Jorgensen et al., 2007), but recently more studies have been performed in other model organisms such as *Xenopus* and zebrafish. The benefits of using these organisms are numerous: their pancreases develop faster, the number of animals is

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TABLE 1. Comparison of Major Morphological Events in Pancreas Development Between *Xenopus*, Mouse, Chick, and Zebrafish*

	Xenopus laevis	*Mus musculus*	*Gallus gallus*	*Danio rerio*
Regional specification of endoderm	NF11–20	E7.5–8	HH5–8	
Pre-pancreatic region specified	NF27–30 expression of Pdx1[a–c]	E8.5–8.75 (8–10 ss)[g]	HH10 (10 ss)[g]	14 hpf, expression of Pdx1[j,k]
Dorsal pancreatic bud emerges	NF35/36[d]	E9.5–10.5 (20–25 ss)[h]	HH16 (26 ss)[g,i]	24 hpf[l]
Ventral pancreatic buds emerge	NF37/38[d]	E10.25 (30 ss)[h]	HH22–23[g,i]	40 hpf[l]
Ventral pancreatic buds fuse	NF40[d,c]	n/a (one bud regresses)		n/a (only one ventral bud)
Dorsal and ventral pancreatic buds fuse	NF40[d,e]	E12.5		52 hpf[l]
β-cell clusters/islets formed	NF66[f]	E18.5[h]		5.5 dpf[d]

*NF, Nieuwkwoop and Faber stages of *Xenopus laevis* development; ss, somite stage; E, embryonic day; HH, Hamburger and Hamilton stages of chick development; hpf, hours post-fertilization; dpf, days post-fertilization.
[a]Horb and Slack (2001). [b]Zeynali et al. (2000). [c]Afelik et al. (2006). [d]Kelly and Melton (2000). [e]Afelik et al. (2004). [f]Maake et al. (1998). [g]Kim et al. (1997a). [h]Slack (1995). [i]Kim et al. (1997b). [j]Biemar et al. (2001). [k]Ober et al. (2003). [l]Field et al (2003).

not limiting as large numbers of embryos can be injected and processed in a single day, early development is more accessible allowing easy isolation of endodermal or pancreatic tissue, overexpression studies are accomplished by simple microinjection of mRNA (targeting to specific regions of the endoderm is done by injection into single blastomeres at the 8-, 16-, or 32-cell stage), transgenic tadpoles can be produced quickly, gene knockdown studies can be performed quickly using antisense morpholinos, and earlier patterning and specification events can be addressed more easily. Indeed, recent results demonstrate that early pancreas development in *Xenopus* closely resembles that of mice and humans, and is applicable to mammalian cells as we previously demonstrated (Horb et al., 2003; Cao et al., 2004; Li et al., 2005; Afelik et al., 2006; Blitz et al., 2006; Jarikji et al., 2007). In fact, it is becoming clear that the same genes used in mammalian pancreas development are involved in *Xenopus* pancreas development (Horb et al., 2003; Afelik et al., 2006; Jarikji et al., 2007).

With the emergence of *Xenopus* as a model system for pancreas development, now is the appropriate time for a comprehensive review of *Xenopus* pancreas development to be written. Although there have been several recent reviews that have touched on some aspects of *Xenopus* pancreas development (Blitz et al., 2006; Pieler and Chen, 2006; Spagnoli, 2007; Zorn and Wells, 2007; Pearl and Horb, 2008), none has comprehensively covered pancreas development in *Xenopus* from gastrulation through metamorphosis. This review aims to give detailed insight into pancreas development in *Xenopus laevis* from the end of gastrulation, when the endoderm becomes regionalized, through the emergence of pancreatic buds, to the cellular rearrangements that take place at metamorphosis resulting in a mature pancreas. We have tried to make this review differ from the numerous other pancreas reviews that have already been published, and have focused on the current state of pancreas development in *Xenopus*. Since pancreas development in *Xenopus* is a new topic, some aspects have not been covered in depth, and for more detail we refer the reader to some of the recent excellent reviews that have been written on pancreas development (Jensen, 2004; Cano et al., 2007; Murtaugh, 2007; Gittes, 2009).

MORPHOLOGICAL DEVELOPMENT

As with other vertebrates, the pancreas in *Xenopus* first arises as separate dorsal and ventral pancreatic buds, which subsequently fuse to give rise to the mature pancreas (Fig. 2). The dorsal pancreatic anlage is first visible at NF35/36 on the dorsal surface of the endoderm at the level of the pronephros (Table 1) (Nieuwkoop and Faber, 1967; Kelly and Melton, 2000). In this review, we routinely refer to the stages described by Nieuwkoop and Faber as NF followed by the stage number (Nieuwkoop and Faber, 1967). At NF35/36, the shape of the dorsal pancreas resembles a crescent straddling the dorsal endodermal midline. In contrast, the ventral pancreas develops from two symmetrical (left and right) anlagen that first become apparent at NF37/38, at the junction of the liver bud and developing duodenum. The two ventral buds fuse together around NF40 (Table 1) and look like two half circles joining together; their fusion, however, is not complete as a space between these two ventral buds remains for a short period (Kelly and Melton, 2000). It is here that the hepatopancreatic duct emerges. By stage 40, the dorsal and ventral pancreatic anlagen have fused; the fusion occurs at the junction of the right side of the dorsal pancreatic bud with the dorsal aspect of the fused ventral buds. From NF42 to 48, the intestine coils dramatically, and as a result the pancreas is displaced to the right side of the embryo (Fig. 2D). After fusion, the pancreas can now be isolated, along with the liver, separate from the intestine; the morphology of an isolated pancreas at NF40 is thin and long, it is also slightly curved in a horseshoe-type shape (Fig. 3A). By NF44, the pancreas appears more compact; it is shorter and wider than when it had just fused (Fig. 3B). This reduction in the length of the pancreas continues as the tadpole ages to NF46 when the pancreas has transformed from a rectangular shape to a square (Fig. 3C). The main difference between *Xenopus* and mammalian gut

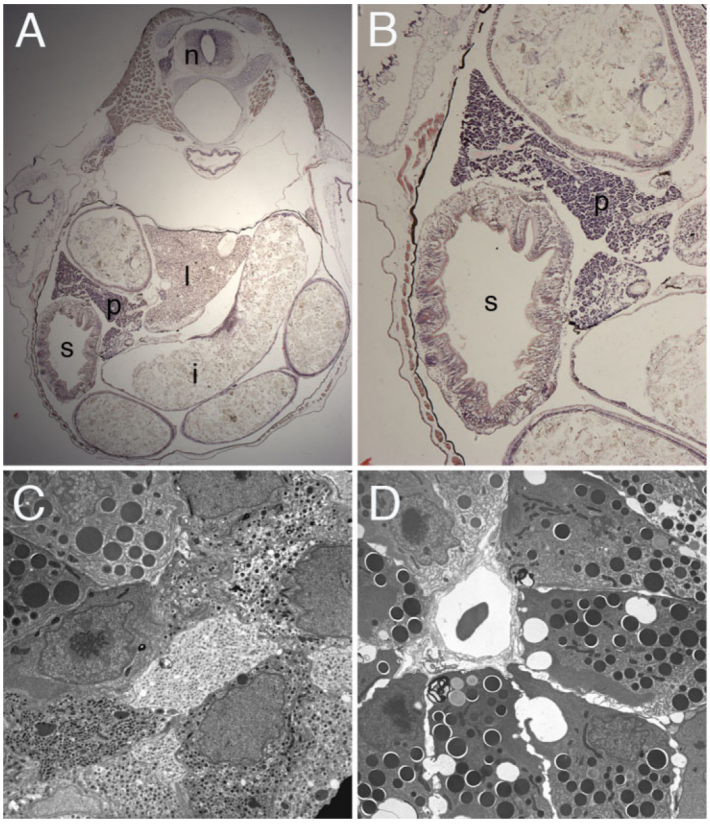


Fig. 1.

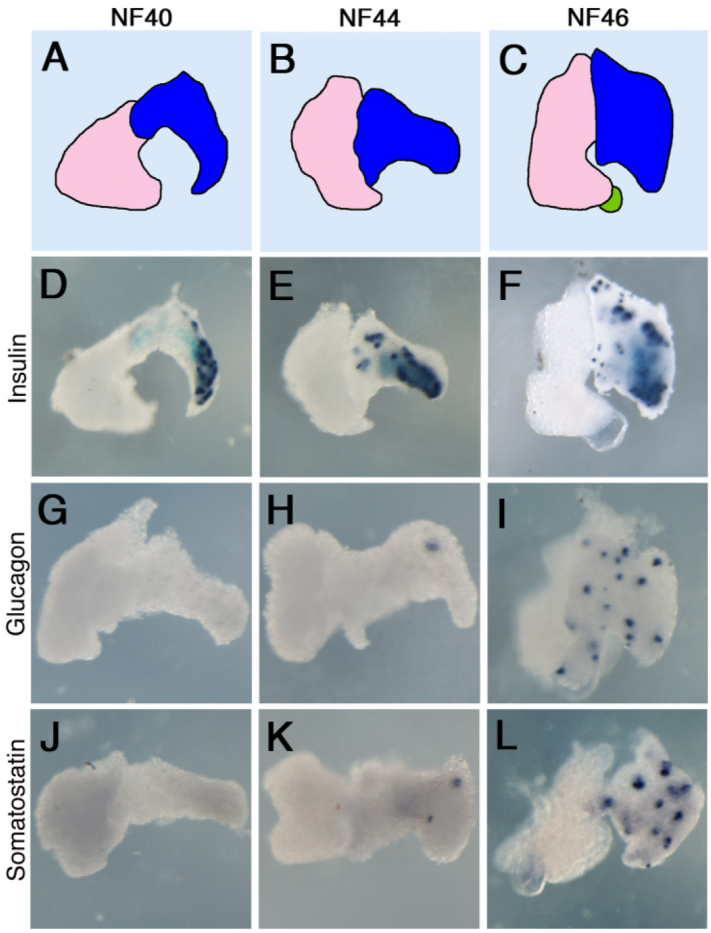


Fig. 3.

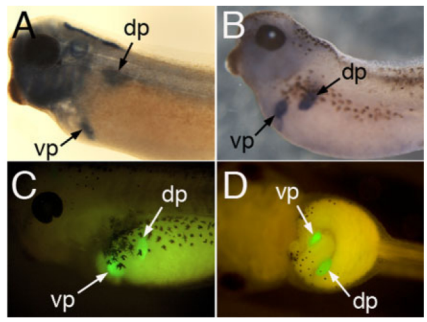


Fig. 2. Developing pancreatic buds. **A:** NF32 tadpole showing *Ptf1a* RNA expression in the dorsal and ventral pancreatic buds prior to overt morphogenesis. **B:** Ptf1a expression in a NF40 tadpole, after buds have fused. **C,D:** Transgenic tadpoles expressing GFP under the control of the elastase promoter at (C) NF40 and (D) NF42. A–C: Anterior to the left, dorsal up (lateral view). D: Anterior to the left, ventral view. vp, ventral pancreas; dp, dorsal pancreas.

development is that the mammalian gut forms a tube early in development, from which the pancreatic buds emerge (reviewed in Jorgensen et al., 2007), whereas in *Xenopus* the gut does not form a tube until after the pancreatic buds have emerged and fused, around NF40 (Chalmers and Slack, 1998). The early morphological differences may be due to the different ways the embryos feed at these stages. *Xenopus* embryos develop ex vivo and require an external nutrient source (which is the yolk in the endoderm at this stage of development), while mice develop in vivo, gaining nutrients from their mother's blood supply.

Fig. 1. Tadpole and froglet pancreas histology. **A:** Transverse section of a tadpole (NF55), showing the positioning of the pancreas relative to the rest of the gut. n, notochord; s, stomach; p, pancreas; l, liver; i, intestine. **B:** Higher magnification of the lower left-hand corner of A. **C:** Electron micrograph image (×4,500) of froglet pancreas endocrine cells. At least three different endocrine cells are visible, with two acinar cells in the top left corner. **D:** EM image (×2,500) of exocrine cells from the same froglet pancreas, illustrating the floral/floret pattern of acinar cells, surrounding a duct.

Fig. 3. Endocrine marker expression in isolated liver and pancreas tissue. **A–C:** Diagrammatic representations of the livers and pancreases: pink, liver; bright blue, ventral pancreas; dark blue, dorsal pancreas; green, gall bladder. **D–L:** Whole mount in situ hybridization for insulin, glucagon, and somatostatin at NF40, NF44, and NF46.

Endocrine Cells

The endocrine pancreas is composed of 5 different cell types—α, β, δ, ϵ, and PP—that secrete glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide hormones into the bloodstream. In *Xenopus*, four of these cell types (α, β, δ, and PP) have been identified in the pancreas, and we will describe their development and differentiation in the next few paragraphs.

The first endocrine marker to be expressed in the pancreas at NF32 is *insulin*. RNA expression is first detected in the dorsal endoderm (where the future dorsal pancreatic anlage emerges) in a punctate manner at NF32. As the pancreas develops, expression initially remains localized to the dorsal pancreas, even after fusion of the dorsal and ventral pancreatic buds at NF40 (Fig. 3D,E). However, by NF46/47 insulin is expressed throughout the entire pancreas, including the ventral pancreas (Fig. 3F) (Kelly and Melton, 2000; Horb and Slack, 2002). Similarly, insulin protein expression is first detected only in the dorsal pancreas at NF39. At these early stages, insulin-expressing cells are found grouped as single cells or clusters of 2–4 cells.

Glucagon and somatostatin, unlike insulin, are not expressed in the pancreas until after fusion of the dorsal and ventral buds, but show similar expression profiles (Maake et al., 1998; Kelly and Melton, 2000; Horb and Slack, 2002). Expression is first detected at NF44/45 marking individual cells, initially in the dorsal portion of the pancreas (Fig. 3H,K). By NF46/47, expression of both markers is now detected throughout the entire pancreas (Fig. 3I,L). However, prior to expression in the pancreas, both *somatostatin* and *glucagon* are initially expressed at NF41 within the stomach and duodenum in a punctate pattern.

The two other endocrine cell types present within the pancreas are PP cells, producing pancreatic polypeptide (PP) and ϵ cells, producing ghrelin. In *Xenopus*, the cDNAs for these two genes remain to be cloned, although genomic sequence for the PP gene is found in the *X. tropicalis* genome. Antibodies to mammalian PP do detect scattered expression in mature *Xenopus* pancreas beginning at NF46 (Maake et al., 1998); the majority (79%) of these PP-expressing cells also co-express *glucagon*. Ghrelin-producing cells are yet to be discovered in *Xenopus*.

Exocrine Cells

The mammalian exocrine pancreas is a lobulated branched tissue, consisting of acinar and ductal cells that secrete and transport digestive enzymes into the duodenum. The expression of acinar differentiation markers follows a temporal and spatial distribution at the early stages of pancreas development, opposite to that seen with *insulin*. Expression of most exocrine markers, including *amylase, trypsinogen* and *elastase*, is first detected at NF41 exclusively in the ventral pancreas (Fig. 4A). Shortly thereafter, their expression diffuses into the dorsal pancreas, such that by NF45 expression is detected throughout the entire pancreas (Fig. 4B) (Horb and Slack, 2002). A fourth exocrine marker, carboxypeptidase A, was detected by immunostaining throughout the entire pancreas at NF44 (Kelly and Melton, 2000). In contrast to these markers, expression of a fifth exocrine marker, *pancreatic protein disulfide isomerase (XPDIp)*, is first observed at NF39 in both dorsal and ventral pancreatic buds (Afelik et al., 2004). The reason for these differences is unclear, although it may be that *XPDIp* is simply expressed at an earlier stage in acinar cell differentiation, and its expression in the dorsal pancreas may be marking exocrine precursor cells that will subsequently express the other late differentiation markers as indicated above. As with other model systems, there is very little known about the development of the ductal system in *Xenopus*.

INDUCTION OF THE PANCREAS (NF11–30)

The pancreas, like organs such as the lungs, liver, stomach, and colon, develops from the endodermal germ layer. One of the first things to occur once the endoderm layer is formed is the specification of anterior and posterior regions of the endoderm. Although this occurs before the end of gastrulation, stable regional specification is not achieved until much later (Table 1) (Zeynali et al., 2000; Horb and Slack, 2001; McLin et al., 2007). Thereafter, during neurula and tail bud stages, these regions are further subdivided into discrete organ domains. It is only after stable regional specification occurs that specific organs, such as the pancreas, develop.

Early investigations into regional specification of the gut endoderm in amphibians were performed in *Triturus pyrrhogaster* (a urodele amphibian); these studies demonstrated that signals from the mesoderm were necessary and sufficient to specify regions of the gut endoderm (Okada, 1960). Initial molecular studies in *Xenopus* seemed to suggest otherwise, that endoderm differentiation did not require input from other cell layers (Gamer and Wright, 1995; Henry et al., 1996). These studies examined the differentiation of endoderm in vegetal explants from blastula stage embryos and found that at tail bud and tadpole stages, expression of pancreas and liver markers was detectable in the absence of axial mesoderm (Gamer and Wright, 1995). However, when these vegetal explants were examined for expression of other mesoderm markers, they were found to express more lateral mesoderm markers (Horb and Slack, 2001). The presence of mesoderm in these vegetal explants was due to the fact that at blastula stages, the mesoderm and endoderm are not cleanly separable. When endoderm explants were isolated at later neurula and early tail bud stages (NF15–25), endodermal differentiation did not occur; at this stage, the mesoderm is completely separate from the endoderm (Horb and Slack, 2001). Further evidence for the importance of mesoderm was provided by endoderm-mesoderm recombinations (i.e., anterior endoderm with posterior mesoderm) wherein the differentiation state of the endoderm (anterior vs. posterior) depends on the character of the mesoderm (Horb and Slack, 2001; McLin et al., 2007). Stable regional specification of the endoderm (independent of mesoderm signals) is not established until late tail bud stages, between NF28 and 32 (Zeynali et al., 2000; Horb and Slack, 2001; McLin et al., 2007). However, many of

the important inductive signals involved in specifying anterior and posterior foregut occur prior to stable regional specification, during gastrula and neurula stages.

In the next section, we briefly review the data implicating that some of these signaling pathways are involved in early specification of the endoderm leading to a pancreatic cell fate. For more detail about these early signaling pathways and the similarities between *Xenopus* and mammals, we refer the reader to a recent review on this topic (Zorn and Wells, 2007).

RA Signaling

One of the earliest molecules required for regional specification of the endoderm leading to a pancreatic fate is retinoic acid (RA). Animal caps treated with high doses of Activin A contain both mesoderm and endoderm, but even at the highest doses of Activin A, pancreatic tissue is only rarely induced. (Animal cap tissue is naive ectoderm removed from blastula stage embryos that can be induced to form every tissue in the tadpole, and is similar to embryonic stem cells.) Addition of RA to the Activin-treated animal caps increased the amount of pancreatic tissue substantially (Moriya et al., 2000; Asashima et al., 2008). Interestingly, the timing of RA addition was found to be critical to the induction of pancreas; only when RA was added several hours after Activin A treatment was a large amount of pancreatic tissue induced in the animal caps. However, caution should be taken when interpreting data from in vitro induction of genes in animal caps or ES cells, as these in vitro events may not necessarily be representative of the in vivo reality. It is, therefore, important to note the effects of RA on pancreas development in the whole animal.

Indeed, whole animal experiments confirmed the animal cap results showing that RA is required for regional specification in vivo, although it has different effects on dorsal versus ventral pancreas. Inhibition of RA at gastrula stages causes a loss of both endocrine and exocrine marker expression later in development, whereas excess RA stimulates endocrine cell differentiation in the dorsal pancreas (at the expense of exocrine cells), and exocrine cell differentiation in the ventral pancreas (Chen et al., 2004). In agreement with the animal cap data, the timing of RA signaling is critical; treatments at gastrula stages (NF11–13) affect pancreas development, but in embryos treated after NF13, the pancreas develops normally.

It is interesting to note that RA only affects dorsal-anterior and not ventral-posterior endoderm (Pan et al., 2007). This differential sensitivity to RA signals between dorsal and ventral endoderm explants can be explained by the spatial restriction of certain RA receptors (RARs). RARγ2.1 has high expression in the dorsal mesoderm, but low expression in the ventral mesoderm, and overexpression of RARγ2.1 in ventral endoderm-mesoderm explants makes them responsive to exogenous RA (Pan et al., 2007). Another factor acting in opposition to RA is BMP; BMP signaling is high ventrally and low dorsally. When BMP signaling was inhibited in ventral endoderm-mesoderm explants, RA was then able to induce pancreatic tissue. Furthermore, the BMP inhibitor Noggin induces the transcription of *RALDH2*, a gene involved in the biosynthesis of RA (Pan et al., 2007). These results all show that RA is required for the specification of endoderm at early stages, but is not sufficient to promote pancreatic fates on its own.

Wnt Signaling

Following the requirement for RA signaling at late gastrula stages in specifying anterior endoderm, the next pathway implicated in regional specification is the Wnt signaling pathway. In contrast to the positive requirement for RA signaling in promoting development of the anterior endoderm, it is the inhibition of Wnt signaling at neurula stages that is essential for proper foregut development; in contrast, activation of Wnt signaling is necessary for posterior endoderm development. Ectopic activation of Wnt signaling in anterior endoderm (β-catenin) is sufficient to prevent foregut specification, while inhibition of Wnt signaling in posterior endoderm (Gsk3β) promotes ectopic foregut development (McLin et al., 2007). Furthermore, the temporal requirement for Wnt was found to differ for anterior versus posterior endoderm. Wnt signaling must be inhibited in anterior endoderm between NF11–20, whereas activation of Wnt signaling in posterior endoderm is required between NF11–13 (McLin et al., 2007). These studies, however, do not directly address whether Wnt signals act directly on the endoderm or whether they respecify mesoderm first.

To address the question of which germ layer Wnt is acting in, McLin et al. (2007) used the Einsteck method. In this procedure, a piece of labeled tissue is implanted into the blastocoelic cavity of a gastrula-stage embryo, and the final fate of the donor tissue is examined at a later stage. In this instance, the donor tissue was either anterior or posterior endoderm (expressing β-catenin or Gsk3β). In control conditions, anterior endoderm contributed to liver and stomach, while posterior endoderm contributed to intestine. However, when anterior endoderm expressing β-catenin was transplanted, the final fate of these cells was now intestine. In contrast, posterior endoderm expressing Gsk3β contributes to liver and stomach (McLin et al., 2007). In agreement with this, the Wnt antagonist *secreted frizzled-related protein 5* (*Sfrp5*) is expressed in the anterior endoderm beginning at NF15, is required for proper foregut specification, and acts directly to prevent signaling by Wnt11 (Li et al., 2008).

TGF-B/BMP

Prior to gastrulation, TGF-β signaling plays an important role in endodermal germ layer induction (Henry et al., 1996; Zorn et al., 1999; Afouda et al., 2005), but its role in later events of endodermal specification has only recently been investigated. TGF-β induced factor 2 (TGIF2) was identified as a modifier of early endoderm, and required for pancreas development (Spagnoli and Brivanlou, 2008). TGIF2 was identified in a microarray screen for targets of GATA5, which acts downstream of TGF-β in promoting an endoderm fate (Weber et al., 2000; Spagnoli and Brivanlou, 2008). TGIF2, a BMP antagonist, was found to act as a modifier of the endoderm to promote more anterior fates; overexpression of *TGIF2* in ventral vegetal explants is sufficient to activate pan-

creatic gene expression. This is consistent with the results of Pan et al. (2007); both studies demonstrated that inhibition of BMP signaling plays an important role in endodermal patterning, which leads to pancreas development. The exact stage and in what germ layer TGIF2 acts is currently unknown. In agreement with this, recent studies using activin-treated animal caps have identified genes acting in the TGFβ pathway to effect endoderm regionalization (Weber et al., 2000; Afouda et al., 2005). In addition to the early role of TGFβ signaling in endoderm induction prior to gastrulation, these results implicate TGF-β signaling in later events of endodermal patterning and regionalization during neurula and tail bud stages.

Intracellular Factors

One of the benefits of *Xenopus* embryos is the ability to identify new genes (or known genes not previously implicated in pancreas development) without a priori knowledge of either them or their role in pancreas development through gain-of-function screens. For example, using an expression cloning screen and injecting pools of mRNAs into vegetal blastomeres and looking for ectopic *insulin* expression the 3′UTR of *Shirin* was found to be sufficient to induce ectopic *insulin* expression (Spagnoli and Brivanlou, 2006). Secondarily, they found that the RNA binding protein, Vg1RBP, bound the 3′UTR of shirin. (*Vg1RBP* is best known for being expressed early in embryogenesis and regulating *Vg1* mRNA localization.) In the context of pancreas development, Vg1RBP was found to be essential for proper development of insulin-expressing cells, but its necessity for acinar cell development was not examined. Although on its own, Vg1RBP is unable to promote endoderm development, it respecifies more posterior-ventral endoderm to become more dorsal-anterior.

Regional Specification of the Endoderm: Summary

At the end of gastrulation, once the endoderm has been established, regionalization subdivides the endoderm into discrete anterior and posterior domains. To produce stable regional identity, continuous endoderm-mesoderm interactions are required until late tail bud stages. Distinct signaling pathways operate in subsequent stages to pattern the endoderm. Initially, RA signaling immediately at the end of gastrulation between NF11 and NF13 is critical for proper pancreas development. Subsequently, Wnt (and BMP) signaling must be inhibited between NF11 and NF20 for proper development of anterior endoderm; active Wnt signaling promotes posterior endoderm development. Thus, although the signals required for patterning the endoderm occur relatively early, pancreatic cell fates are not established until after NF30 when pancreas-specific genes are activated.

PANCREATIC SPECIFICATION (AFTER NF30)

As outlined above, the majority of research into *Xenopus* pancreas development has been focused on the early patterning and specification events that occur prior to NF30. The fact that *Xenopus* can be used to investigate the function of "late-acting" pancreas-specific genes has only recently become appreciated. From these studies, it is becoming clear that *Xenopus* is a valuable model system for elucidating the molecular mechanisms underlying pancreatic cell fate specification; including identifying new genes, determining their function, defining transcriptional regulatory networks, and more specifically identifying candidate genes for diabetes.

Ptf1a and Pdx1

Two important genes in pancreas development that have been studied in *Xenopus* include *Pdx1* and *Ptf1a*. Both genes are expressed in pancreatic progenitors, and are necessary and sufficient for pancreas development (Horb et al., 2003; Afelik et al., 2006; Jarikji et al., 2007). In fact, ectopic expression of both simultaneously converts posterior endoderm into pancreatic tissue (Afelik et al., 2006). The bHLH pancreas-specific transcription factor 1a (PTF1a) is one of the earliest pancreas-specific markers. Loss of Ptf1a function in mice and humans results in the complete absence of acinar cells and a large reduction in insulin expression (Krapp et al., 1996; Kawaguchi et al., 2002; Sellick et al., 2004), whereas in zebrafish it is required mainly for exocrine pancreas development (Zecchin et al., 2004; Lin et al., 2004). In *Xenopus*, ptf1a is detected as early as NF27/30 in the developing dorsal and ventral pancreatic anlagen (Fig. 2A) (Afelik et al., 2006). It is expressed in the tadpole acinar cells until the climax of metamorphosis when the PTF1a gene is shut off. Its expression returns at the end of metamorphic climax and remains acinar-specific (Mukhi et al., 2008). This expression is identical to that seen in mice, where *Ptf1a* is expressed in all pancreatic progenitors at the earliest stages of pancreas development, but becomes localized to acinar cells shortly thereafter (Krapp et al., 1996; Kawaguchi et al., 2002).

Two studies examined the function of *Xenopus Ptf1a* in early pancreas development and found that *Ptf1a* was both necessary and sufficient for pancreas development. Both studies found that knockdown of *Ptf1a* resulted in a complete loss of acinar cells; however, phenotypic differences regarding endocrine cell development were found (Afelik et al., 2006; Jarikji et al., 2007). In the first study, a single morpholino was used to knockdown *Ptf1a,* and early expression of *insulin* at NF35 was unaffected, while the late expression of both *insulin* and *glucagon* was lost at NF48 (Afelik et al., 2006). The second study, using two morpholinos to target distinct regions of the Ptf1a transcript, found the exact opposite: knockdown of *Ptf1a* resulted in an absence of early *insulin* expression, while low levels of *insulin* were detected at later stages (Jarikji et al., 2007). The reason for this discrepancy is currently unclear. Nevertheless, these studies show that the function of Ptf1a in *Xenopus* pancreas development is quite similar to that observed in other vertebrates where Ptf1a is required for development of all acinar cells and a subset of endocrine cells.

The homeobox gene *Pdx1* was originally cloned in *Xenopus* 20 years ago and called *XlHbox8* (Wright et al., 1989). It is expressed in a broad domain in the anterior endoderm beginning at NF27, encompassing the de-

veloping dorsal and ventral pancreatic buds as well as parts of the stomach and duodenum (Wright et al., 1989; Afelik et al., 2006). Low levels of *Pdx1* are detected by RNase protection analysis as early as NF12.5, and by RT-PCR at NF19/21 (Gamer and Wright, 1995; Horb and Slack, 2001). Morpholino knockdown of *Pdx1* in *Xenopus* resulted in the complete loss of exocrine markers, but had little effect on *insulin* expression (Afelik et al., 2006). These results are in contrast to the defects observed in mice and humans, where lack of Pdx1 resulted in pancreas agenesis (Ohlsson et al., 1993; Jonsson et al., 1994; Stoffers et al., 1997; Schwitzgebel et al., 2003). The defect seen in *Xenopus* is similar to that observed in mice where although loss of Pdx1 leads to pancreatic agenesis, there is a small dorsal bud present that produces insulin and glucagon (Ahlgren et al., 1996).

One of the benefits of *Xenopus* is the relative ease in which gain-of-function studies can be performed. In addition to these loss-of-function studies showing that Ptf1a and Pdx1 are necessary for pancreas development, ectopic overexpression experiments revealed that both were also sufficient to promote ectopic pancreatic cell fates. Overexpression of *Pdx1* in naïve endoderm or transgenic overexpression of *Pdx1* in the liver after NF44 did not result in the activation of pancreatic differentiation markers (Horb et al., 2003; Afelik et al., 2006). However, it should be noted that some studies in mammals have found that Pdx1 alone is sufficient to promote ectopic pancreatic cell fates (Ferber et al., 2000; Ber et al., 2003; Sapir et al., 2005; Shternhall-Ron et al., 2007). In contrast, overexpression of *Pdx1-VP16*, an activated form of *Pdx1*, was sufficient to promote ectopic endocrine and acinar cell fates in the developing liver; no effect was seen in the intestine (Horb et al., 2003). The relevance to mammals was shown by the fact that this same transgene was able to convert mammalian HepG2 cells to pancreas (Horb et al., 2003; Li et al., 2005). Subsequent studies have confirmed these results. In particular, *Pdx1-VP16* was found sufficient to convert rat liver cells into immature beta cells that were able to restore euglycemia in diabetic mice (Cao et al., 2004; Tang et al., 2006). The reasons for this difference between Pdx1 and Pdx1-VP16 are unclear, although it is reasonable to assume that the addition of the VP16 activation domain allows for interactions with other proteins creating a favorable environment in which Pdx1 can activate transcription of its downstream targets.

Overexpression of *Ptf1a*, on the other hand, was found sufficient to promote ectopic pancreatic cell fates. Injection of *Ptf1a* mRNA into the two dorso-vegetal blastomeres at the eight-cell stage (targeting the anterior endoderm) resulted in the conversion of stomach/duodenum to pancreas (Afelik et al., 2006; Jarikji et al., 2007). Using a hormone-inducible version (*Ptf1a-GR*), it was found that when *Ptf1a* was activated at NF27 or earlier, it was able to promote ectopic pancreatic cell fates, whereas when activated after NF36, it had no effect (Afelik et al., 2006). Activation of *Ptf1a* between NF30 and 35 did not convert stomach/duodenum to pancreas, but did result in an enlarged pancreas. In contrast, *Ptf1a-VP16* was found to have greater activity than the unmodified Ptf1a in naive endoderm. In addition to being able to convert prospective stomach/duodenum to pancreas as seen with unmodified Ptf1a, Ptf1a-VP16 was also able to convert prospective liver to pancreas. However, only the acinar cell fate was promoted (Jarikji et al., 2007). No effect was seen with either *Ptf1a* or *Ptf1a-VP16* when overexpressed in posterior endoderm. Similar results were also found when *Ptf1a* or *Ptf1a-VP16* was ectopically expressed at later stages after the organs had formed. Transgenic overexpression of *Ptf1a-VP16* in *Xenopus* tadpoles was sufficient to convert liver to acinar cells (but had no effect in the stomach or duodenum), while *Ptf1a* converted stomach/duodenum to endocrine and acinar cell fates (but had no effect in liver) (Jarikji et al., 2007). These results confirm earlier data in mice where it was shown that loss of Hes1 induces ectopic expression of Ptf1a in the stomach, duodenum, and common bile duct resulting in the production of ectopic patches of pancreatic tissue (Fukuda et al., 2006).

Other Factors

There are numerous other transcription factors that have been well studied in pancreas development in other species, but little is known about their role in *Xenopus* pancreas development. Many of these genes, however, have been cloned in *Xenopus* and below we briefly describe what is known about their expression patterns in the developing *Xenopus* pancreas. We also provide a short description of their function in pancreas development based on results from other animals.

HNF6.

In mice, *Hnf6* is one of the earliest markers of the developing anterior endoderm, and is expressed in a broad domain of the anterior endoderm marking the developing pancreas and liver (Jacquemin et al., 2003). In Hnf6 mutant mice, *Pdx1* expression is delayed and the pancreas was hypoplastic (Jacquemin et al., 2000, 2003). We recently cloned *Xenopus Hnf6* and found it to be expressed in a similar pattern as in mice (data not shown). At NF30, *Hnf6* is expressed in a broaddomain in the anterior endoderm marking the developing pancreas and liver, while at later stages it is found exclusively in the liver.

Sox9.

Sox9 is expressed in several different tissues and is implicated in the regulation of various stem/progenitor cells (Lynn et al., 2007; Seymour et al., 2007). In the pancreas, it is expressed in undifferentiated progenitor cells, and is involved in regulating their proliferation; loss of *Sox9* in the pancreas results in hypoplastic pancreas (Seymour et al., 2007). In *Xenopus,* expression is first detected at NF25 in two regions of the undifferentiated endoderm: dorsally in the prospective foregut and ventrally on each side of the future liver diverticulum (Lee and Saint-Jeannet, 2003). By NF35, its expression is localized to the dorsal and ventral pancreatic buds, similar to *Ptf1a*; expression is also seen in the developing lung buds.

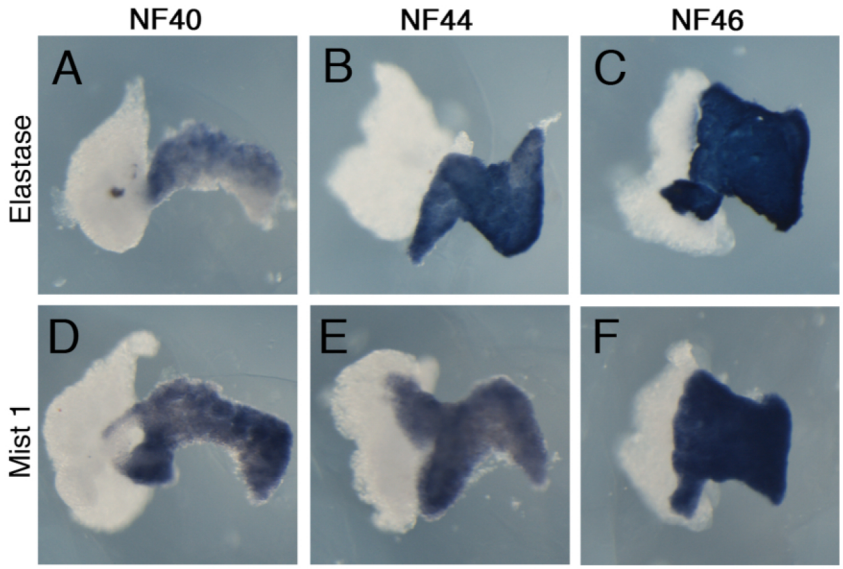


Fig. 4. Exocrine marker expression in isolated liver and pancreas tissue. **A–C:** Elastase expression. **D–G:** Mist1 expression.

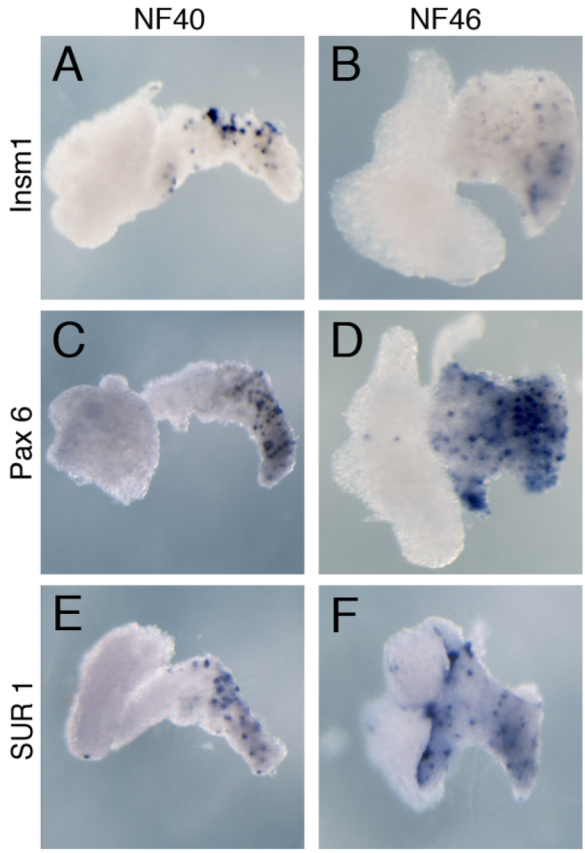


Fig. 5. Expression of endocrine pancreatic factors in isolated liver and pancreas tissue. **A,B:** Pax6 expression. **C,D:** Insm1 expression. **E,F:** Sur1 expression.

Hes1.

Hes-1 is a member of the hairy and enhancer-of-split-like transcriptional repressors that acts in the Notch signaling pathway. In the pancreas, Hes1 is involved in antagonizing the function of B-class bHLH proteins such as Ngn3, and is expressed in undifferentiated precursor cells (Jorgensen et al., 2007). In *Xenopus, Hes1* is known as *hairy2b* and it has been extensively studied in the context of neural development (Yamaguti et al., 2005; Murato et al., 2006). Within the pancreas, it is expressed in a punctate fashion from the earliest stages, most likely marking pancreatic progenitor cells (data not shown).

Ngn3.

Neurogenin3 (ngn3) is a bHLH transcription factor that is the earliest marker of all pancreatic endocrine cells, marking a population of endocrine progenitor cells (Gradwohl et al., 2000; Schwitzgebel et al., 2000; Gu et al., 2002). Mice lacking *ngn3* function fail to generate any pancreatic endocrine cells and die postnatally from diabetes (Gradwohl et al., 2000). In *Xenopus, ngn3* displays a punctate pattern of expression throughout the gastrointestinal tract as seen in mice (Nieber et al., 2009). Within the pancreas, *ngn3* is initially detected only in the dorsal pancreas in a speckled fashion, and at later stages expression extends into the ventral pancreas as well (data not shown). This pattern of expression is identical to Insm1.

Insm1.

Insulinoma-associated protein 1, also known as IA-1, is a zinc finger transcription factor expressed in all endocrine cells, and is necessary for their differentiation (Gierl et al., 2006; Mellitzer et al., 2006). We recently cloned *Xenopus Insm1* and found it to also be expressed in both pancreatic and enteroendocrine cells (Fig. 5A,B). Similar to the mouse knockouts, we found that *Xenopus Insm1* was downstream of *Ngn3* and was required for the differentiation of all endocrine cells (Horb et al., 2009).

NeuroD.

NeuroD is a bHLH transcription factor that is expressed in all endocrine cells. NeuroD mutant mice have reduced numbers of all endocrine cells and develop diabetes, and mutations in NeuroD in humans are associated with maturity onset diabetes of the young (MODY6) (Liu et al., 2006). NeuroD is also possibly a susceptibility marker for type1 in a Japanese study (Iwata et al., 1999) and in a Danish study (Hansen et al., 2000). In

Developmental Dynamics

Xenopus, NeuroD has been studied mainly in the context of eye and neural development (Lee et al., 1995; Chae et al., 2004). In the developing pancreas, *Xenopus NeuroD* is restricted to the dorsal rudiment at stage 40; expression then expands to the intestine as well by NF45 (Kelly and Melton, 2000).

Pax4 and Pax6.

Pax4 and 6 genes contain both a paired box and a homeobox, and both are expressed in a subset of pancreatic endocrine cells. Pax4 is essential for β and δ cell development, whereas Pax6 is involved in α cell development (Sosa-Pineda et al., 1997). As with NeuroD, much of what we know about Pax6 in *Xenopus* is derived from studies on its function in eye and neural development (Hirsch and Harris, 1997; Chow et al., 1999; Zaghloul and Moody, 2007). In the developing pancreas, it is expressed in a punctate fashion in the dorsal pancreas at NF40 (Fig. 5C,D) (Kelly and Melton, 2000). It is also expressed in the developing intestinal tract marking endocrine cells. Pax4, on the other hand, has yet to be cloned in *Xenopus*.

Arx.

Arx encodes a homeodomain protein that is expressed in endocrine pancreas progenitors, but is required only for development of the α cell lineage; Arx-deficient mice do not develop α cells, but have a concomitant increase in β and δ cells (Collombat et al., 2003). In contrast, overexpression of *Arx* in the pancreas converts β cells to α and PP cells (Collombat et al., 2007). In *Xenopus, Arx* has been cloned and studied in the context of forebrain development (El-Hodiri et al., 2003; Seufert et al., 2005). Its expression and function in the pancreas has not been examined.

Nkx2.2.

In mice, Nkx2.2 is an early marker of all endocrine cells, but loss of Nkx2.2 only affects maturation of endocrine cells at the secondary transition (Sussel et al., 1998). In *Xenopus*, Nkx2.2 is first detected in the pancreas beginning at NF40, where it is expressed in both the dorsal and ventral pancreas (data not shown).

Nkx6.1.

In rodents, Nkx6.1 is expressed early in pancreatic progenitors and at later stages is restricted to β cells (Jensen et al., 1996; Oster et al., 1998; Jorgensen et al., 2007; Hald et al., 2008). Although not essential for the initial specification of β cells, it is required for the increase in β-cell number that occurs at the secondary transition (Sander et al., 2000). *Xenopus* Nkx6.1 was only recently cloned; it is first detected in the anterior endoderm at NF36 in the developing pancreas and stomach (Zhao et al., 2007). At NF40, Nkx6.1 is found expressed in a punctate fashion only in the dorsal part of the pancreas (data not shown).

Islet1.

Islet1 is a homeobox transcription factor that is expressed in the dorsal pancreatic mesenchyme at early stages and later in the pancreatic epithelium. Mice lacking islet1 do not develop a dorsal pancreas (Ahlgren et al., 1997). In *Xenopus,* expression of islet1 is detected in the lateral plate mesoderm extending from the dorsal pancreas down to the duodenum, but is absent from the ventral pancreatic region (Kelly and Melton, 2000).

MafA and MafB.

The Maf family of proteins encode leucine zipper transcription factors. *MafA* is a β-cell-specific factor involved in regulating *insulin* gene transcription in the mature cell (Zhang et al., 2005). MafB, on the other hand, is expressed in both α and β cells early in development, becoming localized to only α cells later in development (Nishimura et al., 2006). In *Xenopus*, only *MafB* has been cloned, but its expression in the developing pancreas has not been examined.

SUR1.

SUR1 (sulfonylurea receptor 1), encoded by the gene *ABCC8*, is the regulatory subunit of the K_{ATP} channel in β-cell membranes (Bryan et al., 2007). K_{ATP} channels are octamers consisting of four regulatory subunits (in this case four SUR1 subunits) and four pore-forming subunits (see Kir6.2 below). SUR1 is a target of the sulfonylurea diabetes treatments, which induce insulin release (Rafiq et al., 2008). In *Xenopus, SUR1* is expressed in a punctate pattern in the dorsal pancreas; this pattern is consistent with expression in β cells (Fig. 5E,F).

Kir6.2.

Kir6.2 is the pore-forming subunit of the K_{ATP} channel in the β-cell membrane, encoded by the gene *KCNJ11*. Kir6.2 is also a target for sulfonylurea diabetes treatments, and such treatments have successfully replaced insulin treatment in diabetics with mutations in *KCNJ11* (Pearson et al., 2006). *Xenopus KCNJ11* has been cloned but the expression pattern has not been examined.

Mist1.

Mist1 is a bHLH transcription factor expressed in the exocrine cells late in development (Lemercier et al., 1997). In mice and zebrafish, Mist1 has been shown to be necessary for maturation and maintenance of exocrine cells (Johnson et al., 2004; Guo et al., 2007). In *Xenopus, Mist1* is expressed throughout the pancreas by NF40 and expression is maintained as the pancreas develops (Fig. 4D–F).

Early Development Summary

In this first part of the review, we have attempted to provide a general overview of early pancreas development in *Xenopus*. These results demonstrate that patterning and specification of the endoderm into distinct anterior and posterior regions begins as early as NF11. However, this early patterning is labile, and stable regional specification is not achieved until late tail bud stages. We have also given a summary of various pancreatic transcription factors, with a particular focus on Ptf1a and Pdx1. These results demonstrate one of the strengths of the *Xenopus* model: the ability to determine the sufficiency of specific factors in promoting ectopic pancreatic cell fates, either in naive endoderm, through overexpression of mRNA in vegetal blastomeres at early stages, or in specific organs later in development, using transgenics.

PANCREAS DEVELOPMENT DURING METAMORPHOSIS (AFTER NF55)

Differences Between the Tadpole and Frog Exocrine Pancreas

The anuran pancreas undergoes a major change during metamorphosis (Fig. 6) (Janes, 1937; Bollin et al., 1973; Dodd and Dodd, 1976; Leone et al., 1976; Milano and Chimenti, 1995; Mukhi et al., 2008). The tadpole-to-adult remodeling process is in response to the surge of thyroid hormone (TH) during spontaneous metamorphosis (Leloup and Buscaglia, 1977). The tadpole pancreas is a large spongy, boggy tissue that grows proportionately with the tadpole up to the climax of metamorphosis (NF58). The exocrine tadpole pancreas is rich in acinar cells with typical zymogen granules (Fig. 1D) and expresses very high levels of digestive enzymes. One of the obvious histological differences between the tadpole and adult exocrine pancreas is the absence of a complex ductal arrangement in the tadpole (Mukhi et al., 2008). The ductal system in the frog pancreas comprises all of the types of ductal cells as described by Egerbacher and Bock (1997) in mammals including the intercalcated ducts, intra- and extralobular ducts, and collecting ducts (Fig. 7). The rare tadpole ductal structures are single-cell epithelial tubes without fibrous sheaths surrounding them, morophologically resembling the lobular duct of an adult pancreas. It has been observed that the giant-tadpoles (lacking thyroid function) and tadpoles grown in methimazole (an inhibitor of thyroid hormone synthesis) for long periods of time also lack a complex ductal system (Mukhi et al., 2008).

The frog exocrine pancreas synthesizes many of the same terminally differentiated enzymes as the tadpole pancreas; however, microarray analyses comparing the frog and tadpole pancreas reveal many extreme differences in gene expression. Out of a total of 33,098 microarray 60-mers (Agilent), more than 4,000 entries were upregulated significantly in the tadpole pancreas compared to the frog pancreas and vice versa. The most extreme expression differences revealed by the microarrays are the high activity of carboxyl ester lipases in the tadpole pancreas and trypsin-like serine protease (elastase) in the frog pancreas (Mukhi and Brown, unpublished data). Such huge differences in gene expression profiles and tissue structure (morphology and histology) between tadpole and frog pancreas suggest that the exocrine pancreas in the tadpole is not mature and requires the TH-dependent remodeling process. As we will see, the same can be said for the insulin secreting β cells.

Remodeling of the Exocrine Pancreas During Metamorphosis

Several authors (Janes, 1937; Race et al., 1966; Milano and Chimenti, 1995) have studied remodeling of the pancreas during metamorphosis in different species of frogs. The metamorphic changes have been described as "partial degeneration" followed by "regeneration" (Janes, 1937) and cellular "necrosis" and "dehydration" (Race et al., 1966; Leone et al., 1976). We have observed that at metamorphosis, remodeling begins with the dedifferentiation of the entire exocrine pancreas followed by a redifferentiation phase in the growing frog (Mukhi et al., 2008). One of the first changes in the tadpole exocrine pancreas at metamorphosis in response to the increase in thyroid hormone (TH) is the steady decline of mRNAs that encode the terminally differentiated enzymes (Shi and Brown, 1990; Mukhi et al., 2008). The levels of these mRNAs begin dropping at prometamorphosis (NF56), until they are almost totally absent by the middle of the metamorphic climax (NF62) (Figs. 8 and 9). These transformed exocrine cells have sparse cytoplasm and no zymogen granules. Two months after the end of metamorphosis, the frog's exocrine pancreas once again contains acinar cells but now also a full set of ducts typical of an adult vertebrate pancreas (Fig. 7).

Thyroid Hormone (TH) Induces the Dedifferentiation of the Tadpole Pancreas

The sequence of dedifferentiation events in the *Xenopus* pancreas during climax of metamorphosis is similar to the dedifferentiation process observed in mammalian models. Pancreas acinar cells are considered to be highly plastic in mammals, because they can be induced to dedifferentiate. Exocrine pancreas dedifferentiation and plasticity have been observed in chronic pancreatitis (Tezel et al., 2004), upon exposure to the pharmacological agent, cerulein (Jensen et al., 2005; Strobel et al., 2007), and when the cells are placed in culture (Means et al., 2005). The uniqueness in *Xenopus* is that the changes occur as a part of the normal developmental process as a response to a hormone (Race et al., 1966). The changes to the acinar cells during the climax of metamorphosis result in the cells dedifferentiating and entering a progenitor state (Mukhi et al., 2008). This is evident by the transient up-regulation of pancreatic progenitor markers (*Pdx1, Notch-1*, and *Hes1*) and the down-regulation of the acinar-specific transcription factor *Ptf1a* as well as exocrine-specific pancreatic enzyme mRNAs such as *amylase* and *trypsin.* The more stable proteins persist through climax. This proves that the surviving cells at climax are dedifferentiated acinar cells. In the redifferentiaion phase, the expression of *Pdx1* and *Notch-1* is down-regulated and the mRNAs encoding the pancreatic enzyme reappear. Activation of the Notch signaling pathway has been reported in mammalian pancreas that has been induced to dedifferentiate (Jensen et al., 2005; Rooman et al., 2006). The molecular events involved in this chemically induced dedifferentiation and subsequent regeneration of the mammalian pancreas was described as a "recapitulation of embryonic development" (Jensen et al., 2005). Amphibian metamorphosis provides a new model for pancreatic dedifferentiation and redifferentiation with the major advantage that it can be controlled by TH (Janes, 1937; Race et al., 1966; Milano and Chimenti, 1995; Mukhi et al., 2008). While the dedifferentiation of acinar cells is triggered by TH, the hormone does not seem to be involved in the redifferentiation of the exocrine pancreas that occurs after metamorphosis.

Transgenic tadpoles expressing a dominant-negative form of the *thyroid hormone receptor alpha* (*TRDN*) controlled by the rat *elastase* promoter do

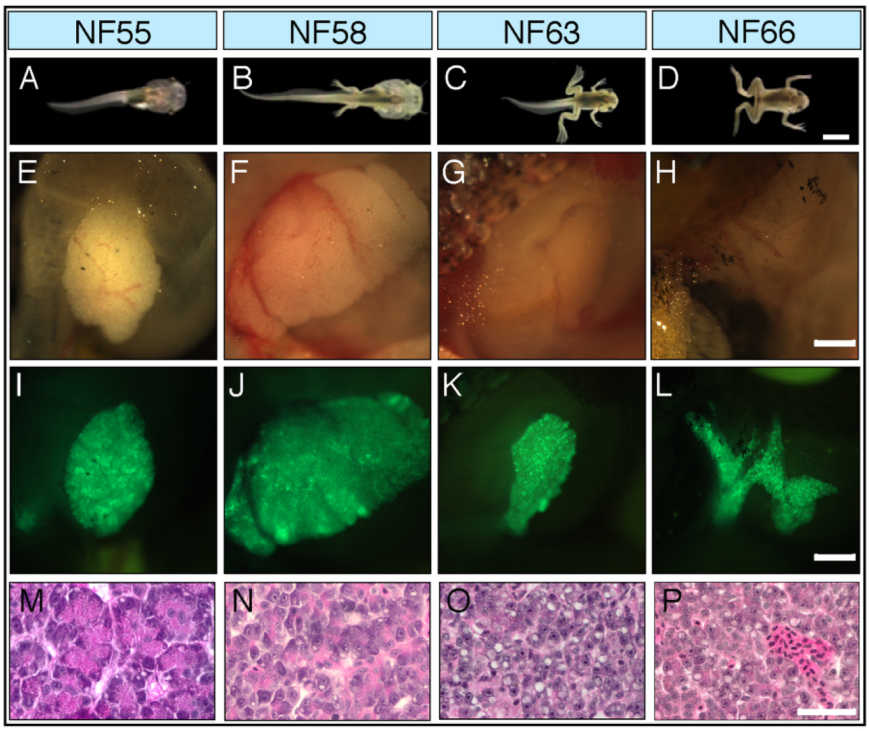
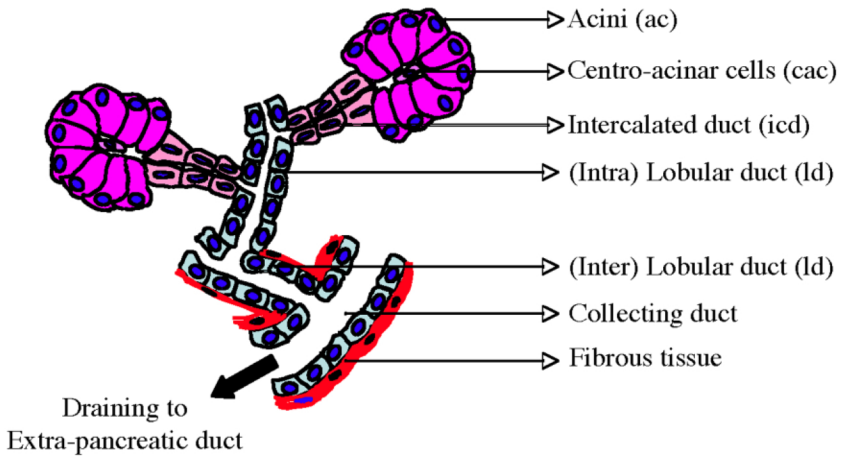


Fig. 6.



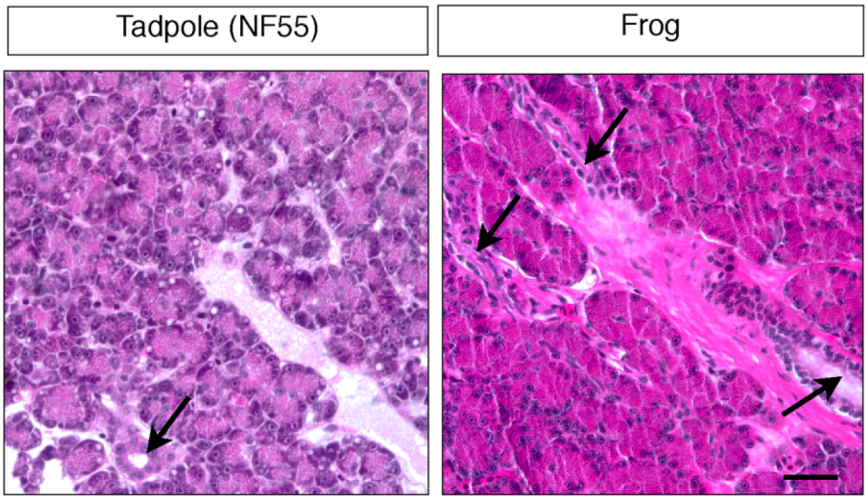


Fig. 7.

not dedifferentiate their exocrine pancreas at climax (Mukhi et al., 2008). The transient up-regulation of *Pdx1* and *Notch-1* and the down-regulation of *amylase* at climax were inhibited. This genetic interference in the spontaneous metamorphosis process inhibited the maturation of the tadpole to the frog pancreas. Even after 2 months of growth, the transgenic pancreas of these frogs has only a rudimentary ductal system. These abnormal pancreases have unusual inclusions that look nothing like pancreas cells. Despite the abnormal pancreas, the transgenic frogs are healthy and grow to sexual maturity. This is not surprising since pancreatectomy in frogs has been reported not to be lethal (Frye, 1964). A summary of the changes that occur in the exocrine pancreas at metamorphosis is presented in Figure 9.

Remodeling of the Endocrine Pancreas at Metamorphosis

In the adult *Xenopus* pancreas, the islets consist of only insulin-producing β cells; the other endocrine cells are scattered around in the pancreas as single cells sometimes loosely associated with the islets (Maake et al., 1998; Horb and Slack, 2002). The shrinkage of the pancreas that occurs at climax brings the various endocrine cells closer together (Maake et al., 1998). In addition, a "maturation" of the pancreatic system has been proposed to occur at climax (Maake et al., 1998; Accordi and Chimenti, 2001), which involves limited cell death and changes in insulin expression. In the

Fig. 6. The development of the *X. laevis* pancreas through metamorphosis. **A–D:** Pictures of four development stages. **E–H:** The pancreases at each stage are below the pictures. **I–L:** Transgenic animals expressing GFP controlled by the rat elastase promoter. **M–P:** Histology of the pancreas at each stage stained with H and E. Scale bars = (A–D) 4 mm; (E–L) 1 mm; (M–P) 40 μm. Redrawn from Mukhi et al. (2008).

Fig. 7. Diagram of a typical mature acinar assembly with its collecting ducts (after Egerbacher and Bock, 1997). Histological sections of (left) premetamorphic pancreas and (right) frog pancreas. The arrows point to ducts. Scale bars = 40 μm. Redrawn from Mukhi et al. (2008).

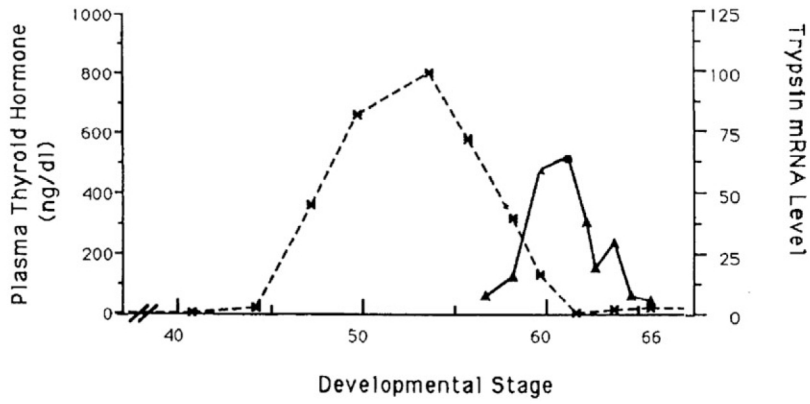


Fig. 8.

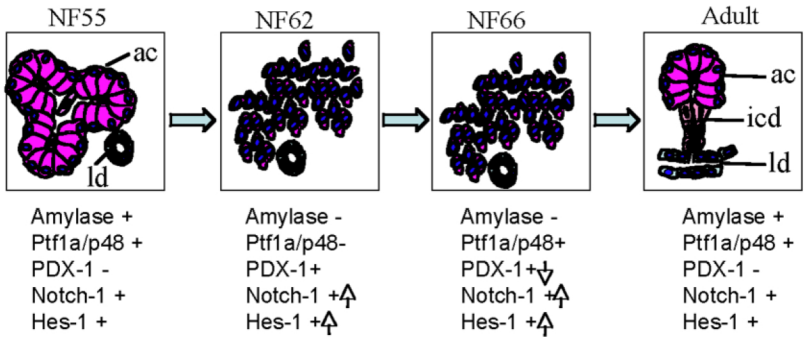
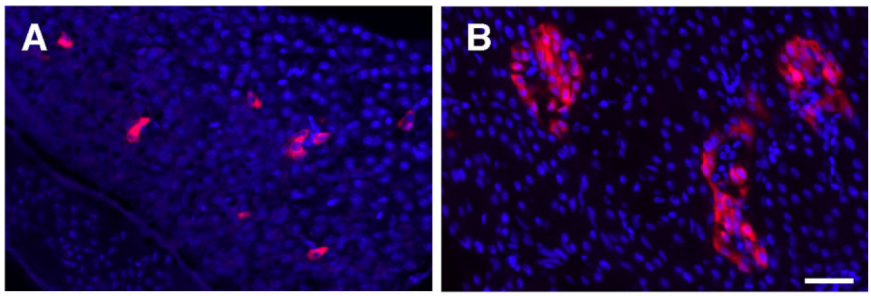


Fig. 9.



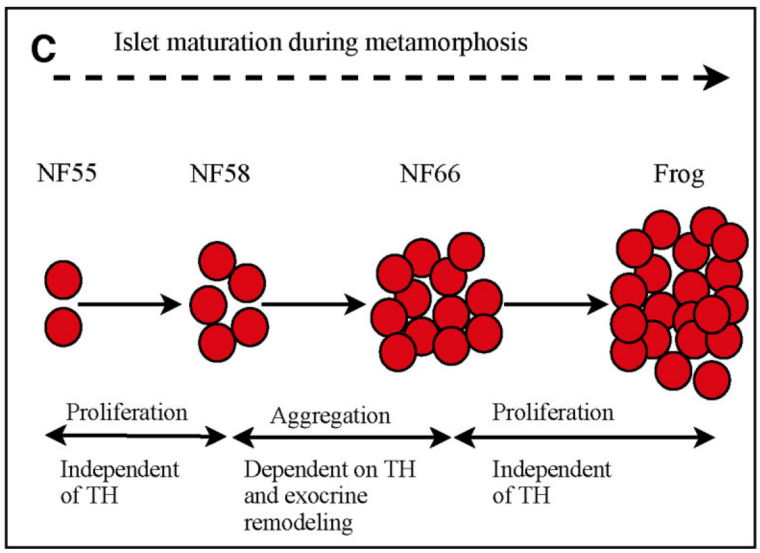


Fig. 10.

tadpole, β cells are present in small clusters (Kelly and Melton, 2000), but during the eight days of metamorphic climax, while the exocrine pancreas dedifferentiates, these clusters are induced by TH to become larger (Fig. 10). The reason for this increase in islet size during climax is due to the aggregation of pre-existing β cells (Mukhi et al., 2009). During climax, β cells do not replicate, and the absolute number of β cells does not change. In addition, acinar to β cell transdifferentiation was not observed. In the months following metamorphosis, the islets grow larger at least in part by replication of existing β cells.

Unlike the gene expression changes seen in the exocrine pancreas, insulin protein is detected in the β cells throughout metamorphic climax. However, there is a brief loss of *insulin* mRNA during early climax (NF62). *Insulin* mRNA is synthesized again several days later at the end of climax (NF66). No known β cell progenitor genes have been found to be activated when the β cells stop synthesizing insulin mRNA, leaving no explanation for this brief down-regulation of *insulin* mRNA (Mukhi et al., 2009).

This aggregation of β cells at climax is not cell-autonomous but requires interactions with the surrounding dedifferentiated acinar cells. In transgenic *elastase-TRDN* animals, where acinar cells do not dedifferentiate, there is no aggregation of β cells. However, once metamorphosis is complete, the small (nonaggregated) islets in these transgenic frogs begin to replicate so that in two months they are equal in size to the control islets (Mukhi et al., 2009). This finding adds evidence that the role of TH in pancreas remodeling

Fig. 8. Developmental profile of trypsin mRNA (x) and endogenous T3 (triangles). The period called climax is bracketed. Redrawn from Shi and Brown (1990).

Fig. 9. Diagram of exocrine pancreas remodeling during metamorphosis. The expression changes of key genes are indicated below each diagram. Reproduced from Mukhi et al. (2008) with permission of the publisher.

Fig. 10. Islet size in the pancreases of (**A**) premetamorphic tadpoles and (**B**) NF66 froglets at the end of climax. Scale bars = 40 μm. **C:** A summary of remodeling b-cells.

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ends when metamorphosis is complete and does not account for the redifferentiation process. In contrast, the down-regulation of insulin gene expression in beta cells is a cell-autonomous response to TH. In transgenic *pIns-TRDN* animals, *insulin* expression is not down-regulated, while the aggregation of β cells occurs normally (Mukhi et al., 2009). We conclude that changes in β cells during climax result from two separate TH-controlled programs. The aggregation of β cells during climax requires the concomitant dedifferentiation of the exocrine cells, whereas the transient down-regulation of insulin gene expression is a direct response of TH.

Metamorphosis Summary

The remarkable TH-induced changes in the tadpole pancreas are a normal part of the animal's life cycle. Although definitive lineage studies have not been carried out, it is likely that the frog's mature ductal system as well as the new acinar cells derived from dedifferentiated acinar cells at climax. The first islets formed at climax are derived by aggregation of preexisting β cells. By analogy, mammalian islets are generated in a multistep process involving differentiation, migration, and aggregation of progenitor cells into islets (Deltour et al., 1991; Kim and Hebrok, 2001). The mechanisms that control these processes are unknown.

The pancreas is just one of many amphibian organs and cell types that remodel during metamorphosis. The tadpole intestine undergoes extensive shortening, and each of its cell types change during metamorphic climax (McAvoy and Dixon, 1978a,b). The brain remodels by essentially rewiring its connections (Alley and Barnes, 1983). Tadpole red cells undergo globin switching (Weber, 1996); the B- and T-cells are renewed (Du Pasquier et al., 2000). The liver (Paik and Cohen, 1960) and the skeleton (Berry et al., 1998) also remodel. Each of these changes is controlled by thyroid hormone. Organs like the pancreas can have more than one TH-controlled program. Some of these are cell autonomous, others require the influence of an adjacent cell type just as the aggregation of β cells depends upon the dedifferentiation of the exocrine cells.

SUMMARY

We have covered many of the most important aspects of pancreas development, including regional specification, early morphological events, induction of pancreas, and the changes that occur during metamorphosis. What is obvious from this review is that although early pancreas development and pancreatic remodeling during metamorphosis are quite distinct events, they have much in common, and the same signaling molecules that promote early pancreas development are re-expressed during metamorphosis as the cells dedifferentiate.

In this review, we have attempted to provide a broad overview of pancreas development that would be beneficial not only to the *Xenopus* community, but also researchers studying pancreas development in other animals. Although some morphological events are unique to *Xenopus*, it is becoming increasingly obvious that the early stages of Xenopus pancreas development are quite similar to mammalian pancreas development. The same genes that control major developmental decisions in the developing pancreas of a frog also control them in a mouse or indeed a human. And although metamorphosis is not found in mammals, the changes that occur in the *Xenopus* pancreas at metamorphosis can be studied to understand how remodeling of a "mature" pancreas occurs.

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