2009-11-17

Endoderm Patterning in Zebrafish: Pancreas Development: A Dissertation

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ENDODERM PATTERNING IN ZEBRAFISH: PANCREAS DEVELOPMENT

A Dissertation Presented

By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

November 17, 2009

Interdisciplinary Graduate Program

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Interdisciplinary Graduate Program

November 17, 2009

DEDICATION

To my husband Michael-Ryan, who is my inspiration, my rock, my everything.

ACKNOWLEDGEMENTS

First I would like to thank Charles. He provided a wonderful environment that allowed me to grow and develop as a student. He was always very patient with me but yet pushed when I needed to be pushed. I always knew his door was open and that I could go to him with any problem and he would listen. I appreciate the time he spent with me trying to figure out my mapping data. He always believed in me and made me think that I could do this. Thank you.

I would like to thank my TRAC committee, Zheng-Zheng Bao, Brian Lewis and Nathan Lawson for their years of coming to my IGP talks and committee meetings. Thank you for providing helpful ideas and having time for me. I would like to thank two new people that have joined my committee. Thank you to Jaime Rivera for giving his time to be part of my DEC as well as Iain Drummond for agreeing to be my outside member and taking his time to come to UMass for my defense. I really appreciate everything.

I would like to thank the Sagerström Lab including past and present members. They provided such a fun and caring atmosphere to be in so that going to work didn't always seem like work. In particular, I would like to thank two former postdocs Mako and Nick who were here at the beginning. I could always go to them with questions and they took the time to answer them and help with whatever I needed help with. Next I would like to thank Kyu for all the time and effort he gave me with mapping ideas, presentations, and papers. I would like to thank my fellow grad students Xiaolan and Steve for always coming

to my practice talks and presentations. To Steve for always being able to make a joke out of anything and make us all laugh. And Denise who just joined the lab I really enjoyed our talks. And last but not least Letitiah who has been one of my biggest supporters in lab and in life. She is the best lab manager I have ever worked with and such a great friend. Thank you to Kyu, Denise and Letitiah for reading my thesis and for their feedback.

I have made some wonderful friends at UMass through the years, Mary, Melonnie, Vivian, Laura, Adriana and Sara. Mary and Vivian made excellent roommates and even better friends. I always enjoyed my weekly dinners with Mary, Melonnie and Letitiah. I especially want to thank Sara whom I went to high school with and met up again in grad school. You have become such a great friend. We tried to keep each other sane through the years not sure if we succeeded but we tried!! I have some wonderful friends from home I would like to thank; Maura, Erin, Meghan, Marsha, Jill, Annie and Jocelyn have been such great friends. I would like to thank Maura for her cards, emails and texts. For always being there for me no matter when or what I needed her for.

I certainly could not have done this without my family. To my in-laws; Kathi, Donnie, Melissa, Derek, Micaela and Thommy, Donna, Bobby, Luma, Lindsay, Matt, AJ, Shani, Greg and my niece Nathalia and nephew Jared whom have all known me for a very long time and probably thought I would never finish school but have always supported me through everything and believed in me.

Especially these past few months, thank you for the emails, cards, phone calls and texts. I really appreciate everything and I love you guys.

To the Davidson's, Robin's, O'Hagan's, my grandmother and my Godmother, thank you for always being there for me, for sending cards, emails, phone calls, texts, fixing my computer and just listening. I love you.

To my brother Jay and his family especially my nephews and niece; Coby, Jaycob and Kali who just make life fun and make me feel good. Thank you to my brother Michael who has been there for me through the years as a brother but also from the science aspect. He helped me through core and gave feedback on my thesis. Thank you and I love you guys.

I would like to thank my parents John and Darryl for their unconditional love and support in everything that I do. They always believed in me even when I didn't believe in myself and made me feel like I can do anything. Thank you for always being part of my life, for wanting me to always be happy, and for always loving me and I love you.

Last but certainly not least I would not have been able to do this without my husband Michael-Ryan. Words cannot express what he means to me and how instrumental he has been over the years. He is my rock that I can always lean on, has always loved me even when I wasn't so lovable, and always tried so hard to make things easier on me. His love and support truly got me through everything. I am so lucky to have you in my life. I love you and thank you for being you.

ABSTRACT

The pancreas is located below the liver and adjacent to the small intestine where it connects to the duodenum. It consists of exocrine and endocrine components. The exocrine portion makes enzymes which are deposited in the duodenum to digest fats, proteins, and carbohydrates. Exocrine tissue also makes bicarbonates that neutralize stomach acids. The endocrine portion produces hormones such as insulin and glucagon which are released into the blood stream. These hormones regulate glucose transport into the body's cells and are crucial for energy production. The pancreas is associated with diseases such as cancer, diabetes, Annular pancreas and Nesidioblastosis. Annular pancreas and Nesidioblastosis are congenital malformations associated with excess endocrine tissue of the pancreas and its structures. Understanding the development of the pancreas might lead to insight of these diseases.

The pancreas arises from the endoderm. In zebrafish, Nodal signaling activates *mix-type* and *gata* genes that then function together to regulate *sox32* expression which is necessary and sufficient to induce endoderm formation. Interestingly, *sox32* is exclusive to zebrafish and works synergistically with *pou5f1* to regulate its own expression and turn on *sox17* expression. *sox17* is evolutionarily conserved from zebrafish to mouse and is necessary for endoderm formation.

Signals from within the endoderm and the surrounding mesoderm specify regions in the endoderm to develop into the pancreas and other endodermal

organs. *Sonic hedgehog* (*shh*) expression in the foregut establishes the anterior boundary of the pancreas primordium while *cdx4* expression establishes the posterior boundary, but what regulates these factors is unclear. We determined that two Three Amino Acid Loop Extension (TALE) homeodomain transcription cofactors, Meis3 and Pbx4, regulate *shh* expression in the anterior endoderm. Disrupting either *meis3* or *pbx4* reduces *shh* expression in the anterior endoderm. As a result, anterior ectopic *insulin* expression occurs outside the normal pancreatic domain. Therefore, we discovered upstream regulatory factors of *shh* expression in the anterior endoderm, which is necessary for patterning the endoderm and pancreas primordium.

We performed an ENU (*N*-ethyl-*N*-nitrosurea) haploid screen to look for endocrine pancreas mutants and to find other factors involved in pancreas development and patterning. From the screen, we characterized two mutants. We identified an *aldh1a2* mutant, *aldh1a2*^{um22}, which blocks the production of Retinoic Acid (RA) from vitamin A. While RA is known to be necessary for differentiation of the pancreas and liver, we also found it to be necessary for intestine differentiation. Two other *aldh* family genes exist in the zebrafish genome, but our data suggests that *aldh1a2* is the only Aldh that functions in endoderm differentiation and it is maternally deposited.

From the screen, we discovered a second mutant, 835.4, that spontaneously arose within the background. *pou5f1* expression is normal in mutant embryos, but *sox32* expression is reduced and *sox17* expression is lost.

Downstream endoderm genes of *sox17* are also lost and as a result no endodermal organs develop. Rescue experiments indicate that the mutation is located between *sox32* and *sox17* in the endoderm pathway. We currently have not been successful at mapping this mutation and therefore are unable to rule out the possibility that it lies in the *sox17* gene. However, our data suggest that the mutation occurs in a new gene that is necessary for *sox17* expression, potentially working with *sox32* and/or *pou5f1*.

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LIST OF ABBREVIATIONS

µL	microliter
32c	32 cell injections
ae	Anterior endoderm
alb	*albino*
aldh	retinaldehyde dehydrogenase
amy	amylase
A-P	anterior-posterior
Arg	Arginine
BAC	Bacterial artificial chromosomes
bhLH	basic helix loop helix
BMP	Bone Morphogenic Protein
bon	*bonnie and clyde*
carb a	*carboxypeptidase A*
cas	*casanova*
cdx	*caudal*-type homeodomain *transcription factor*
cM	centimorgans
CNS	central nervous system
cyc	*cyclops*
DEAB	Diethylaminobenzaldehyde
dpf	days post fertilization
EGF	epidermal growth factor
EGF-CFC	epidermal growth factor -Cripto/FRL-1/Cryptic
EK	Ekkwill
EMS	Ethyl methanesulfonate
EnR	Engrailed repressor domain
ENU	(*N*-ethyl-*N*-nitrosourea)
eomes	*eomesodermin*
EST	Expressed sequence tag
Exd	Extradenticle
Fb	Fin bud
FGF	fibroblast growth factor
Fl-MO	Fluorescent control morpholino
fox	forkhead transcription factor
Gb	gigabases
GCKD	Glomerulocystic kidney disease
GFP	Green fluorescent protein
gluc	glucagon
Gly	*Glycine*
gol	*golden*
her5	*hairy/enhancer of split related transcriptional regulator*
hg	*hatching gland*
Hhex	Hematopoietically-expressed homeobox protein

HNF	hepatocyte nuclear factor
hox	*homeodomain transcription factor*
hpf	hours post fertilization
HSTC	*Autologous nonmyeloablative*
Hth	Homothorax
i	*islet*
ifabp	*intestinal fatty acid binding protein*
Ihh	Indian hedgehog
IN	India
ins	insulin
isl1	*islet-1*
IVP	in vitro fertilization
Kb	kilobases
krx20	*krox20*
L	Ladder
LG	Linkage group
LPM	lateral plate mesoderm
L-R	left-right
Lys	Lysine
lzr	*lazarus*
M	maternal
Meis	myeloid *ecotropic viral integration site*
mez	*mezzo*
MGH	Massachusetts General Hospital
MHB	midhindbrain boundary
mhc	*myosin heavy chain*
mM	milimolar
mm	mismatch
MO	morpholino
MOCO	Control morpholino
MODY	Maturity onset diabetes of the young
mol	*monorail*
Mt	mutant
Mxtx1	mix-type homeobox gene 1
Mxtx2	mix-type homeobox gene 2
MZ	maternal zygotic
NAD	Nucleotide binding domain
ndr1	*nodal related protein 1*
ndr2	*nodal related protein 2*
ngn3	*neurogenin3*
nL	nanoliter
nls	*neckless*
nof	*no fin*
oep	*one eyed pinhead*

PAC	P1-derived artificial chromosome
Patched	Ptc
Pbx	Pre-B cell leukemia transcription factor
PCR	Polymerase chain reaction
pdx1	*pancreatic and duodenal transcription factor 1*
Phe	Phenylalanine
PHHI	Persistent hyperinsulinemic hypoglycemia of infancy
PISCES	Pancreatic islet cell enhancer sequence
PP	Polypeptide
prox1	*prospero-related homeobox 1*
ptf1a	*pancreas transcription factor 1*
r	rhombomere
RA	Retinoic Acid
RAR	retinoic acid receptor
RARE	retinoic acid response elements
rdh	*retinol dehydrogenase*
Rho dex	*Rhodamine dextran*
RXR	retinoid X receptor
s	somite
Shh	Sonic hedgehog
SMAD	Combination of *Drosophila* protein, mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein SMA
sMO	Splice morpholino
Smoothened	Smo
SNP	Single nucleotide polymorphism
spa-1	*kit receptor a*
spaw	*southpaw*
spg	*spiel-ohne-grenzen*
sqt	*squint*
sst	somatostatin
Std	standard
TALE	Three amino acid loop extension
TARAM-A	Type 1 TFG-β receptor
TGF-β	transforming growth factor β
Thr	Threonine
TL	Tupfel long fin
tMO	Translational morpholino
transf	*transferrin*
tryp	trypsin
TU	*Tuebingen*
twhh	*tiggywinkle hedgehog*
UV	Ultraviolet
val	*valentino*
VegT	novel T-box protein

vhnf1	*variant hepatocyte nuclear factor 1*
Wt	wildtype
Xnr	*Xenopus* nodal related protein
XSox17	*Xenopus* Sox17
YAC	Yeast artificial chromosome
YSL	yolk syncytial layer
Z	zygotic
ΔCpbx4	Deletion of C-term of pbx4

STATEMENT OF CONTRIBUTION

In Chapter II, Philip diIorio from the Rossini lab and Seong-Kyu Choe performed the dominant negative Meis injections and Phillip diIorio injected tMO1 (Table 2.1 tMO1 and MOCO; Table 2.2 tMO1 and MOCO) and did the *in situs* for Figures 2.1A-K; 2.2A-F,O,P; 2.6A-K; 2.7A-D; 2.8A-H; 2.9A-K. The Sagerström lab manager Letitiah Etheridge did *in situs* with *islet1* probe in Figures 3.2; 4.1A,B 5.1A,B. ENU treatment of Ekkwill zebrafish was performed by Elizabeth Laver, Michael Karcergis, Nicolas Hirsch and Seong-Kyu Choe from the Sagerström and Lawson labs. I am solely responsible for all other experiments presented in this thesis.

CHAPTER I

INTRODUCTION

The digestive system

The digestive system plays an important role in all vertebrates. Functions of the digestive system include taste, gas exchange, digestion, nutrient absorption, glucose homeostasis, detoxification, blood clotting, and hematopoiesis (Conlon, Lyons et al. 1994; Waldrip, Bikoff et al. 1998; Wells and Melton 1999). Also, the digestive organs themselves establish a barrier to external toxins, provide essential immune functions and have essential roles in metabolism and salt and water absorption (Johnson 1994).

Formation of the vertebrate digestive system begins during gastrulation with the development of a primitive endodermal gut tube. Gastrulation is the process whereby cells of the epiblast divide, differentiate, and rearrange into three germ layers; the ectoderm, the mesoderm, and the endoderm. The ectoderm gives rise to the central nervous system (CNS) and epidermis. Development of the mesoderm creates muscle, mesenchyme, blood and bone. It also contains the notochord which serves as a signaling source during development. Finally, the endoderm gives rise to the respiratory system and digestive system along with the associated organs and glands including pharynx, esophagus, stomach, intestine, and colon (Kimmel, Ballard et al. 1995; Sampath, Rubinstein et al. 1998; Wells and Melton 1999; Bruce, Howley et al. 2003).

The organization of organs within the digestive system is conserved among vertebrates and develops in a specific temporal and spatial pattern within

the endoderm (Bellairs 1998; Kaufman 1999). The developing gut tube spans the length of the embryo and the anterior portion, also known as the foregut, develops first and the hindgut forms soon after (Wallace and Pack 2003). Organs such as the liver and the pancreas develop as endodermal buds from the gut tube. These buds grow into the surrounding condensed mesenchyme which originates from the lateral plate mesoderm (LPM). Growth of muscle and connective tissue that surrounds the organs then follows (Wallace and Pack 2003). Cross talk between the mesoderm (the mesenchyme in particular) and the endoderm is essential in the development of endodermal organs and for patterning them (Kedinger, Lefebvre et al. 1998; Wells and Melton 2000).

The pancreas

The word pancreas is derived from the Greek word “pan” meaning all and “creas” meaning flesh (Slack 1995). The pancreas is linked to many diseases such as diabetes and pancreatic cancer. Therefore, understanding how the pancreas forms may provide insight into possible treatments for these diseases. The pancreas is both an exocrine and endocrine gland. The exocrine pancreas consists of acinar cells that release digestive enzymes such as trypsin (tryp), amylase (amy) and carboxypeptidase A (carbA) into the small intestine. The exocrine ductal system develops as branches of the main pancreatic duct that insert into the intestine (Yee, Lorent et al. 2005)(Figure 1.1A).

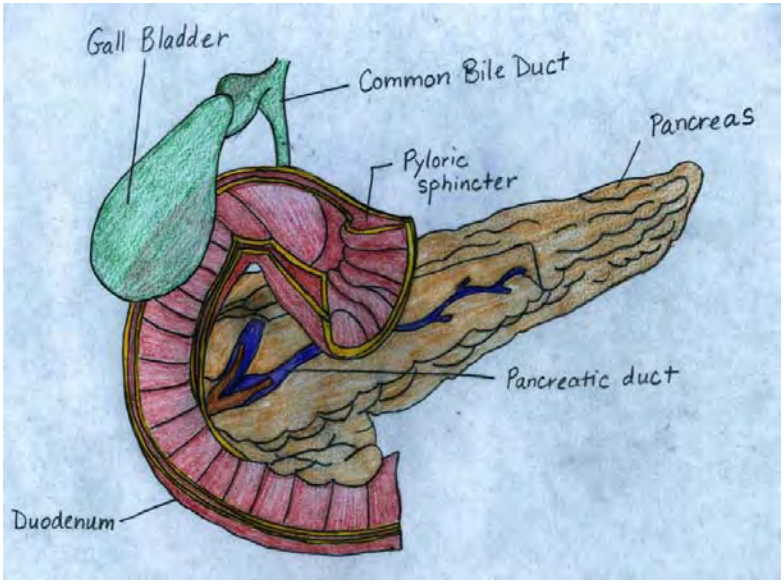
Figure 1.1 – The pancreas

A. The pancreas contains both an endocrine portion and an exocrine portion. The exocrine pancreas consists of acinar and duct cells. The acinar cells produce digestive enzymes and constitute the bulk of the pancreatic tissue. They are organized into grape-like clusters at the end of the branching duct system. The ducts, which add mucous and bicarbonate to the enzyme mixture, form a network of increasing size, filing into a main pancreatic duct that empty into the duodenum.

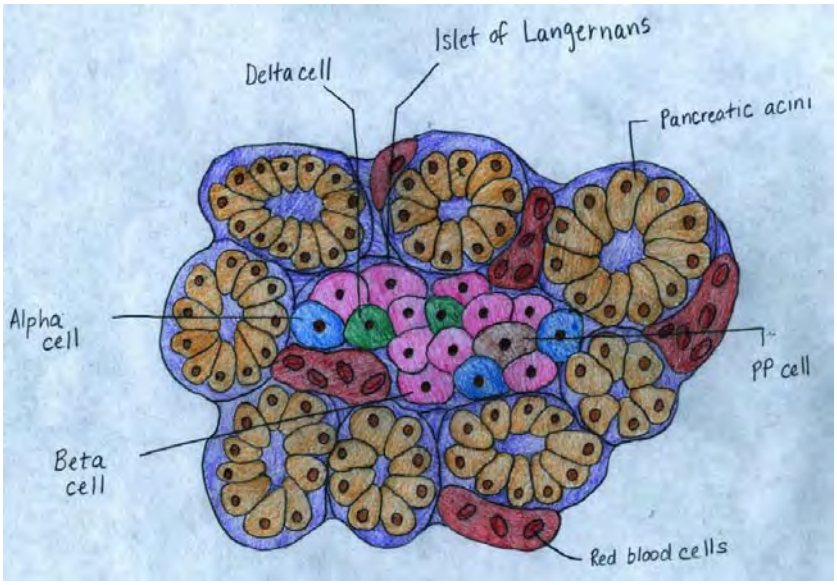
B. The endocrine pancreas, consisting of four specialized cell types that are organized into compact islets embedded within acinar tissue, secretes hormones into the bloodstream. The α- and β-cells regulate the usage of glucose through the production of glucagon and insulin. Pancreatic polypeptide and somatostatin that are produced in the PP and δ-cells modulate the secretory properties of the other pancreatic cell types.

Figure 1.1

A.


Gall Bladder
Common Bile Duct
Pancreas
Pyloric sphincter
Pancreatic duct
Duodenum

B.


Islet of Langernans
Delta cell
Pancreatic acini
Alpha cell
PP cell
Beta cell
Red blood cells

The endocrine pancreas is composed of five cell types that secrete hormones directly into the blood vessels that circulate throughout the pancreas (Figure 1.1B). These hormone producing cells include: β-cells that secrete insulin (ins), δ-cells that secrete somatostatin (sst), α-cells that secrete glucagon (gluc), Pancreatic Polypeptide-secreting (PP)-cells that release PP hormone and ε-cells that secrete ghrelin (not shown). These cells are all grouped together (Slack 1995) and form round clusters called Islets of Langerhans (Figure 1.1B). The Islets are always in contact with capillaries, which allows for continuous monitoring of blood glucose levels and efficient hormone secretion (Slack 1995; Kumar and Melton 2003).

Initially, a portion of the endoderm is patterned to generate tissue that is competent to make pancreas. Then, signaling between the endoderm and neighboring tissues induces pancreas-specific gene expression and finally the pancreatic domain is specified in relation to its surrounding organs (Kumar, Jordan et al. 2003). The three pancreatic lineages (endocrine, exocrine and ductal) segregate within the pancreatic epithelium as it undergoes branching morphogenesis (Gu, Brown et al. 2003).

Overview of endoderm formation in zebrafish

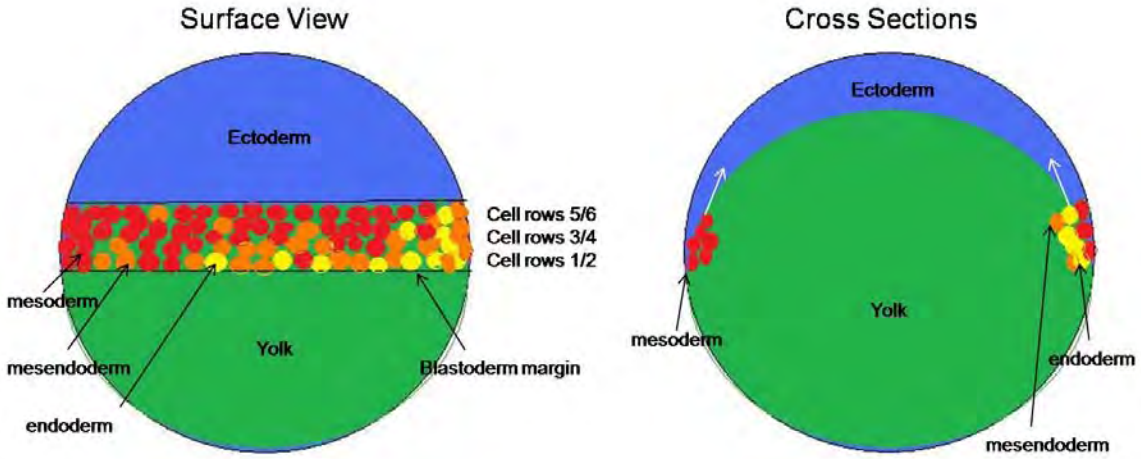
Cell movement: Proper endoderm formation is necessary for development of the pancreas. Mesoderm and endoderm originate from common progenitor cells (called mesendoderm) located where the blastoderm meets the yolk; called the blastoderm margin (Kimmel, Warga et al. 1990) (Figure 1.2). Cells located further than 4 cell diameters from the margin give rise to mesoderm only. However, cells within a 4 cell diameter primarily give rise to endoderm and a few mesoderm progenitors. Following internalization, endodermal cells take on a distinct appearance and gradually flatten and extend filopodial processes. By 6 hpf, they form a noncontiguous inner layer of cells that migrate over the extraembryonic yolk syncytial layer (YSL), a source of mesendoderm-inducing signals (Warga and Kimmel 1990; Mizuno 1996; Rodaway, Takeda et al. 1999; Warga and Nusslein-Volhard 1999; Kimelman and Griffin 2000).

Live imaging demonstrated that the internalized endodermal cells display a nonoriented/noncoordinated movement called “random walk” that disperses the cells over the yolk surface. This behavior was found to be cell-autonomous and induced by signals from the YSL. At midgastrulation, the cells change to a convergence movement in response to environmental cues (Pezeron, Mourrain et al. 2008). Endoderm has completed its migration by 18 hpf.

Figure 1.2 – Gastrulating zebrafish embryo

Gastrulating zebrafish embryos: Surface view (left) and cross section (right). Yolk is represented in green, the ectoderm progenitors in blue, the mesoderm progenitors in red, the endoderm progenitors in yellow and the mesendoderm progenitors in orange. White arrows refer to cell movement. Endoderm and mesoderm cells originate near the blastoderm margin. Endoderm progenitors arise no more than 4 cell diameters away from the yolk. Cells positioned more dorsally and laterally give rise to endoderm. Early involuting cells form endoderm and later involuting cells form mesoderm.

Figure 1.2


Surface View
Ectoderm
Cell rows 5/6
Cell rows 3/4
Cell rows 1/2
mesoderm
mesendoderm
endoderm
Yolk
Blastoderm margin
Cross Sections
Ectoderm
Yolk
mesoderm
endoderm
mesendoderm

This occurs later than in amniotes where the digestive tract (gut) begins to form early at the 1-2 somite stage. The zebrafish gut forms through rearrangements of contiguous cells as they polarize while the mammalian endoderm folds to form the gut tube.

Endoderm cells separate from mesoderm cells: Involuted endoderm and mesoderm progenitors form the hypoblast, while the ectoderm is derived from cells that remain in the outer layer, which is named the epiblast (Warga and Kimmel 1990; Warga and Nusslein-Volhard 1999). The majority of endoderm progenitors are derived from the earliest deep involuting cells from the blastoderm margin. At 4 hpf, endoderm progenitors express endodermal markers such as *sox32* and *sox17* (Figure 1.3), separate themselves from mesoderm progenitors and move dorsally. By 6 hpf, the majority of endoderm progenitors lie at the blastoderm margin (Melby, Warga et al. 1996; Warga and Nusslein-Volhard 1999). As a result, an asymmetric distribution of endoderm progenitors is observed at the margin (Figure 1.2) and at 10 hours hpf they form a sparse but uniform monolayer (Warga and Nusslein-Volhard 1999).

Figure 1.3 – Time line of endoderm and pancreas formation in zebrafish

Figure 1.3

Time	Event
4 hpf	•*sox32/sox17* expression begins
5 hpf	•Gastrulation begins •Endodermal cells involute •Cells are committed
6 hpf	•Majority of endodermal cells are found at the margin
10 hpf	•Endodermal cells form a uniform layer at the margin
14 hpf	•First pancreas gene is expressed
18 hpf	•Endodermal rod is positioned midline
20 hpf	•Endodermal rod is positioned midline
24 hpf	•Dorsal pancreatic bud forms
40 hpf	•Ventral pancreatic bud forms
42 hpf	•Clear endodermal lumen has formed
52 hpf	•Two pancreatic buds merge to form one organ

Cell commitment: Fate commitment of endoderm occurs after the onset of gastrulation (Ho and Kimmel 1993; David and Rosa 2001). Transplantation of marginal cells from late blastula (about 5 hpf) into animal blastomeres demonstrated that only a small portion of transplanted cells contributed to endoderm. Most contributed to neuroectodermal tissue which corresponds to the region in the fate maps where the cells were transplanted (David and Rosa 2001) demonstrating that these cells were committed to the endoderm lineage. Cells contributing to the endoderm increased when marginal cells were transplanted (David and Rosa 2001) demonstrating that these were cells are already committed to the endoderm lineage by 5 hpf.

Antero-posterior patterning of the endoderm: In zebrafish, gut precursors form an endodermal rod, rearrange, polarize and form a lumen. By 20 hpf, a solid multicellular midline rod has formed and a clear lumen can be seen throughout most of the endoderm by 42 hpf (Ober, Field et al. 2003). Graded levels of various signaling factors from other germ layers during gastrulation establishes the Antero-posterior (A-P) patterning of the endoderm (Stafford and Prince 2002; Tiso, Filippi et al. 2002). In particular, the notochord, mesenchyme and the YSL contain positional cues for endodermal organs (Wallace and Pack 2003). Overall, the zebrafish gut is an endoderm derived structure and is divided similarly to other vertebrates: pharynx, esophagus, liver, pancreas, intestinal bulb, and a posterior intestine (Field, Ober et al. 2003).

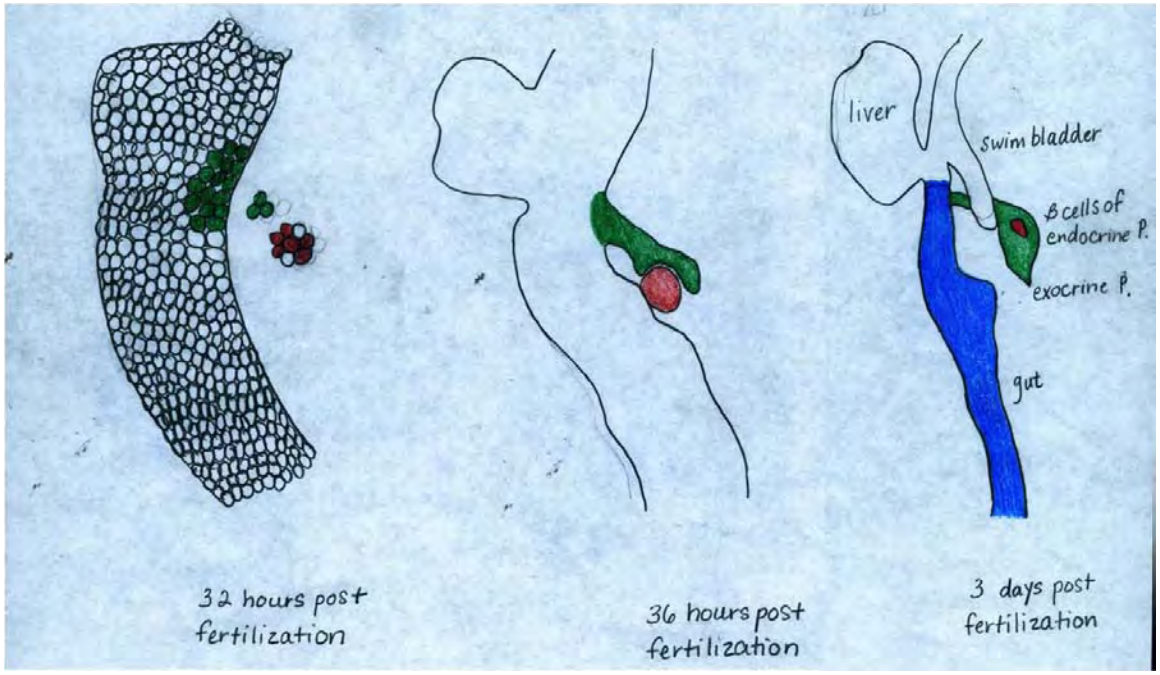
Pancreas morphogenesis in zebrafish

The zebrafish pancreas develops from a dorsal and ventral bud projecting from the gut tube (Figure 1.4). The dorsal bud is positioned slightly posterior to the ventral bud. The buds form from a monolayer of early anterior endodermal cells (Field, Dong et al. 2003; Ober, Field et al. 2003; Wallace and Pack 2003). The dorsal bud forms at approximately 24 hpf (not shown) and is comprised of endocrine pancreas. The ventral bud is formed by 36 hpf and primarily consists of exocrine cells and a few endocrine cells (Field, Dong et al. 2003; Wallace and Pack 2003). Between 24 and 40 hpf the dorsal bud maintains contact with the dorsal aorta and posterior cardinal veins (not shown). The ventral bud is not in contact with any endothelial cells, but rather the surrounding mesenchyme (Field, Dong et al. 2003). By 52 hpf, the two buds have merged to form one organ on the right side of the embryo and by 3 dpf, a single islet surrounded by exocrine pancreas can be observed (Figure 1.4)(Field, Dong et al. 2003; Wallace and Pack 2003).

Figure 1.4 – Endocrine and exocrine pancreas formation in zebrafish

By 32 hpf, the dorsal bud has formed and expresses endocrine markers (red). A second domain, ventral bud (green), is established adjacent to the islet and expresses exocrine markers. ABy36 hpf, the exocrine pancreas comes in contact with the islet. By 52 hpf, the two buds merge and after 3 dpf, exocrine cells can be observed surrounding the islet.

Figure 1.4


liver
swim bladder
β cells of endocrine P.
exocrine P.
gut
32 hours post fertilization
36 hours post fertilization
3 days post fertilization

Pancreas differentiation: endocrine and exocrine lineage specification

Pancreas progenitors are specified before the gut tube forms (Roy, Qiao et al. 2001; diIorio, Moss et al. 2002; Wallace and Pack 2003). The endocrine islet is composed of a core domain where β-cells and δ-cells are located and an enclosing outer layer consists of α-cells and ε-cells (Argenton, Zecchin et al. 1999; Biemar, Argenton et al. 2001); this distribution of endocrine cells is different than in other vertebrates where δ-cells are found in the surrounding layer not the core (Ober, Field et al. 2003). *insulin* (*ins*) expressing cells are the first to appear at 16 hpf followed by *somatostatin* (*sst)* and *glucagon* (*gluc)* between 16-18 hpf. All the endocrine cells appear in a similar scattered formation within the pancreas primordium indicating that the islet arises from the aggregation of differentiated cells rather than by individual progenitors. This is most likely regulated by lateral inhibition. The endocrine precursors then migrate to form a single islet midline at somite 4 by 24 hpf (Argenton, Zecchin et al. 1999; Biemar, Argenton et al. 2001). The exocrine pancreas develops later surrounding the single islet on the right side of the body (Biemar, Argenton et al. 2001). Following larval development, additional endocrine islets develop and by adulthood multiple islets can be observed in the pancreatic parenchyma (Pack, Solnica-Krezel et al. 1996; Milewski, Duguay et al. 1998).

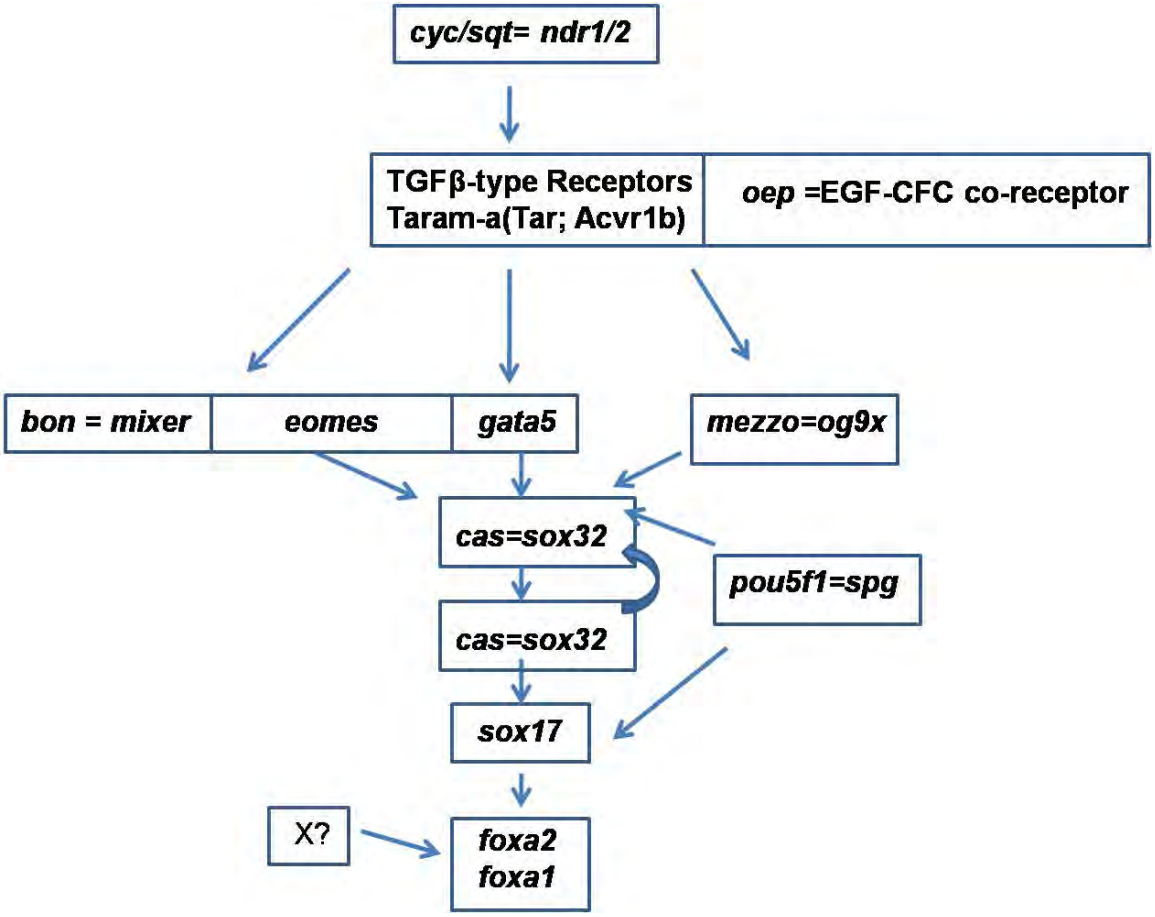
Molecular genetics of endoderm formation in zebrafish

Nodal signaling initiates endoderm formation: Nodals are TFG-β family members and include three proteins in zebrafish but only (Squint [Sqt], Cyclops [Cyc], now known as *ndr1* and *ndr2* (*nodal related protein 1 and 2*) function in early endoderm formation (Figure 1.5). *ndr1* and *ndr2* single mutants form endoderm and appear essentially normal. However, *ndr1; ndr2* double mutants lack all endoderm demonstrating that these genes act redundantly (Feldman, Gates et al. 1998). By 3 hpf, *ndr1* and *nrdr2* are expressed at the blastoderm margin where mesendoderm progenitors form. They act on the progenitors to initiate the endoderm signaling cascade (Rebagliati, Toyama et al. 1998; Sampath, Rubinstein et al. 1998; Warga and Stainier 2002). Cells that are exposed to high doses of Nodal signaling become endoderm and those cells exposed to lower doses take on a mesoderm fate, demonstrating that nodal proteins may act as a morphogen in endoderm specification (Schier, Neuhauss et al. 1997; Thisse and Thisse 1999). In support of this model, Ndr1 has been shown to function as a morphogen during mesoderm formation whereas Ndr2 does not appear to share this feature (Chen and Schier 2001).

Figure 1.5 – Early endoderm pathway in zebrafish

Nodal signaling lies upstream in the endoderm pathway in zebrafish and directly activates *bon* (*bonnie and clyde,* mixer), *gata5*, and *mezzo* (mix-type gene). Bon and Gata5 bind to Eomes (Eomesodermin), a protein that increases the efficiency of these two proteins binding the *sox32* promoter. Another factor, *mezzo*¸ does not interact with *bon, eomes,* or *gata5*, but has proven to be important in activating *sox32* gene expression. *sox32* is the earliest gene specific to the endoderm. It is necessary and sufficient to initiate endoderm formation. Sox32 and Pou5f1 act together to regulate *sox32* expression and both bind conserved regulatory regions that lie upstream in the *sox17* promoter. *foxa2* and *foxa1* expression are dependent on *sox17* as well as another unknown factor, X.

Figure 1.5


cyc/sqt= ndr1/2
TGFβ-type Receptors
Taram-a(Tar; Acvr1b)
oep =EGF-CFC co-receptor
bon = mixer
eomes
gata5
mezzo=og9x
cas=sox32
pou5f1=spg
cas=sox32
sox17
X?
foxa2
foxa1

However, some cells closest to the marginal zone still become mesoderm, indicating that other regulators are acting in germ layer determination (Warga and Nusslein-Volhard 1999) but both *ndr1* and *ndr2* are essential for endoderm formation.

Ndr1 and Ndr2 act via a TGF-β receptor called TARAM-A, which is co-expressed at the blastoderm margin (Figure 1.5). Overexpression of Antivin (specific to zebrafish, is structurally and highly related to mouse lefty, an inhibitor of Nodal signaling), a competitive inhibitor of the Activin (TGF-β receptor) and therefore the Nodal signaling pathway (Thisse and Thisse 1999; Thisse, Wright et al. 2000), results in a complete loss of endoderm (Alexander and Stainier 1999; Thisse and Thisse 1999). Opposite to this, a constitutively active form of type-I TGF-β receptor TARAM-A upregulates endodermal gene expression throughout the zebrafish embryo (Alexander and Stainier 1999) and can cell-autonomously convert embryonic cells to an endodermal fate (Peyrieras, Strahle et al. 1998). This data demonstrates that TARAM-A is essential and sufficient for endoderm formation.

Ndr1 and Ndr2 interact with an EGF-CFC (epidermal growth factor - Cripto/FRL-1/Cryptic) protein that functions as a co-receptor to bind TARAM-A (Figure 1.5)(Gritsman, Zhang et al. 1999; Kumar, Novoselov et al. 2001; Yeo and Whitman 2001). One-eyed Pinhead (OEP) is a member of the EGF-CFC family of membrane-associated proteins (Feldman, Gates et al. 1998) and is expressed both maternally and zygotically. MZ*oep* mutants (lacking both maternal and

zygotic copies) fail to develop endoderm and most mesoderm structures (Zhang, Talbot et al. 1998), which is similar to *ndr1;ndr2* double mutants (Stainier 2002) confirming that Oep is necessary for Ndr1 and Ndr2 to bind TARAM-A and therefore for Nodal signaling.

Nodal signaling functions upstream of Bonnie and Clyde and Gata5 proteins

Bonnie and Clyde: There are three Mixer genes found in zebrafish, but *bonnie and clyde* (*bon*, formerly known as mixer) is expressed at the margin and is the only mixer involved in endoderm formation (Figure 1.5)(Alexander, Rothenberg et al. 1999; Kikuchi, Trinh et al. 2000). *bon* mutants have reduced endodermal gene expression (Alexander and Stainier 1999; Kikuchi, Trinh et al. 2000) confirming the role of *bon* in endoderm formation. Meanwhile, *ndr1;ndr2* double mutants do not display *bon* expression demonstrating that *bon* is downstream of *ndr1/ndr2*. In support of this, *bon* expression is lost in *antivin*-injected embryos indicating that TFG-β signals act upstream of *bon* (Alexander and Stainier 1999).

Gata: Gata factors are zinc-finger transcriptional activators that bind the consensus sequence (A/T)GATA(A/G). There are six evolutionary conserved *gata* genes but only *gata4, gata5* and *gata6* are expressed in extraembryonic tissues, heart, and endoderm and play a role in endoderm development (Figure 1.5)(Charron and Nemer 1999). Transfection of nonendodermal cells with *gata4,*

gata5, or *gata6* activates downstream endodermal genes (Maeda, Kubo et al. 1996; Gao, Sedgwick et al. 1998; Morrisey, Tang et al. 1998; Fukuda and Kikuchi 2005) demonstrating their role in endoderm formation.

In particular, *gata 4* and *6* are expressed in the posterior endoderm in zebrafish after gastrulation has begun (Reiter, Kikuchi et al. 2001). Meanwhile, *gata5* expression is initiated before gastrulation in endoderm progenitor cells and is regulated by Nodal signaling (Alexander and Stainier 1999; Reiter, Alexander et al. 1999; Rodaway, Takeda et al. 1999; Reiter, Kikuchi et al. 2001). When compared to *bon* expression, *gata5* expression is more marginally restricted (Reiter 2001). *gata5* mutants display myocardial defects and defects in gut morphogenesis that range from lack of gut looping to severely reduced endoderm formation (Reiter, Alexander et al. 1999; Reiter, Kikuchi et al. 2001). Overall, *gata5* mutants develop less endodermal cells while overexpression of *gata5* results in expanded endodermal gene expression. Furthermore, injecting *gata5* into *Zoep* mutants, *bon* mutants and *bon*-injected *Zoep* mutants leads to an increase in endodermal gene expression (Alexander and Stainier 1999; Kikuchi, Trinh et al. 2000; Reiter, Kikuchi et al. 2001) suggesting that *gata5* could function downstream and/or parallel of Nodal or Bon.

Eomesodermin is required for endoderm gene expression: Overexpression of *bon* and *gata5* induces endodermal gene expression in marginal blastomeres only, while overexpression of *sox32* (downstream of *bon* and *gata5)* induces

overexpression of endoderm markers throughout the blastoderm. This indicates that another factor must act with Bon and Gata5 to initiate *sox32* expression outside of the blastoderm margin (Peyrieras, Strahle et al. 1998; Kikuchi, Agathon et al. 2001; Sakaguchi, Kuroiwa et al. 2001; Poulain and Lepage 2002). Eomesodermin (Eomes) is a T box transcription factor and a maternal determinant specific to zebrafish that is localized to marginal blastomeres. It has previously shown a role in mesoderm formation and patterning and more recently in endoderm development (Ryan, Garrett et al. 1996; Russ, Wattler et al. 2000; Bruce, Howley et al. 2003; Bjornson, Griffin et al. 2005). Overexpression of *eomes* did not induce ectopic expression of *ndr1* but did induce *sox32* expression in *MZoep* embryos suggesting that it acts downstream of Nodal signaling (Bjornson, Griffin et al. 2005). Knockdown of *eomes* by Morpholinos (MOs, antisense oligos that block the translation start site or block splicing) results in endoderm deficiency confirming their role in endoderm formation. Furthermore, MOs targeting *eomes* and *bon* together significantly reduce endoderm expression and development (Bjornson, Griffin et al. 2005) suggesting that Eomes and Bon work together in endoderm formation. Interestingly, Eomes pulled down both Bon and Gata5 in an immunoprecipitation assay, indicating that Eomes works with Gata5 as well. Reciprocally, Bon and Gata5 were able to pull down Eomes as well as bind one another (Bjornson, Griffin et al. 2005). Specifically, direct interaction between Eomes, Bon and Gata5 on the *sox32* promoter is necessary for proper activation of *sox32* expression indicating that

Eomes acts by assembling a transcriptional activating complex on the *sox32* promoter (Figure 1.5). As a result, Eomes along with Bon and Gata5 induce downstream genes even in animal pole blastomeres. Also, Eomes bind a consensus T site upstream of the *bon* start site and point mutations in this site result in reduced promoter activation suggesting that Eomes regulate *bon* expression.

Mezzo is necessary for endoderm gene induction: In zebrafish, *mezzo* (*mez*) is another MIX-like paired homeobox protein involved in endoderm development (Poulain and Lepage 2002). *mez* is expressed in the mesendoderm progenitors and is an immediate-early target of Nodal signaling. It acts in parallel and cooperates with Bon and Gata5 to turn on and maintain downstream endoderm markers and it regulates its own expression (Figure 1.5). *mez* can partially rescue *bon* mutants and injecting MO targeting *mezzo* into *bon* mutants completely abolishes all endoderm gene expression. Mez plays an important role in endoderm formation by influencing the mesendoderm progenitors to choose between endoderm and mesoderm. In conjunction with this, overexpression induces ectopic endoderm gene expression and reduces mesoderm (Poulain and Lepage 2002). Therefore, *mez* is necessary in zebrafish endoderm formation and works in parallel to *bon* and *gata5*.

***sox32* and *sox17* are necessary and sufficient to induce endoderm:** *sox32 (casanova, cas*) acts upstream of *sox17* and plays a critical role in early endoderm formation in zebrafish (Alexander, Rothenberg et al. 1999; Alexander and Stainier 1999; Dickmeis, Mourrain et al. 2001; Kikuchi, Agathon et al. 2001). *sox32* mutants do not form a gut tube, never express regional markers of endoderm differentiation (for example lack *sox17* expression) and lack endoderm at the onset of gastrulation. Furthermore, mosaic analysis indicates that *sox32* acts cell-autonomously within the endodermal progenitors to initiate *sox17* expression (Alexander, Rothenberg et al. 1999; Stainier 2002). Bon, Eomes, Gata5 and Mezzo activate *sox32* directly (Figure 1.5)(Alexander and Stainier 1999; Reiter, Kikuchi et al. 2001). As a result, *sox32* is regulated indirectly by nodal signaling. However, *sox32* restores endoderm formation in *bon* and *gata5* mutants (Alexander and Stainier 1999; Kikuchi, Agathon et al. 2001) and induces *sox17* expression in *MZoep* mutants, suggesting that it induces *sox17* expression without Nodal signaling (Dickmeis, Mourrain et al. 2001; Kikuchi, Agathon et al. 2001). Furthermore, Sox32 is sufficient to drive mesodermal cells to an endodermal fate (Dickmeis, Mourrain et al. 2001). As a result, cells normally fated to become endoderm, in the absence of Sox32, will become mesoderm (Dickmeis, Mourrain et al. 2001). Thus, *sox32* is necessary and sufficient to induce endoderm.

The role of *sox17* in endoderm development is evolutionarily conserved. *sox17* expression in zebrafish is first detected before gastrulation in a dorsally

located group of marginal cells which later form the forerunner cells (non-involuting cells that will form the Kupfer's vesicle-fluid filled sac and become part of the mesoderm as notochord and muscle). It is also expressed in endoderm precursor cells throughout gastrulation and disappears after somitogenesis. However, expression does reappear around 48 hpf in cells located in the upper left trunk that will contribute to the swim bladder (Cooper and D'Amico 1996; Melby, Warga et al. 1996; Alexander, Rothenberg et al. 1999; Alexander and Stainier 1999; Warga and Nusslein-Volhard 1999). Overexpressing *bon* induces *sox17*-expressing cells in wildtype embryos and promotes expression in MZ*oep* mutants suggesting that *sox17* is downstream of *bon*. HMG binding domains exist in the *sox17* promoter, suggesting that it is regulated by another Sox factor, specifically *sox32* (Figure 1.5)(Ober, Field et al. 2003). In support of this, overexpression of TARAM-A promotes *sox17* expression in wildtype and MZ*oep* mutants but not in *sox32* mutants, suggesting that another factor regulates *sox17* (Alexander and Stainier 1999). Overexpressing *sox17* or knocking down *sox17* by MO injections has not been performed in zebrafish. Therefore, the role of *sox17* in zebrafish has not been further analyzed.

***pou5f1* regulates *sox32* and *sox17* expression:** The *pou5f1* gene encodes a POU domain transcription factor and is mutated in *spiel-ohne-grenzen (spg)* embryos (Schier, Neuhauss et al. 1996; Belting, Hauptmann et al. 2001; Burgess, Reim et al. 2002; Reim and Brand 2002). *pou5f1* is both maternally

and zygotically expressed (Takeda, Matsuzaki et al. 1994; Hauptmann and Gerster 1995; Howley and Ho 2000). Zygotic transcription is involved in neural development, in particular establishing the midbrain-hindbrain boundary, but Z*spg* mutants also have minor defects in mesoderm and endoderm formation (Reim and Brand 2002; Lunde, Belting et al. 2004; Reim, Mizoguchi et al. 2004). Maternal *pou5f1* plays a role in endoderm formation since MZ*spg* do not develop mesoderm and expression of early endoderm markers cannot be detected. However, *sox32* is initially detected at the blastula stage in mesendodermal cells, but is not maintained during the gastrula stage, suggesting that Pou5f1 is necessary to maintain *sox32* expression (Figure 1.5)(Lunde, Belting et al. 2004; Reim, Mizoguchi et al. 2004). Endoderm does not form in MZ*spg* mutants when *sox32* is injected, suggesting that *pou5f1* and *sox32* together are both necessary and sufficient to activate endoderm development by turning on downstream endodermal genes (Figure 1.5). In support of this, *sox17* promoter-luciferase reporter assays have shown that Sox32 and Pou5f1 synergistically regulate *sox17* expression. Nodal signaling does not regulate *pou5f1* expression, but rather *pou5f1* acts in parallel to *sox32*. Therefore, both maternal and zygotic *pou5f1* acts cell-autonomously to induce expression of *sox17* and to maintain *sox32* expression (Reim, Mizoguchi et al. 2004).

The endoderm pathway: *Xenopus* vs. Zebrafish

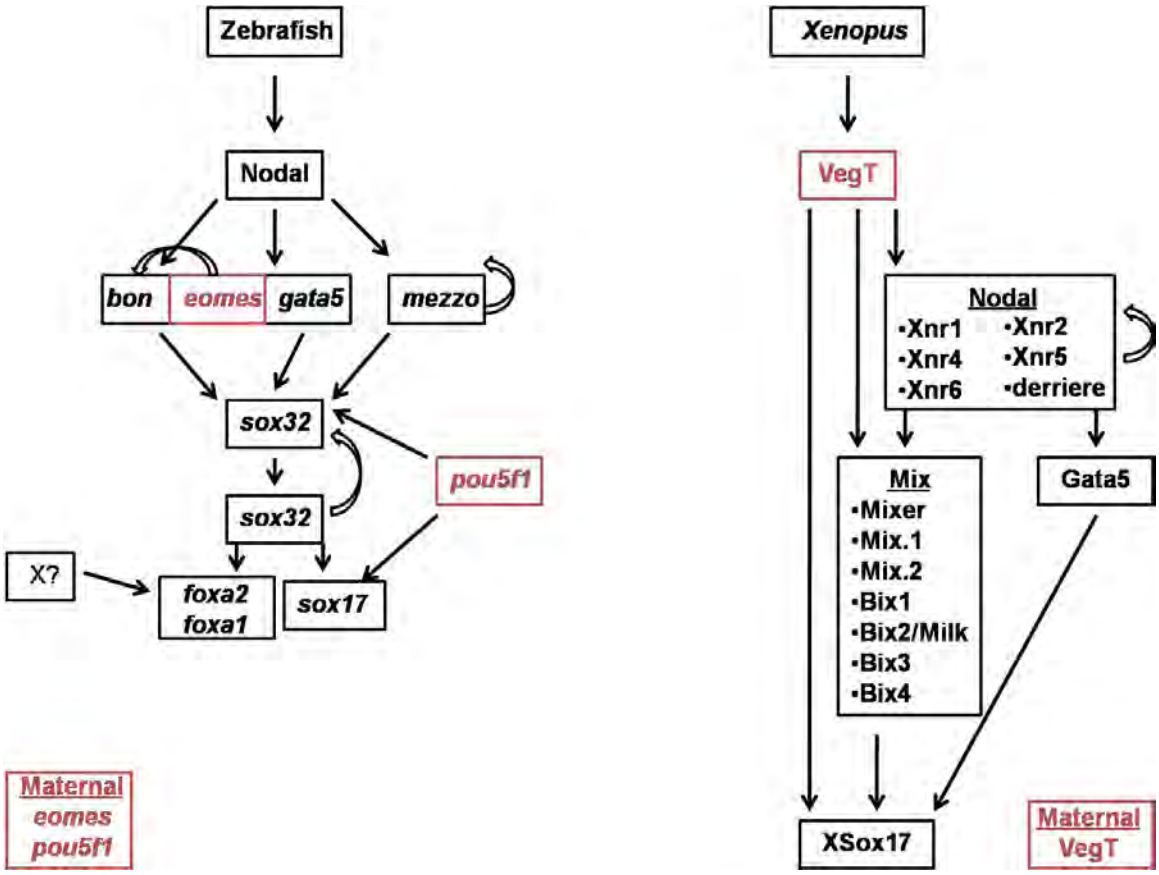
Some elements in the endoderm pathway are evolutionarily conserved (such as *sox17*), but other elements differ between organisms. In *Xenopus*, VegT, a T-box protein that is maternally and zygotically expressed, plays a critical role in inducing Nodal signaling, which is involved in mesoderm and endoderm formation. In addition, maternal VegT activates *XSox17* expression, which in turn suppresses mesodermal markers in the vegetal blastomeres and predisposes these cells to respond to endodermal signals (Figure 1.6). Accordingly, misexpressing XSox17 in the marginal zone inhibits the expression of mesodermal genes (Engleka, Craig et al. 2001). Once *XSox17* is activated, Nodal signaling maintains *XSox17* expression, as well as its own expression, and the expression of endoderm genes including *Mixer* and *Gata5* (Figure 1.5). Therefore, VegT activation of *Sox17* plays an important role in establishing the endoderm in *Xenopus* (Engleka, Craig et al. 2001).

In zebrafish, *tbx16/spadetail* is the ortholog of VegT. However, *tbx16* is zygotically expressed and is not involved in endoderm formation. Also, no similarity can be found when comparing the *sox17* upstream regulatory sequences between the two organisms and they are regulated differently. In particular, *VegT* induces *XSox17* and *XSox17* autoregulates its own expression in *Xenopus*, while it is *sox32* that regulates *sox17* expression in zebrafish and *sox17* may in turn regulate *sox32* (Figure 1.6)(Chan, Chao et al. 2009).

Figure 1.6 – Zebrafish and *Xenopus* endoderm pathways

Factors that function in early endoderm differentiation in zebrafish and *Xenopus*. To date, Mix-like transcription factors, *bonnie and clyde* (*bon*) and *mezzo*, function in early zebrafish endoderm formation, in contrast to seven Mix proteins in *Xenopus*. Orthologs for zebrafish *sox32* and *Xenopus Veg T* remain unidentified. In *Xenopus*, Pou5f1 functions to maintain embryonic stem cell pluripotency rather than in endoderm formation like it does in zebrafish.

Figure 1.6


Zebrafish
Nodal
bon
eomes
gata5
mezzo
sox32
sox32
pou5f1
X?
foxa2
foxa1
sox17
Maternal
eomes
pou5f1
Xenopus
VegT
Nodal
•Xnr1
•Xnr2
•Xnr4
•Xnr5
•Xnr6
•derriere
Mix
•Mixer
•Mix.1
•Mix.2
•Bix1
•Bix2/Milk
•Bix3
•Bix4
Gata5
XSox17
Maternal
VegT

The *sox32* gene, found only in zebrafish, and is necessary and sufficient for endoderm formation. Like *sox17*, *sox32* can repress mesodermal markers (Aoki, David et al. 2002). Also, similar to VegT regulating *XSox17*, *eomes* (VegT homologous T-box gene) positively regulates *sox32* expression in zebrafish (Bjornson, Griffin et al. 2005; Chan, Chao et al. 2009). It appears that *sox32* takes on part of the role of *sox17* in zebrafish.

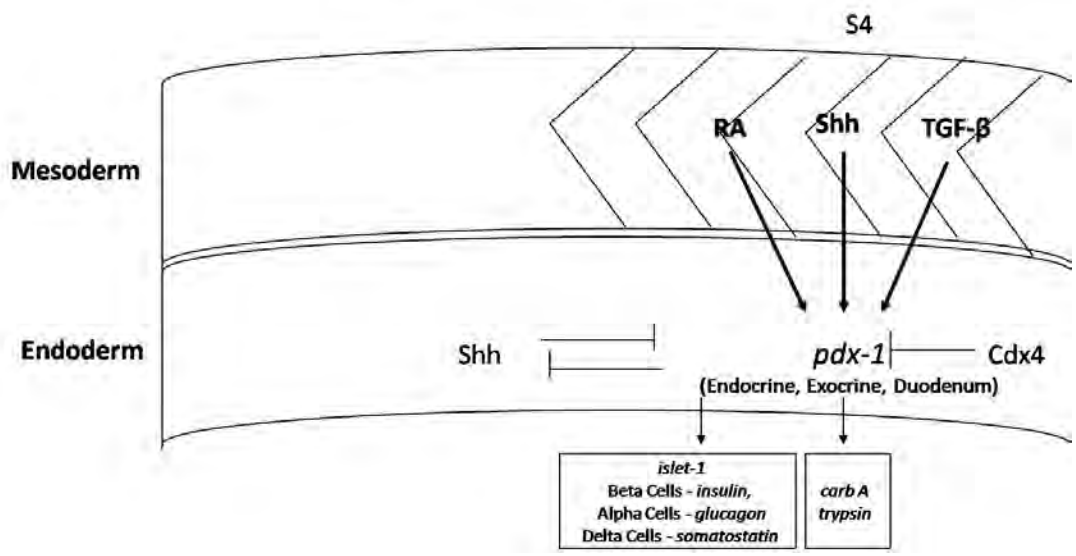
Specifying the pancreas from the endoderm

Once the endoderm is formed, there are various signals from the mesoderm that are necessary for development and patterning the pancreas (Figure 1.7). When endoderm fragments from gastrulating embryos are cultured with various adjacent mesoderm tissues, the adjacent tissue determines which genes are expressed (Wells and Melton 2000).

Figure 1.7 – Early pathway in pancreas patterning

Signals from the mesoderm (upper layer represented by somites), Retinoic Acid (RA), Sonic Hedgehog (Shh), and TGF-β, are necessary to activate *pdx1* expression which is necessary for pancreas formation. Furthermore, *pdx1* can then activate downstream pancreas markers specific to endocrine and exocrine cells. *Shh* expression in the anterior endoderm establishes the anterior border for the pancreas primordium. *Pdx1* and *shh* cannot be co-expressed but a gradient of Shh is established in the anterior endoderm and is further involved in patterning the pancreas, in particular endocrine versus exocrine fate. A RA gradient is formed in the anterior mesoderm which signals to the endoderm and pancreas. Posteriorly, *cdx4* is expressed and establishes the posterior border for the pancreas primordium. RA induces pancreas markers and Cdx4 represses them. These factors act together to maintain the pancreas primordium at somite 4 during early embryogenesis.

Figure 1.7


S4
RA
Shh
TGF-β
Mesoderm
Endoderm
Shh
pdx-1
Cdx4
(Endocrine, Exocrine, Duodenum)
islet-1
Beta Cells - insulin,
Alpha Cells - glucagon
Delta Cells - somatostatin
carb A
trypsin

Sonic Hedgehog plays a dual role in pancreas development

Sonic hedgehog (Shh) is one of three proteins in the mammalian signaling pathway family called hedgehog. Shh protein interacts with two transmembrane proteins; Patched (Ptc) and Smoothened (Smo). Ptc binds with Shh whereas Smo does not bind Shh, but rather acts as a signal transducer. Ptc inhibits Smo function in the absence of Shh and therefore transcription downstream of the Shh pathway does not occur. If Shh is present, then Ptc binds to Shh and the inhibition on Smo lifted and transcription can occur. Shh plays a key role in regulating vertebrate organogenesis, such as the growth of digits on limbs and organization of the brain and it also plays a role in pancreas development. Using cyclopamine (inhibits Smo by direct binding) to block Shh signaling before 4 hpf abolishes *ins* expression, where later treatment results in an anterior shift of *ins* and multiple ectopic spots of *ins*. Thus, *shh* expression (from the dorsal mesoderm) is required before gastrulation to induce pancreas formation and is required during gastrulation in the pharyngeal endoderm to maintain expression of *ins* at somite (s) 4 (dilorio, Moss et al. 2002).

Retinoic Acid (RA) signaling is necessary for pancreas formation

Retinoic Acid is the oxidized form of vitamin A. Retinol (alcohol form of vitamin A) is converted to an aldehyde (retinaldehyde), which is further converted to a carboxylic acid (retinoic acid). The first oxidation step is performed by retinol dehydrogenases (RDHs), which have widespread overlapping expression

patterns. The second oxidation step is made possible by retinaldehyde dehydrogenases (Raldh or Aldh), which have a more tissue specific expression pattern. Retinoic acid acts by binding to heterodimers of the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), which then bind to retinoic acid response elements (RAREs) in the regulatory regions in DNA, thereby activating gene transcription (Ang, Deltour et al. 1996; Mic, Haselbeck et al. 2002; Sandell, Sanderson et al. 2007; Wingert, Selleck et al. 2007; Zhang, Hu et al. 2007; Duester 2008).

In zebrafish RA acts as a posteriorizing agent in mesoderm and neural patterning (Maden 1999; Gavalas and Krumlauf 2000) and has a similar effect on endoderm development (Kumar and Melton 2003). In particular, RA acts at the end of gastrulation (9-13 hpf) in patterning the endoderm. Blocking RA signaling with BMS493 (RAR antagonist) results in loss of pancreas and liver markers but thyroid and pharyngeal endoderm expression is unaffected. Overexpressing RA expands liver and pancreas markers anteriorly and as a result thyroid and pharynx expression is decreased (Stafford and Prince 2002). Markers only expand anterior because endodermal *cdx4* expression regulates the posterior limit of the pancreatic domain, blocking it from expanding posteriorly (Kinkel, Eames et al. 2008). Blocking *cdx4* and overexpressing RA results in expansion of the pancreas both anteriorly and posteriorly (Stafford and Prince 2002).

To date, *aldh1a2*, *aldh1a3,* and *aldh8a1* have been identified in zebrafish. *aldh1a3* is expressed in the developing eye and ear after gastrulation and

aldh8a1 is expressed in the liver and intestine around 48 hpf (Liang, Zhang et al. 2008; Pittlik, Domingues et al. 2008). *aldh1a2* is expressed in the mesendoderm before gastrulation and in the posterior and lateral mesoderm during gastrulation (Begemann, Schilling et al. 2001). The expression pattern is consistent with the findings that the paraxial mesoderm is the source of RA involved in pancreas development. Three RAR (two RARα and RARγ) are expressed in the endoderm, indicating that RA signals can be received in the endoderm (Stafford, White et al. 2006) supporting the role of RA in endoderm patterning and pancreas development.

Notochord is required for pancreas development but not differentiation

The notochord is a flexible, rod-shaped structure found in embryos. The notochord forms during gastrulation and induces the formation of the floor plate and the development of the neural tube. Initially, the notochord is located between the neural tube and the endoderm, but soon becomes separated from them by the mesoderm, which grows medially and surrounds it. The role of the notochord in pancreas formation has been revealed by the analysis of several mutations. For example, *floating head/Xnot* mutant embryos, which lack notochord, develop only a few pancreas cells. Interestingly, *no tail/T* mutants, which have disrupted notochord differentiation, have normal pancreas expression suggesting that the notochord is required for pancreas development and expansion in zebrafish, but not its differentiation (Biemar, Argenton et al. 2001).

The endoderm plays multiple roles in development

Not only does the endoderm give rise to the digestive tract, which is necessary for survival of the organism, it also secretes signals that pattern the mesoderm and the ectoderm. For example, the zebrafish cardiac primordia are contained within the anterior LPM (a bilateral population) and these two populations meet and fuse at the midline to form the definitive heart tube. In *gata5, sox32, oep,* and *bon* mutants, this migration does not occur and the two cardiac primordia develop in their original lateral positions resulting in a phenotype called cardia bifida. This suggests a role for zebrafish endoderm in cardiac development within the mesoderm (Chen, Haffter et al. 1996; Stainier, Fouquet et al. 1996).

The endoderm also plays a role in patterning the neural crest cells in the ectoderm. Pharyngeal endoderm pouches meet the ectoderm and piercing of the thin membrane gives rise to gill slits (individual openings to gills that act in respiration). The individual gill slits develop in an A-P pattern and are separated by pharyngeal arches with ectoderm tissue on the outside and endoderm on the inside. The endodermal pouches are necessary for keeping neural crest cells of neighboring arches separate. *sox32* and *oep* mutants lack segmentally arranged endodermal pouches which leads to the fusion of neural crest-derived pharyngeal cartilages as well as patterning defects in the pharyngeal mesoderm (Piotrowski and Nusslein-Volhard 2000). Tissue culture experiments

demonstrate that neural crest cells can differentiate into chondroblasts (gives rise to cartilage) if they are cocultured with endoderm (Balinsky 1939; Hall 1980). This is consistent with reduced cartilage observed in *sox32* mutants. This indicates that endoderm is responsible for the initial segmentation of the pharyngeal region, but the neural crest cells specify the positioning of the different arches (Piotrowski and Nusslein-Volhard 2000).

Endodermal organs can also contribute signaling factors that pattern the mesoderm surrounding them. For example, the pancreas primordium participates in cross talk between germ layers. *fgf24* expression in the pancreas triggers patterning of the LPM which lies adjacent to the ventral pancreatic bud. In turn, the LPM then expresses *isl1, fgf10,* and *meis* as well as *fgf24* to then further specify the ventral pancreas (Manfroid, Delporte et al. 2007). Therefore, cross talk between germ layers is essential to proper patterning and development of the organism.

In summary, the endoderm pathway consists of some evolutionary conserved factors among vertebrates while including nonconserved elements necessary for endoderm formation and each component plays a specific role in endoderm formation. Signals from the mesoderm such as Shh and RA are necessary to direct the endoderm to form the pancreatic buds. The buds are then further patterned by activation of pancreatic gene expression to develop endocrine and exocrine lineages and eventually to form one functioning organ.

CHAPTER II

TALE-FAMILY HOMEODOMAIN PROTEINS REGULATE ENDODERMAL *sonic hedgehog* EXPRESSION AND PATTERN THE ANTERIOR ENDODERM

Abstract

sonic hedgehog (*shh*) is expressed throughout the endoderm but its expression is excluded from the pancreas primordium. Specifically, *shh* expression in the anterior endoderm blocks pancreatic markers and therefore patterns the anterior endoderm, but the pathway controlling endodermal *shh* expression is unclear. Using loss of function experiments, we found that expression of Meis3, a TALE class homeodomain protein, acts upstream of *shh* expression in the anterior endoderm of zebrafish embryos. Use of a dominant negative construct or anti-sense morpholino oligos (MOs) to disrupt Meis3 function, led to ectopic *insulin* expression in anterior endoderm. This phenotype is also observed when *meis3* MOs are targeted to the endoderm suggesting that Meis3 acts within the endoderm to restrict *insulin* expression. Notably, loss of Meis3 function results in decreased *shh* and *foxa2* expression in the anterior endoderm. Loss of Pbx4, a TALE homeodomain family member encoding a Meis cofactor, results in the same phenotype. This is consistent with a conserved role of Meis3 acting in a complex with Pbx4. Our data indicate that Meis3 and Pbx4 regulate *foxa2* and *shh* expression in anterior endoderm, thereby influencing patterning and growth of the foregut. Lastly, we observe a

progressive anterior displacement of endodermal organs when disrupting Meis3 or Pbx4.

Background

The vertebrate pancreas is a gland with both endocrine and exocrine functions. The pancreas is primarily composed of exocrine tissue (95-99%) that produces and secretes digestive enzymes into the pancreatic ducts to be secreted into the small intestine. The endocrine pancreas consists of islets of Langerhans which are comprised of clusters of hormone secreting cells; insulin secreting β-cells, surrounded by glucagon–secreting α-cells, somatostatin-secreting δ-cells, pancreatic polypeptide hormone-secreting cells and ghrelin-secreting ε-cells that all aiding in blood homeostasis (Slack 1995; Kumar and Melton 2003). The pancreas develops from two distinct buds from the ventral and dorsal foregut endoderm. The dorsal bud is specified by signals secreted from the notochord which is in close contact with the dorsal bud during initial phases of development. *Pdx1* (paraHox transcription factor, *pancreas duodenal transcription factor 1*) expression is initiated during this time period (Kim, Hebrok et al. 1997; Hebrok, Kim et al. 1998). The ventral bud is specified by signals secreted from the lateral plate mesoderm and then by the cardiac mesoderm (Lammert, Cleaver et al. 2001; Kumar, Jordan et al. 2003). The ventral and dorsal buds both interact with the aorta dorsally and the two vitelline veins ventrally. Both regions have been shown to be necessary and sufficient to determine expression of *Pdx1* (Lammert, Cleaver et al. 2001; Lammert, Cleaver et al. 2003).

Signaling from various pathways is necessary to initiate pancreas development, as well as pattern the endoderm. These include Hedgehog, Wnt, transforming growth factor (TGF)-β, and receptor tyrosine kinase pathways; including fibroblast growth factors (FGF) and epidermal growth factors (EGF) (van den Brink 2007). Hedgehog proteins act as morphogens, substances that originate from a localized source and form a concentration or activity gradient through a tissue. Morphogen concentration gradients contain positional information and specify distinct cellular phenotypes within a group of receiving cells. This positional information is dependent on the distance of the cell from the signal source, or in other words exposure time to the signal as well as ligand concentration (Wolpert 1996). The *hedgehog* gene was initially discovered in *Drosophila* (Lee, von Kessler et al. 1992; Mohler and Vani 1992; Tabata, Eaton et al. 1992). In mouse and humans, there are three hedgehog genes, *sonic hedgehog*, *desert hedgehog*, and *indian hedgehog* which are highly conserved (Echelard, Epstein et al. 1993; Marigo, Roberts et al. 1995). In zebrafish, *shh a, shh b* (formerly described as *tiggywinkle hedgehog*), and *indian hedgehog b* (formerly described as *echidna hedgehog)* are expressed (Krauss, Concordet et al. 1993; Roelink, Augsburger et al. 1994; Schauerte, van Eeden et al. 1998). Within a cell, the Hedgehog signal is transmitted by a seven-span transmembrane receptor called Smoothened (Smo). Hedgehogs do not directly bind Smo but bind a second receptor called Patched (Ptc)(van den Brink 2007). There are two vertebrate *Ptc* genes, *Ptc1* (Goodrich, Johnson et al. 1996) and

Ptc2 (Motoyama, Takabatake et al. 1998). The *Ptc* gene encodes a 12-span transmembrane receptor with two large hydrophilic extracellular loops that mediate Hedgehog binding. In the absence of Hedgehog, Ptc inhibits Smo signaling. This process is not completely understood but it is believed that Ptc acts catalytically (Taipale, Cooper et al. 2002) and controls the localization of a secondary Smo-inhibiting molecule (Bijlsma, Spek et al. 2006; Dwyer, Sever et al. 2007).

Pancreatic development requires exclusion of Hedgehog expression from both the pancreatic ventral and dorsal buds (Apelqvist, Ahlgren et al. 1997; Kim, Hebrok et al. 1997; Hebrok, Kim et al. 1998; Deutsch, Jung et al. 2001). In mouse, *Shh* and *Ihh* are expressed uniformly throughout the endoderm of the gut tube except in the pancreatic buds where *Pdx1* is expressed (Apelqvist, Ahlgren et al. 1997; Li, Arber et al. 1999). Overexpression of *Shh* under the control of the *Pdx1* promoter converts the majority of the pancreatic tissue to intestine and intestinal mesenchyme supporting that the exclusion of Hedgehog expression is necessary for pancreas gene expression. Although the pancreatic buds did contain a few endocrine and acinar cells, they failed to form islets and acinar structures and they produced mucins that are typical of intestinal epithelial cells (Apelqvist, Ahlgren et al. 1997). In *Xenopus*, injecting a constitutively active form of the Hedgehog receptor Smo blocks pancreas formation (Zhang, Rosenthal et al. 2001). This phenotype is more severe than what was observed in mouse, most likely due to *Shh* being regulated by *Pdx1* and the pancreatic domain

already being specified by the time *Shh* was overexpressed (van den Brink 2007). Therefore, suppressing Shh signaling from the pancreatic buds early in development is essential for normal pancreatic development.

In zebrafish, the role of Hedgehog signaling has diverged from other vertebrates. Treating zebrafish embryos with cyclopamine (inhibitor of Hedgehog signaling) at various timepoints during development indicates that *shh* (from the dorsal mesoderm) is necessary before gastrulation to initiate pancreas development but must be excluded from the pancreas primordium in order for pancreas genes to be expressed (Roy, Qiao et al. 2001; dilorio, Moss et al. 2002). Notably, injecting *shh* mRNA increases the number of pancreas precursors (Roy, Qiao et al. 2001) and the *Shh* and *Smo* mutants have diminished number of pancreatic precursors confirming the early role of *shh* in pancreas formation (Roy, Qiao et al. 2001; dilorio, Moss et al. 2002). However, after gastrulation, *shh* is expressed in the pharyngeal endoderm and plays a repressive role; thereby blocking pancreas markers and maintaining pancreas expression at somite 4 and as a result, patterning the anterior foregut (dilorio, Moss et al. 2002).

TALE (three amino acid loop extension) homeodomain proteins were originally identified as genes involved in body patterning. They function as cofactors primarily known for their interactions with Hox proteins (Mann 1995; Mann and Chan 1996; Mann and Affolter 1998). The first cofactor discovered was *Drosophila* Extradenticle (Exd) (Peifer and Wieschaus 1990; Rauskolb,

Peifer et al. 1993; Rauskolb and Wieschaus 1994; Rauskolb, Smith et al. 1995). Exd is related to a vertebrate protein called Pbx1, which is linked to human preB cell acute lympoblastic leukemia (Kamps, Murre et al. 1990; Nourse, Mellentin et al. 1990; Rauskolb, Peifer et al. 1993). Other *Pbx* genes are expressed in the vertebrate genome including, *Pbx2*, *Pbx3* and *Pbx4* (Monica, Galili et al. 1991; Popperl, Rikhof et al. 2000). Another cofactor, the MEIS class (*myeloid ecotropic viral integration site*)(Burglin 2005), regulates Hox activity as part of the DNA-bound HOX complex and by regulating Pbx/Exd activity in the absence of DNA (Moskow, Bullrich et al. 1995; Chang, Jacobs et al. 1997; Chen, Rossier et al. 1997; Knoepfler, Calvo et al. 1997; Berthelsen, Zappavigna et al. 1998). The MEIS class includes Homothorax (Hth) in flies and the Meis and Prep proteins in vertebrates (Moens and Selleri 2006). In vertebrates, there are four Meis proteins: Meis1, Meis2, Meis3 and Meis4 and two Prep proteins, Prep 1 and Prep2 (Sagerstrom, Kao et al. 2001; Waskiewicz, Rikhof et al. 2001; Zerucha and Prince 2001). Not only do Meis/Prep proteins act directly with Pbx/Exd and participate in DNA-bound Hox complexes (Mann and Affolter 1998; Jacobs, Schnabel et al. 1999; Ryoo and Mann 1999; Ferretti, Marshall et al. 2000; Ebner, Cabernard et al. 2005), they also promote nuclear localization (Rieckhof, Casares et al. 1997; Berthelsen, Zappavigna et al. 1998; Abu-Shaar, Ryoo et al. 1999; Mercader, Leonardo et al. 1999; Jaw, You et al. 2000) and stability of Pbx proteins (Jaw, You et al. 2000; Waskiewicz, Rikhof et al. 2001; Longobardi and Blasi 2003).

Pbx members are widely expressed in the developing embryo including the pancreas and dimerize with a variety of hox and parahox homeodomain transcription factors (Chan, Jaffe et al. 1994; van Dijk and Murre 1994; Chang, Shen et al. 1995). In zebrafish, a subset of motor neurons that originate from rhombomere 4 (r4) in the hindbrain fail to migrate into r6 and r7 in *pbx4* mutants (*lazarus, lzr*) (Pöpperl, Rikhof et al. 2000; Cooper, Leisenring et al. 2003). This phenotype mimics the mouse *Hoxb1* mutant and *hoxb1a* anti-sense morpholino (MO) injected zebrafish embryos (Studer, Lumsden et al. 1996; McClintock, Kheirbek et al. 2002; Arenkiel, Tvrdik et al. 2004) indicating a conserved role in hindbrain development. *pbx4* is expressed both maternally and zygotically and the zygotic ubiquitous expression overlaps with *pbx2*. Injecting *pbx2* MO into MZ*pbx4* (lacks maternal and zygotic expression) results in severe hindbrain phenotype converting the entire hindbrain to r1 identity (Waskiewicz, Rikhof et al. 2002) suggesting that they both function in hindbrain development.

Results

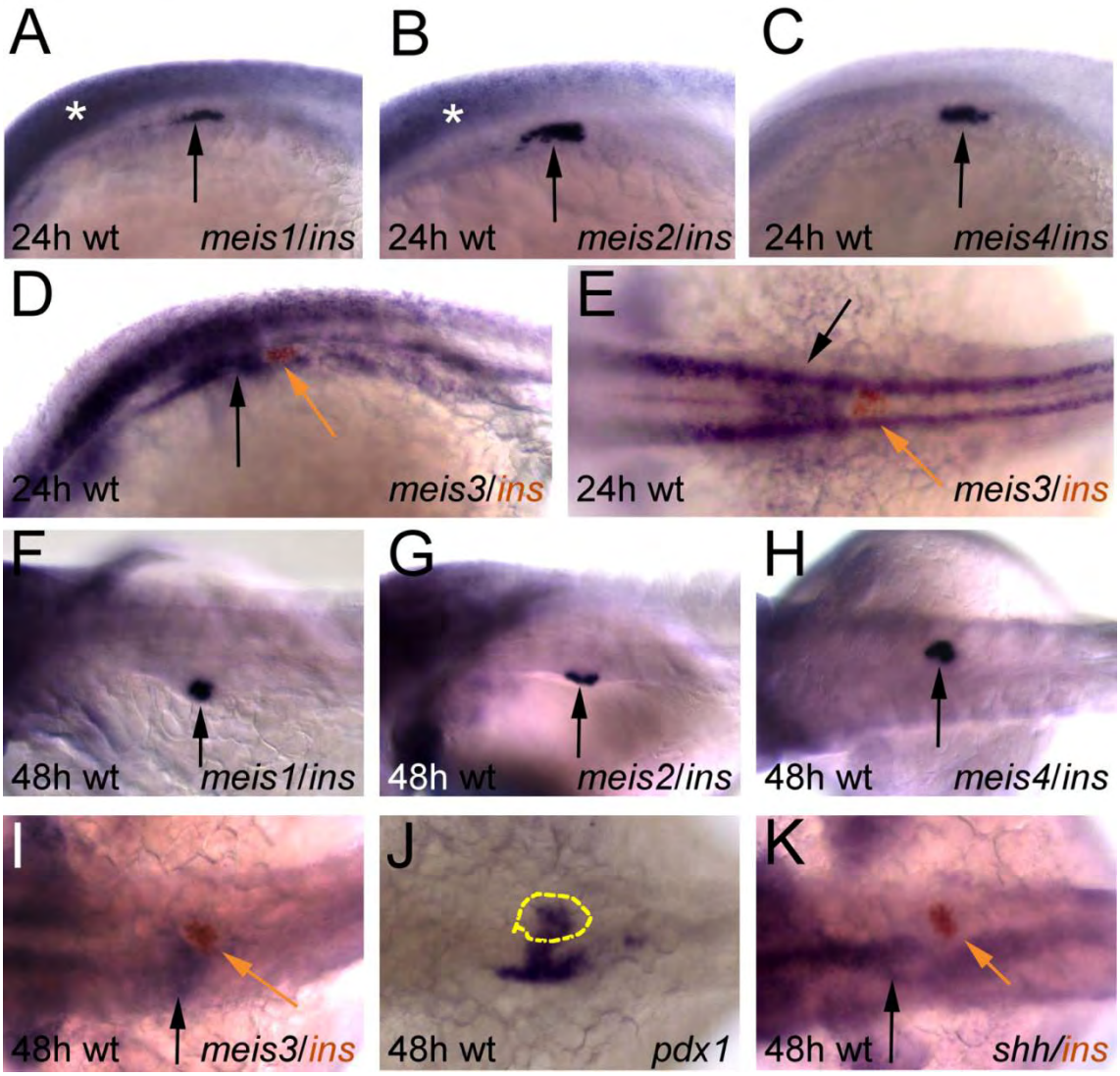
meis3 is expressed in the anterior endoderm

We examined expression of *meis1, meis2, meis3* and *meis4* at 24 and 48 hpf (Sagerstrom, Kao et al. 2001; Waskiewicz, Rikhof et al. 2001; Zerucha and Prince 2001). Only *meis3* shows expression in the endoderm at these stages of development (Figure 2.1D, E). *meis1, meis2* and *meis4* are not expressed in the endoderm at 24 hpf (Figure 2.1A-C) or 48 hpf (Figure 2.1F-H) but rather, *meis1* and *meis2* are expressed in the neural tube (Figure 2.1A, B, white asterisks). However, *meis3* expression (Figure 2.1D, E, purple stain) lies adjacent to *insulin* (*ins*) expression (red stain) in the islet where it remains at 48 hpf in the region that also expresses *pdx1* (pancreas expression outlined by yellow) (Figure 2.1I-J). The expression pattern is similar to *shh* expression in the pharyngeal endoderm (Figure 2.1K). Two other MEIS family members, *prep1*and *prep2* (Waskiewicz, Rikhof et al. 2001; Choe, Vlachakis et al. 2002; Deflorian, Tiso et al. 2004) are broadly expressed throughout the entire embryo and were not examined.

Figure 2.1 – *meis3* is expressed in the endoderm anterior to the islet

(A-C) Double in situ of 24 hpf zebrafish embryos, marking *ins* expression (black arrows) and (A) *meis1*, (B) *meis2* and (C) *meis4* expression in blue, not in the endoderm. *meis1*and *meis2* are both expressed in the neural tube (white asterisks). (D, E) *meis3* expression (purple stain, black arrows) is expressed anterior to *ins* (red stain, red arrows). (F-H) *meis1, meis2* and *meis4* expression is not detected near *ins* (black arrows) or in the endoderm at 48 hpf (purple stain). (I) At 48 hpf, *meis3* (purple stain, black arrow) is expressed adjacent to *ins* (red stain, red arrow). (J) *pdx1* expression in the pancreas (yellow outline) and intestine at 48 hpf. (K) *shh* (purple stain, black arrow) is expressed next to *ins* (red stain, red arrow) in the anterior foregut. Developmental stages are located at lower left; in situ probes are located at lower right. A-D, lateral view; E, H-K, dorsal view; F, G, ventrolateral views. Anterior is to the left in each panel.

Figure 2.1


A
24h wt
meis1/ins
B
24h wt
meis2/ins
C
24h wt
meis4/ins
D
24h wt
meis3/ins
E
24h wt
meis3/ins
F
48h wt
meis1/ins
G
48h wt
meis2/ins
H
48h wt
meis4/ins
I
48h wt
meis3/ins
J
48h wt
pdx1
K
48h wt
shh/ins

meis3 is required for *shh* expression and therefore the repression of *insulin* in the anterior foregut

We have previously reported that ΔCpbx4 construct sequesters Meis proteins in the cytoplasm (Choe, Vlachakis et al. 2002). This inhibits Meis proteins from regulating transcription in the nucleus resulting in a dominant negative phenotype (Choe, Vlachakis et al. 2002; Choe and Sagerstrom 2004; Choe and Sagerstrom 2005). We injected ΔCpbx4 at the one cell stage and ectopic *ins* expression resulted in the anterior endoderm. In particular, 70% (51/77) of 24 hpf and 89% (64/72) of 48 hpf ΔCpbx4-injected embryos exhibited this phenotype (Figure 2.2C, F). Since ΔCpbx4 inhibits all *meis* family members (Choe, Vlachakis et al. 2002; Choe and Sagerstrom 2004), we injected two translational antisense morpholinos (tMO1 and tMO2) targeting *meis3*. In both cases, ectopic *ins* expression was observed in the anterior endoderm in a dose-dependent manner (Table 2.1). This shows that *meis3* is necessary to repress *ins* expression in the anterior endoderm. 200 μM tMO1 and 150 μM tMO2 had the greatest number affected (74% and 48%), while a 5 bp mismatch control MO (MOCO) had little to no effect indicating that tMO is functional.

Figure 2.2 – *meis3* and *pbx4* are necessary to inhibit *insulin* expression in the anterior foregut

(A-F) Ectopic *ins* results in ΔCpbx4-injected embryos and *pbx4* mutant embryos. *ins* expression (ectopic expression, black arrows) in wildtype embryos at 24 and 48 hpf (A, D), *lzr* mutant embryos (B, E) and ΔCpbx4-injected embryos (C, F). Anterior shift in *ins* expression can be seen at 48 hpf. (G-I) Embryos injected with *sox32* into one cell at the 32 cell stage. (G) Wildtype embryo injected with *sox32* mRNA and rhodamine-dextran. Image is a confocal stack of approximately 125 μm that extends halfway through the embryo. (H, I) Gut GFP transgenic embryos (Field, Ober et al. 2003) injected with *sox32* mRNA and rhodamine dextran at the 32 cell stage. (I) Overlay of 24 hpf Gut GFP embryo with rhodamine-dextran signal. Signal is observed in the region next to the yolk, same as *foxa3:gfp* expression in the endoderm (white arrows in H,I) but does not overlap with expression in the hatching gland (white asterisks). (J,K) Wildtype embryos injected with *sox32* mRNA along with *gfp* mRNA (J, green) and rhodamine dextran (K, red). mRNA co-localizes in the endoderm at 24 hpf (white arrows). (L-N) Wildtype embryos injected with *sox32* mRNA, rhodamine-dextran, and fluorescent control MO (Fl-MO). 24 hpf embryos were monitored for Fl-MO expression (L) and rhodamine dextran (M), and (N) is an overlay of the two expression patterns in the endoderm (white arrows). (O,P) Embryos injected with *sox32* mRNA, tMO1, and rhodamine dextran were assayed for *ins* expression (purple) at 24 hpf. Ectopic *ins* expression is marked by black arrow in

the anterior endoderm (P). All panels are lateral views with anterior to the left. s1-s4 indicates somite position determined by Nomarski optics.

Figure 2.2

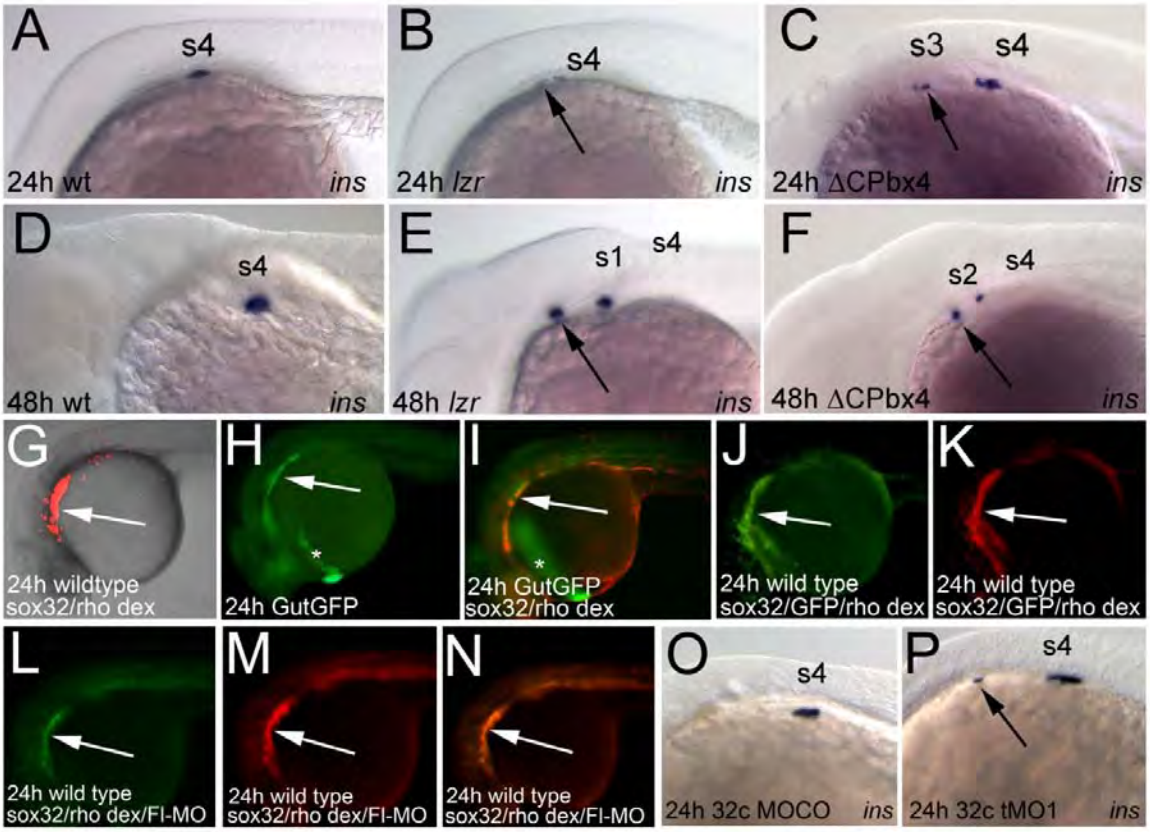


Table 2.1: Dose-response data for *meis3* MOs

Morpholino[1]	Embryos with ectopic *insulin* clusters[2]	Deformed Embryos[3]
tMO1		
100uM	22/38 (58%)	0/38 (0%)
200uM	86/117 (74%)	1/117 (1%)
300uM	22/31 (71%)	3/28 (11%)
400uM	29/52 (56%)	5/56 (9%)
tMO2		
100uM	22/63 (35%)	0/63 (0%)
150uM	46/96 (48%)	0/96 (0%)
MOCO		
100uM	6/48 (12%)	0/48 (0%)
200uM	18/240 (7%)	0/240 (0%)
300uM	3/25 (12%)	2/25 (8%)
400uM	2/29 (7%)	3/29 (10%)

[1]Embryos were injected with the indicated MO concentration at the 1-cell stage, raised to 72hpf and scored for *insulin* expression by in situ hybridization.

[2]Embryos with more than one patch of *insulin* expression were scored as having ectopic *insulin* clusters.

[3]Deformed embryos showed convergence/gastrulation defects and were not scored for *insulin* expression.

Next, we took advantage of a previously published technique to target tMO1 to the endoderm. Overexpressing TARAM-A* at the 1-2 cell stage inhibits epiboly and converts the entire blastoderm into mesendoderm (Renucci, Lemarchandel et al. 1996; Peyrieras, Strahle et al. 1998; Alexander and Stainier 1999). Injection of mRNA encoding TARAM-A* into a single blastomere at the 16 cell stage directs progeny to endoderm fates in cell-autonomous manner. Similarly, *casanova* (*sox32*, acts downstream of TARAM-A) injected into one cell at the 16 cell stage results in the marginal blastomeres failing to express prechordal plate markers and express endodermal markers. As a result, the progeny were incorporated into the digestive tract endoderm (Ober, Field et al. 2003). Also, misexpressing *sox32* at the margin at early stages can redirect cells to endodermal fate in a cell autonomous manner (Alexander and Stainier 1999; Kikuchi, Agathon et al. 2001).

First, we used this technique to determine if *sox32* injected embryos develop normal endoderm and does the technique successfully target reagents to the endoderm. As a result, the endoderm develops normally when we injected *sox32* mRNA into one cell at the 32 cell stage with rhodamine dextran as a tracer (Figure 2.3). Also, we determined that the mRNA co-localizes with rhodamine dextran (Figure 2.4) as well as an injected fluorescent MO (Figure 2.5).

Figure 2.3 – Injecting *sox32* mRNA does not affect normal endoderm development

A single cell was injected with *sox32* mRNA and rhodamine dextran at the 32-cell stage. Embryos were fixed and assayed for expression of *ins* (endocrine pancreas) at 24 hpf (A, B), *p48* (exocrine pancreas) at 30 hpf (C, D), *sid4* (liver) at 48 hpf (E, F), *pdx1* (exocrine and endocrine pancreas, and intestine) at 48 hpf (G, H), *ins* (endocrine pancreas) at 72 hpf (I, J) and *carbA* (exocrine pancreas) at 72 hpf (K, L) by whole mount in situ hybridization. All embryos are in lateral view with anterior to the left and exhibit normal endoderm gene expression.

Figure 2.3

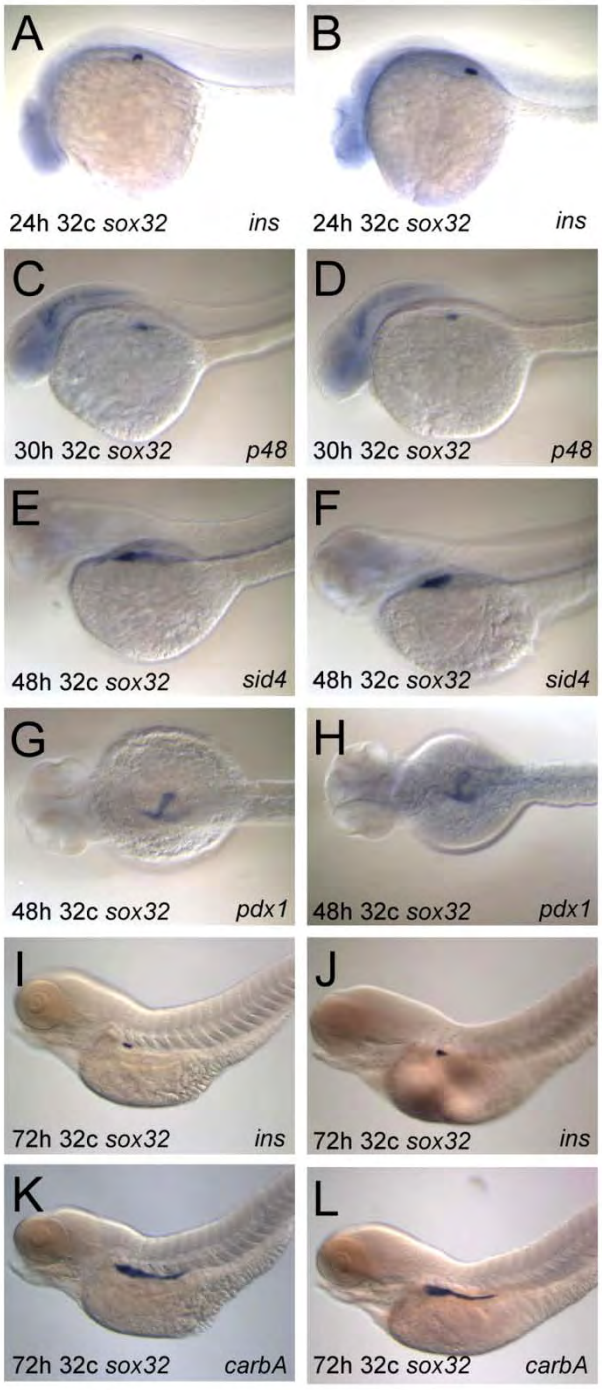

A
24h 32c sox32 ins
B
24h 32c sox32 ins
C
30h 32c sox32 p48
D
30h 32c sox32 p48
E
48h 32c sox32 sid4
F
48h 32c sox32 sid4
G
48h 32c sox32 pdx1
H
48h 32c sox32 pdx1
I
72h 32c sox32 ins
J
72h 32c sox32 ins
K
72h 32c sox32 carbA
L
72h 32c sox32 carbA

Figure 2.4 – *gfp* mRNA and rhodamine dextran co-localize following co-injection

A single cell was injected with a mixture of *sox32* mRNA, *gfp* mRNA and rhodamine dextran at the 32-cell stage. Embryos were monitored by bright field (A, B) and for rhodamine (C, D) and fluorescein (E, F) signals at the 256-cell stage (approximately 2 hpf; A, C, E) and the oblong stage (~3.5 hpf; B, D, F). Rhodamine and fluorescein signals overlap throughout development.

Figure 2.4

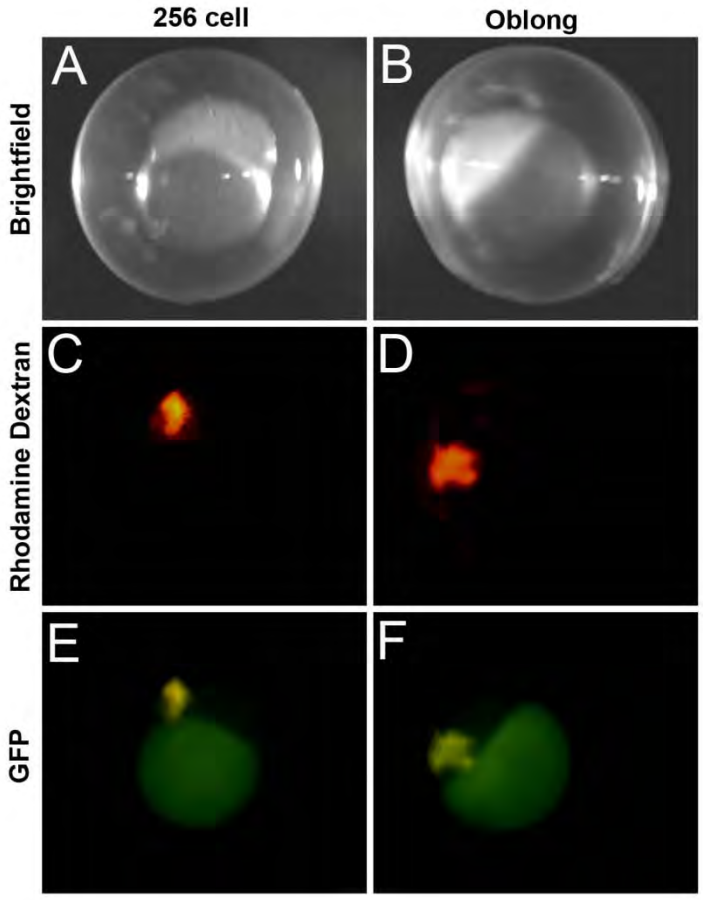


Figure 2.5 – Morpholinos and rhodamine dextran co-localize following co-injection

A single cell was injected with a mixture of *sox32* mRNA, rhodamine dextran and fluorescein-tagged control MO at the 32-cell stage. Embryos were monitored by bright field (A-D) and for rhodamine (E-H) and fluorescein (I-L) signals at the 64-cell stage (2 hpf), the 128-cell stage (2.5 hpf), the 512-cell stage (3 hpf) and the oblong stage (3.5 hpf).

Figure 2.5

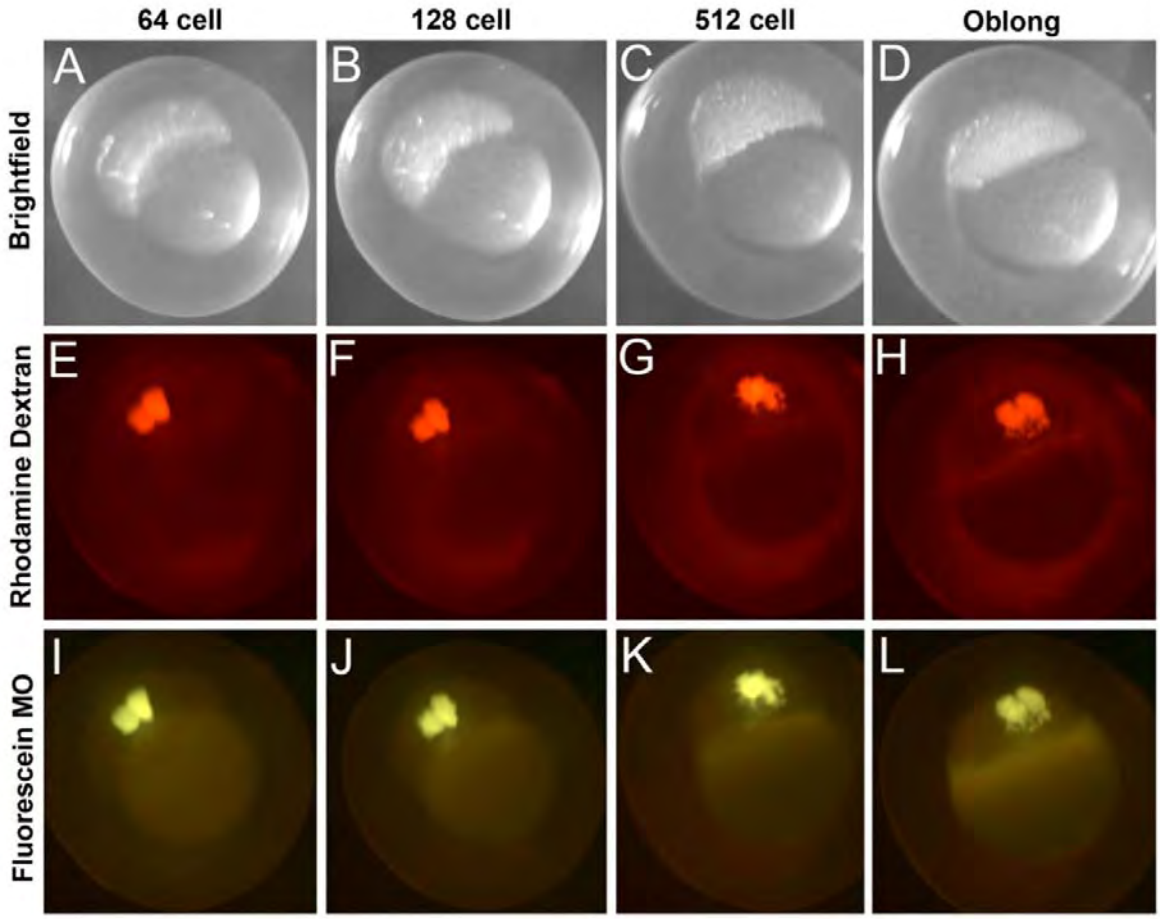

64 cell
128 cell
512 cell
Oblong
Brightfield
Rhodamine Dextran
Fluorescein MO
A
B
C
D
E
F
G
H
I
J
K
L

Table 2.2: Dose response data for targeting of MOs to the endoderm

Morpholino[1]	Embryos with ectopic *insulin* clusters[2]	Embryos with anterior shift of *insulin* clusters[3]
500uM tMO1		
24hpf	14/21 (67%)	0/21 (0%)
48hpf	16/31 (52%)	31/31 (100%)
72hpf	47/89 (53%)	89/89 (100%)
1mM tMO1		
24hpf	9/27 (33%)	0/27 (0%)[4]
48hpf	4/36 (11%)	0/36 (0%)[4]
72hpf	27/59 (46%)	59/59 (100%)
500uM MOCO		
24hpf	0/5 (0%)	0/5 (0%)
48hpf	0/54 (0%)	0/54 (0%)
72hpf	0/30 (0%)	0/30 (0%)
1mM MOCO		
48hpf	1/15 (6%)	0/15 (0%)
72hpf	4/33 (12%)	0/33 (0%)

[1]MOs were injected at the indicated concentration and targeted to the endoderm as outlined in Materials and Methods. *insulin* expression was detected by in situ hybridization at the stage indicated.

[2]Embryos with more than one patch of *insulin* expression were scored as having ectopic *insulin* clusters.

[3]Embryos where the predominant cluster of *insulin* positive cells was found anterior to the level of somite 4 were scored as having an anterior shift.

[4]The predominant cluster of *insulin* positive cells showed an anterior ‘smear’ in many of these embryos (18/27 at 24hpf; 32/36 at 48hpf), possibly indicating a mild anterior shift.

Next, we injected *meis3* tMO1 along with *sox32* mRNA and rhodamine-dextran into a single cell at the 32-cell stage, targeting tMO1 to the endoderm. The mRNA and rhodamine dextran co-segregates during development and contributed primarily to the endoderm (Figure 2.2G-N). Approximately 67% (14/21) of 24 hpf and 52% (16/31) of 48 hpf embryos with tMO1 targeted to the endoderm have ectopic *ins* expression (Table 2.2; Figure 2.2P). Embryos injected with MOCO have normal *ins* expression (59/59; Figure 2.2O) indicating that loss of Meis3 has an effect on islet formation.

shh and _foxa2_ expression in the pharyngeal endoderm is disrupted in _lzr_ mutant embryos and ΔCpbx4-injected embryos

shh (*sonic hedgehog*) is expressed in the endoderm, but is excluded from the pancreatic primordium. Since anterior endoderm expression of *shh* is not co-expressed with pancreatic markers, there is a conserved role for *hedgehog* to pattern the endoderm by preventing pancreatic markers from being expressed in zebrafish (Sun and Hopkins 2001; diIorio, Moss et al. 2002), chick (Kim and Melton 1998), and mouse (Hebrok, Kim et al. 2000). Interestingly, *shh* expression in the anterior endoderm and fin buds is reduced in ΔCpbx4-injected embryos but CNS (central nervous system) expression is unaffected (Figure 2.6H). Upon closer examination, *ins* positive cells were found caudal to *shh* expression in a double in situ (red arrowhead, Figure 2.7A).

Figure 2.6 - *shh* and *foxa2* expression is reduced in the anterior endoderm of *lzr* and ΔCpbx4-injected embryos

(A) At 48 hpf wildtype *shh* expression is found in the anterior endoderm (ae) and fin buds (fb). (B) *lzr* mutants (lack pectoral fin buds) have reduced *shh* expression in the anterior endoderm. (C-E) ΔCPbx4 injected embryos have reduced *shh* expression in the anterior endoderm and fin buds. Variations in the severity of the ΔCPbx4-mediated phenotype were observed and are most likely due to variable distribution of the injected mRNA. (F-H) CNS *shh* expression (black arrows) is unaltered in *lzr* mutant and ΔCPbx4 injected embryos. (I) *foxa2* is expressed in the anterior endoderm of 48hpf embryos. (J, K) lzr mutant and ΔCPbx4 injected embryos have reduced *foxa2* expression in the pharyngeal endoderm, similar to that seen for *shh*. Dashed black lines outline the normal expression domains of *shh* (A-E) and *foxa2* (I-K) in the endoderm. Dorsal views in A-E, I-K; lateral views in F-H. Anterior is to the left in all panels.

Figure 2.6

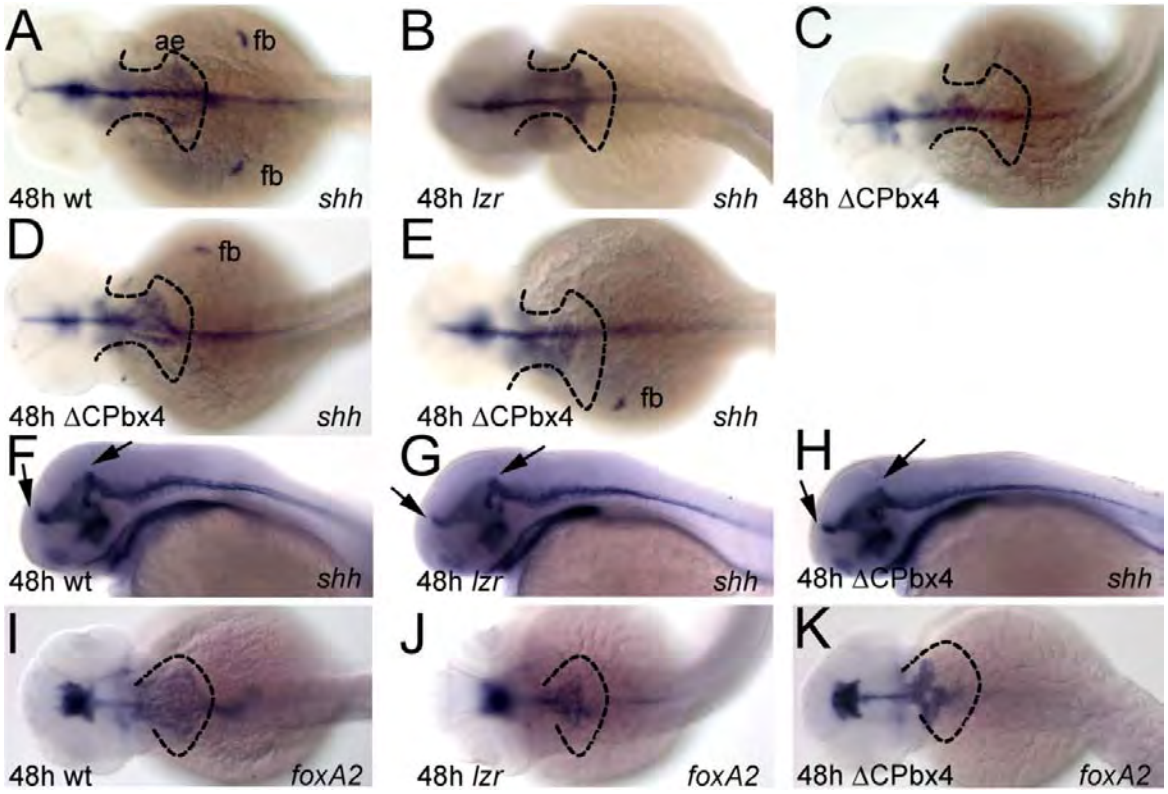
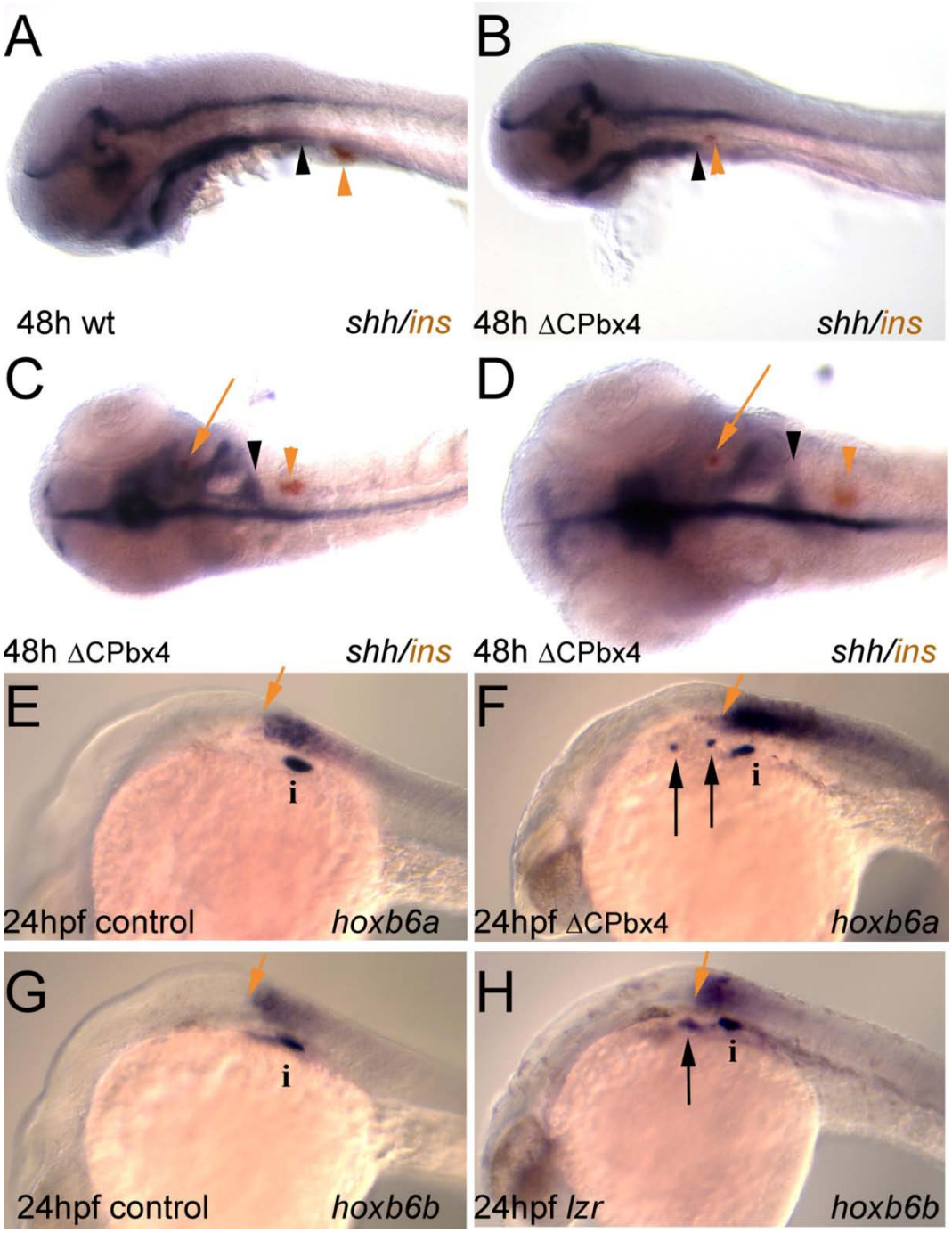

A
ae
fb
fb
48h wt
shh
B
48h lzr
shh
C
48h ΔCPbx4
shh
D
fb
48h ΔCPbx4
shh
E
48h ΔCPbx4
fb
shh
F
48h wt
shh
G
48h lzr
shh
H
48h ΔCPbx4
shh
I
48h wt
foxA2
J
48h lzr
foxA2
K
48h ΔCPbx4
foxA2

Figure 2.7 - Ectopic *insulin* expression in ΔCpbx4-injected embryos occurs in regions that lost of *shh* expression

(A) Double in situ marking *insulin* expressing cells (orange arrowhead) develop just caudal to pharyngeal *shh* expression (black arrowhead) at 48 hpf. (B, C) This spatial relationship is conserved ∆CPbx4 injected embryos exhibiting caudally reduced *shh* expression (compare the position of arrowheads in panel A with the position in panel B, C). (D) Higher magnification of ∆CPbx4 injected embryo in panel C marking *ins* expressing cells (red arrow) in a shh negative region. (E-H) *hox* gene expression in mesoderm is unaffected in ∆CPbx4 injected embryos and *lzr* mutants at 24 hpf. Mesodermal expression of *hoxb6a* (E) and *hoxb6b* (G) normally begins just anterior to *ins* expression (i) at 24 hpf. Ectopic *ins* expressing cells develop anterior of the *hox* expression in the mesoderm in ∆CPbx4 injected embryos (F) and *lzr* (H) embryos. In both cases the anterior limit of *hox* expression is unchanged relative to the principal islet. A, B, E-H are in lateral view; C and D are in dorsal view. Anterior is to the left in all panels. i= islet; black arrow indicates ectopic *insulin*-expressing cells; red arrows in E-H indicate anterior limit of *hox* gene expression.

Figure 2.7


A
48h wt
shh/ins
B
48h ΔCPbx4
shh/ins
C
48h ΔCPbx4
shh/ins
D
48h ΔCPbx4
shh/ins
E
i
24hpf control
hoxb6a
F
i
24hpf ΔCPbx4
hoxb6a
G
i
24hpf control
hoxb6b
H
i
24hpf lzr
hoxb6b

Next we analyzed *shh* expression in ΔCpbx4-injected embryos. As a result, *shh* expression is reduced and ectopic *ins* expressing cells were found in regions where there is little or no *shh* expression (red arrow, Figure 2.7C,D). *foxa2* is expressed in the anterior endoderm and gut in a similar pattern to *shh* (Strahle, Blader et al. 1993) and its expression is reduced in ΔCpbx4-injected embryos (Figure 2.6K).

lzr mutant embryos have reduced _shh_ expression and ectopic _insulin_ expression in the anterior endoderm

Meis proteins function in complexes that involve other transcription factors and cofactors. The common partner for Meis proteins is another cofactor, Pbx (Mann and Affolter 1998). Pbx proteins belong to the TALE class family of homeodomain proteins. There are four Pbx proteins discovered in the vertebrate genome (Popperl, Rikhof et al. 2000; Vlachakis, Ellstrom et al. 2000; Waskiewicz, Rikhof et al. 2002) but only *pbx2* and pbx4 both function during early zebrafish development (Waskiewicz, Rikhof et al. 2002) and are ubiquitously expressed (Popperl, Rikhof et al. 2000; Vlachakis, Ellstrom et al. 2000; Waskiewicz, Rikhof et al. 2002). To determine if Pbx has a similar function to Meis3 in early pharyngeal development, we utilized *lazarus* (*lzr*^{b557}) embryos that lack zygotic *pbx4* faction due to a point mutation in the *pbx4* gene (Popperl, Rikhof et al. 2000). We find ectopic *ins* expression in approximately 25% of embryos from an incross of *lzr* heterozygous fish (Figure 2.2B). Also, *lzr*

embryos have reduced *shh* expression in the anterior endoderm as well as fin buds but normal CNS expression (Figure 2.6B, C, G). *foxa2* expression is also reduced in the pharyngeal endoderm (Figure 2.6J). The similar phenotype observed between ∆CPbx4-injected embryos and *lzr* mutant embryos is consistent with Meis3 and Pbx4 proteins functioning together to pattern the anterior foregut.

∆CPbx4 injected embryos and *lzr* mutant embryos exhibit an anterior displacement of endodermal organs later in development

Ectopic *ins* expression varies somewhat between tMO and ΔCpbx4-injected embryos. We sometimes observed one ectopic cluster (Figure 2.2B,C,F, P) and in other embryos we detected more than one (Figure 2.7C). The position of these clusters varies, however it is never observed posterior to s4 or anterior to the pericardium. Also, at 24 hpf, the most posterior cluster of *ins* is positioned correctly at s4 with the ectopic patches positioned anteriorly. However, at 48 hpf and later, all *ins* clusters shift anteriorly to s1 and s2 for the main cluster and under the hindbrain for the ectopic clusters (Figure 2.2E, F; Figure 2.8; Figure 2.9). A similar phenotype results when tMO1 was targeted to the endoderm (Table 2.2). By 72 hpf, *ins* has shifted anteriorly in 100% of the injected embryos. The shift correlates with the size of the *shh* expression domain. For example, ΔCpbx4-injected embryos have reduced *shh* expression at the

posterior border where the pancreas lies. The *ins* expression has shifted only to where *shh* expression is not (Figure 2.7B-D).

Interestingly, the most severe effect was observed at 72 hpf. The anterior ectopic *ins* expression was observed in multiple clusters just behind the pericardium in *lzr* embryos, ΔCpbx4-injected embryos, tMO1-injected embryos and tMO2-injected embryos and embryos where tMO1 was targeted to the endoderm (Figure 2.8A-H). We examined other endodermal markers to determine if the shift was an overall effect on the endoderm. The caudal *carbA* expression in the exocrine pancreas is lost in *lzr* embryos and ΔCpbx4-injected embryos and the remaining expression is anterior to s1 (Figure 2.9B, C). *sid4* expression can be observed in the liver extending from s1 caudally to s 4 on the left side of the embryo (Figure 2.9D) but in *lzr* embryos and ΔCpbx4-injected embryos the expression is shifted anteriorly (Figure 2.9E,F). The anterior edge of *sid4* expression is in close proximity to the posterior region of the pericardium and the posterior boundary of *sid4* ends at s1.

Upon closer examination, ΔCpbx4- injected embryos results in the occasional loss of *sid4* expression at the expense of *ins* expression (Figure 2.9G-I). Overall, inhibiting *meis3* or *pbx4* results in a progressive anterior shift in the endodermal organ gene expression and on occasion anterior foregut gene expression is lost. Lack of *shh* expression most likely results in an underdeveloped pharyngeal endoderm region, resulting in the anterior shift of organs.

Figure 2.8 - Disruption of *meis3* and *pbx4* function causes an anterior shift of *insulin*-positive cells

Wildtype (A), *lzr* mutant (B), ΔCPbx4-injected (C), MOCO-injected (D), tMO1-injected (E) and tMO2-injected (F) embryos, as well as embryos where MOCO (G) or tMO1 (H) was targeted to the endoderm, were assayed for *ins* expression by in situ hybridization at 72hpf. *ins* positive cells are found anterior to somite 4 in mutant and injected embryos (B, C, E, F, H), but not in control embryos (A, D, G). s1-s4 indicates the position of somites 1-4. All embryos are in lateral view with anterior to the left.

Figure 2.8

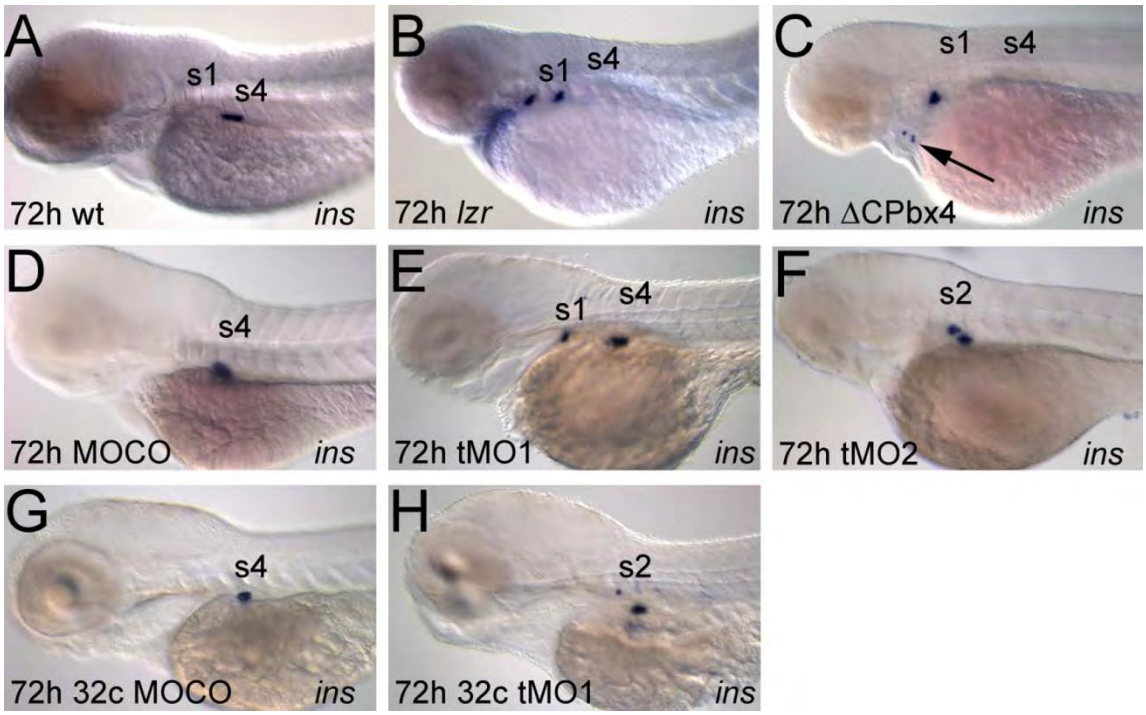
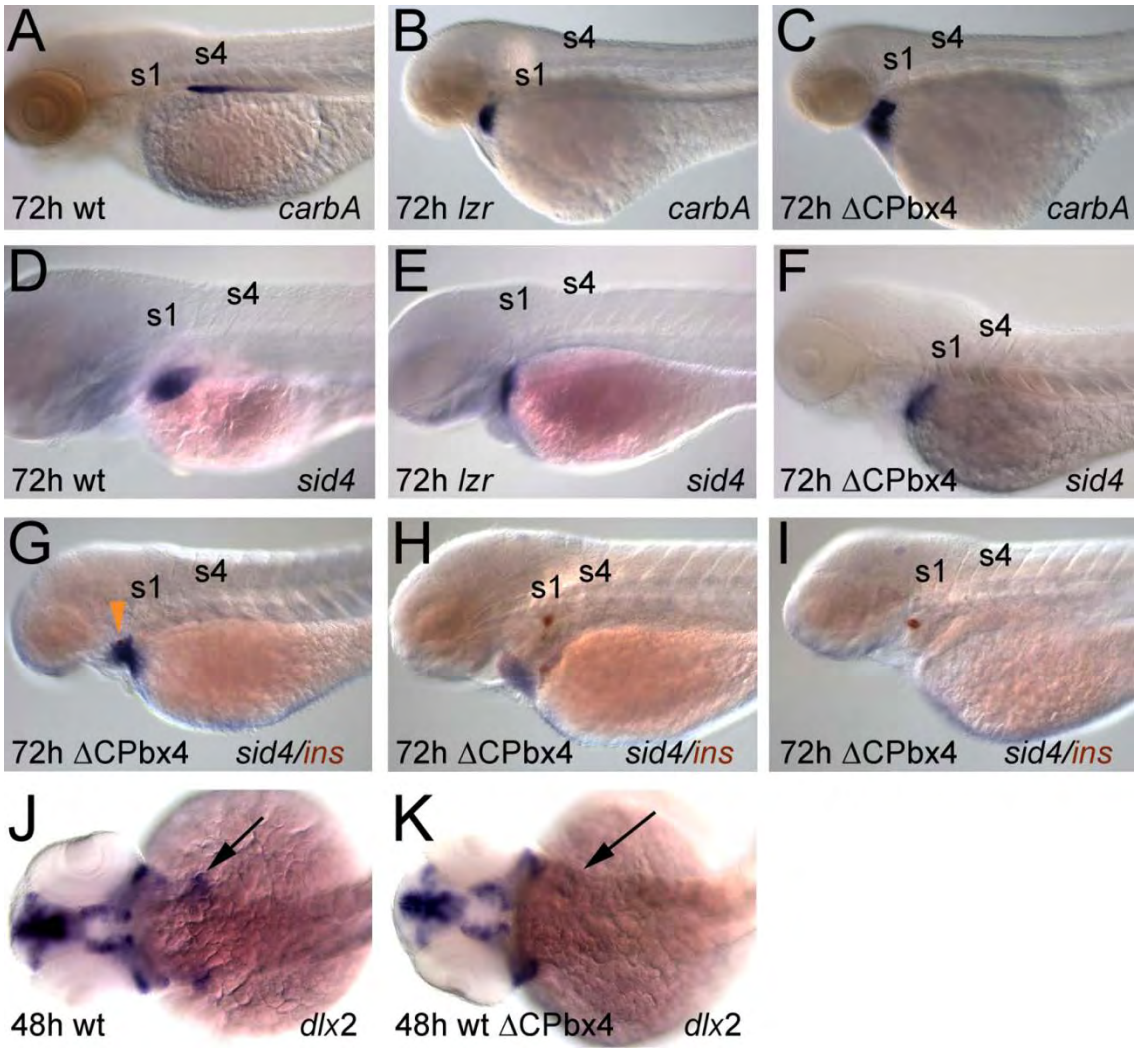

A
s1
s4
72h wt
ins
B
s1
s4
72h lzr
ins
C
s1
s4
72h ΔCPbx4
ins
D
s4
72h MOCO
ins
E
s1
s4
72h tMO1
ins
F
s2
72h tMO2
ins
G
s4
72h 32c MOCO
ins
H
s2
72h 32c tMO1
ins

Figure 2.9 – Multiple organs are displaced anteriorly in *meis*- and *pbx4*-deficient embryos

lzr mutant and ΔCPbx4-injected embryos exhibit anterior displacement of exocrine pancreas (*carbA*, (exocrine pancreas, A-C) and *sid4*, (liver, D-F) gene expression at 72 hpf. (G-I) Anterior shifts in ΔCPbx4-injected embryos can result in loss of liver but not *ins* expression. (J, K) Posterior, *dlx2*-expressing pharyngeal arches are lost in ΔCPbx4-injected embryos. A-I are in lateral view; J, K are in dorsal view. Anterior is to the left in all panels.

Figure 2.9


A
s1
s4
72h wt
carbA
B
s4
s1
72h lzr
carbA
C
s1
s4
72h ΔCPbx4
carbA
D
s1
s4
72h wt
sid4
E
s1
s4
72h lzr
sid4
F
s1
s4
72h ΔCPbx4
sid4
G
s1
s4
72h ΔCPbx4
sid4/ins
H
s1
s4
72h ΔCPbx4
sid4/ins
I
s1
s4
72h ΔCPbx4
sid4/ins
J
48h wt
dlx2
K
48h wt ΔCPbx4
dlx2

Underdevelopment of these arches could result in the anterior shift observed. We analyzed *dlx2* expression in the pharyngeal arches and found it is lost in ΔCpbx4-injected embryos (arrow, Figure 2.9J). The reduction in branchial arches could be the result of neural crest defects since *meis3* and *pbx4* function in hindbrain development (Popperl, Rikhof et al. 2000; Waskiewicz, Rikhof et al. 2001; Choe, Vlachakis et al. 2002) where neural crest cells originate. However, in *smoothened* (*smu*) mutants, pharyngeal arches are underdeveloped indicating the role of hedgehog signaling in their formation (Chen, Burgess et al. 2001). Thus, loss of *shh* expression in the pharyngeal endoderm in *lzr* and ΔCpbx4-injected embryos is the most likely cause in the ectopic *ins* expression and subsequent anterior displacement of the pancreas and liver.

Does *foxa2* act upstream of *shh* in the endoderm?

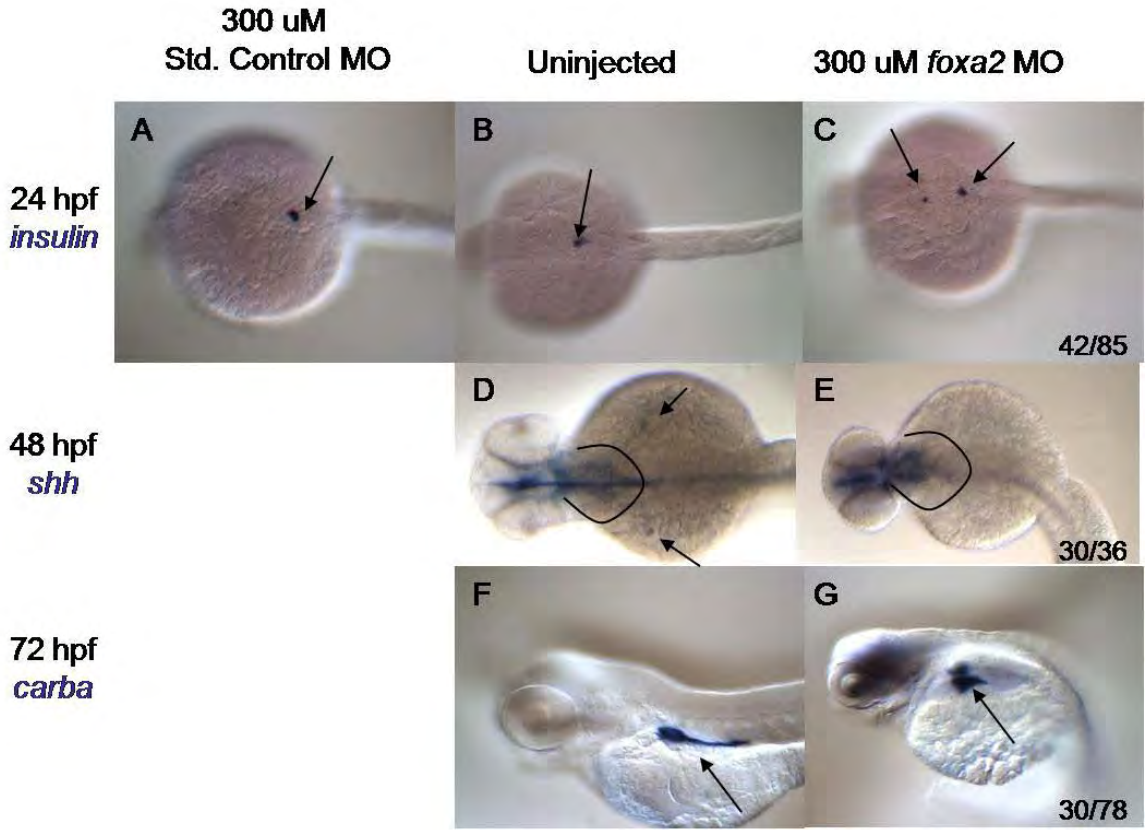
Foxa2 acts upstream of *shh* in the notochord and overexpression of *foxa2* leads to ectopic expression of *shh* in the yolk, endoderm, neural tube and the tail in zebrafish. These results suggest that Foxa2 activates *shh* expression in both the mesoderm and neuroectoderm (Chang, Blader et al. 1997; Strahle, Lam et al. 2004). Specifically, *foxa2* mutant embryos, *monorail* (*mol*^{st20} or *mol*^{tv53a}), do develop floorplate, but it fails to differentiate and *hedgehog* expression is not maintained in the floor plate and as a result lateral expansion of the floorplate fails to occur (Norton, Mangoli et al. 2005). Also, *mol* mutant embryos have defective motor neuron development in the CNS supporting the role of Foxa2 in

ectoderm (Norton, Mangoli et al. 2005). However, it has not been confirmed if Foxa2 acts upstream of *shh* in the endoderm (Norton, Mangoli et al. 2005). In order to test this, we first injected *foxa2* tMO and assayed for *ins* expression at 24 hpf (Figure 2.10A-C) and observed ectopic *ins* expression, similar to *lzr* and ΔCpbx4-injected embryos. Next, we wanted to determine if this ectopic expression is due to loss in *shh* expression. We assayed *shh* expression in the *foxa2* morphants at 48 hpf and observed decreased expression in the endoderm (Figure 2.10D, E), but expression is completely lost in the notochord and fin buds.

Figure 2.10 – *foxa2* MO reduces *shh* expression in the pharyngeal endoderm

(A) 300 μM standard control MO was injected at the one cell stage into wildtype embryos. *ins* is expressed in a single cluster at s4 (black arrow). (B) Uninjected control with *ins* expressed in a single islet at s4 (black arrow). (C) Wildtype embryo injected with 300 μM *foxa2* MO and assayed for *ins* expression. Ectopic *ins* expression can be found anterior to the main cluster at s4 (black arrows). (D,E). Wildtype uninjected control embryos with *shh* expression in the pharyngeal endoderm (black outline) and fin buds (arrows)(D). (E) Wildtype embryo injected with 300 μM *foxa3* MO, *shh* expression is lost in the fin buds and notochord and is reduced in the pharyngeal endoderm (black outline). (F,G) *carbA* expression is lost in the caudal tip of the exocrine pancreas and has shifted anteriorly in *foxa2* MO injected embryos (G) when compared to the uninjected control (F).

Figure 2.10


300 uM
Std. Control MO
Uninjected
300 uM foxa2 MO
24 hpf
insulin
48 hpf
shh
72 hpf
carba
A
B
C
D
E
F
G
42/85
30/36
30/78

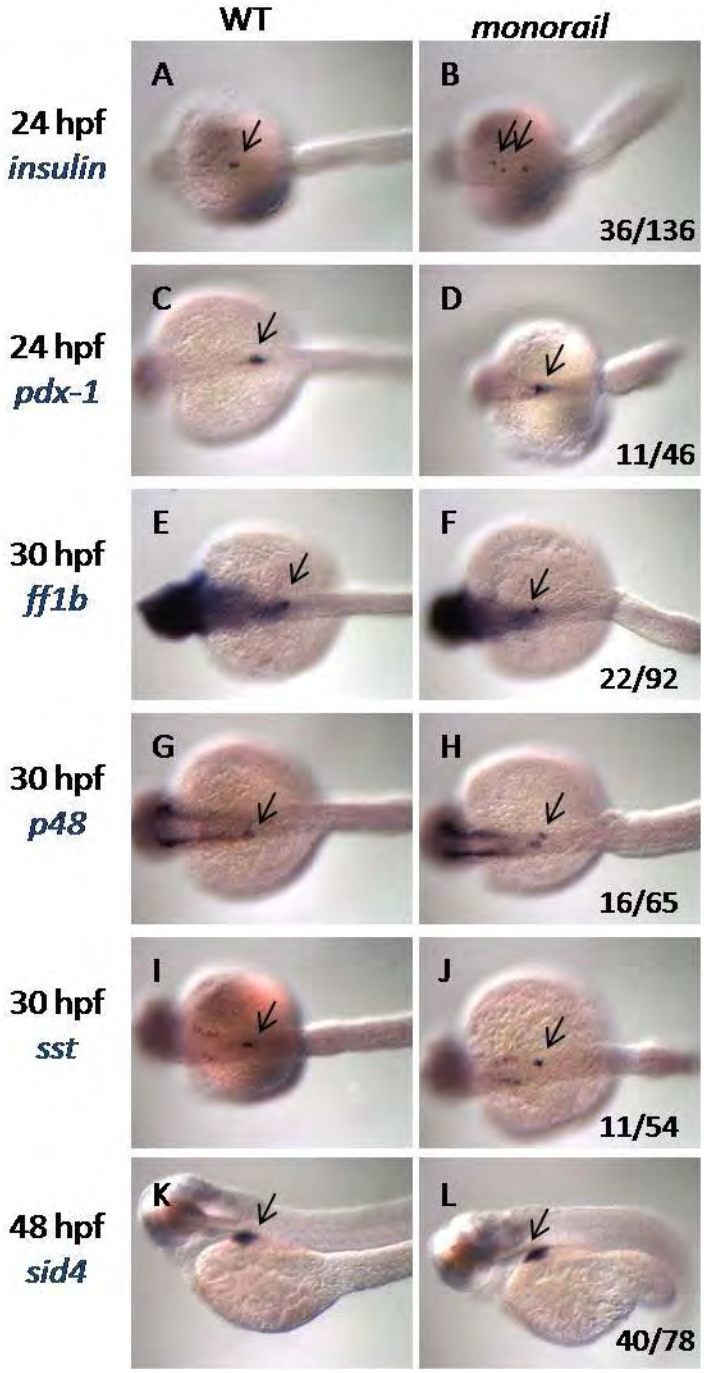
To determine if *foxa2* mutant embryos exhibit an anterior shift in endoderm markers, we assayed for *carbA* expression (Figure 2.10F) and the caudal tip of the exocrine pancreas is lost and the pancreas has shifted anteriorly (Figure 2.10G).

Next, we analyzed various endodermal markers in *mol*^{st20} mutant embryos. Similar to *foxa2* morphants, we observed ectopic *ins* expression in mutant embryos at 24 hpf (Figure 2.11B). We next assayed for *pdx1* at 24 hpf (Figure 2.11C, D), *ff1b* (islet) expression at 30 hpf (Figure 2.11E, F), *p48* (exocrine pancreas) at 30 hpf (Figure 2.11G,H), *sst* (*somatostatin,* endocrine pancreas) at 30 hpf (Figure 2.11I, J), and *sid4* at 48 hpf (Figure 2.11K, L). As a result, an anterior shift of endodermal marker expression occurs in *mol* mutant embryos. However, we did not conclude that Foxa2 functions upstream of *shh* in the pharyngeal endoderm. Notably, *mol* and *foxa2* morphants become deformed by 24 hpf and as a result, the shift observed might be a secondary effect due to the malformation of the embryo. Also, *shh* endodermal expression is not completely lost in *foxa2* MO injected embryos, although it is reduced. Therefore, *foxa2* might act upstream to regulate *shh* expression in the endoderm but could require a partner to regulate expression. Therefore, blocking *foxa2* would not completely abolish *shh* expression.

Figure 2.11 – Endodermal organ gene expression has shifted anteriorly in *monorail* mutant embryos

mol mutant embryos appear to have ectopic *ins* (black arrows) expression at 24 hpf (B vs. A) and an anterior shift in *pdx1* expression (black arrow) (D) at 24 hpf, *ff1b* expression (black arrow) at 30 hpf (F), *p48* expression (black arrow) at 30 hpf (H), *sst* expression (black arrow) at 30 hpf (J), and *sid4* expression black arrow) at 48 hpf (L). This shift does not occur in wildtype embryos (A, C, E, G, I, and K, black arrows).

Figure 2.11


WT
monorail
A
B
24 hpf
insulin
36/136
C
D
24 hpf
pdx-1
11/46
E
F
30 hpf
ff1b
22/92
G
H
30 hpf
p48
16/65
I
J
30 hpf
sst
11/54
K
L
48 hpf
sid4
40/78

Discussion

Pbx and Meis function together during vertebrate development

Pbx4 is required for segmentation of the zebrafish hindbrain and proper expression and function of Hox genes. Expression of *pbx4, meis3* and *hoxb1b* overlap in the caudal hindbrain and in vitro, form a trimeric complex indicating that these three proteins function together in hindbrain development (Vlachakis, Ellstrom et al. 2000). Moreover, Hoxb1b, Pbx4 and Meis3 induce ectopic expression of numerous hindbrain factors including *hoxb1a, hoxb2, krox20,* and *valentino* and transforms the forebrain and midbrain into hindbrain fates (Vlachakis, Choe et al. 2001). Therefore, this interdependent complex consisting of Hoxb1b, Pbx4 and Meis3 has a much stronger effect on hindbrain patterning than just Pbx4 and Hoxb1b alone. Also, these results confirm that Meis functions in the same pathway as Pbx in hindbrain development. Specifically, injecting dominant negative Meis results in phenotypes similar to *lzr* and injecting *meis* mRNA partially rescues the *lzr* phenotype. Also, Pbx4 protein levels increase when Meis is overexpressed in wildtype embryos. Therefore, Meis is a DNA-binding partner of Pbx proteins and it acts as a post-transcriptional regulator of Pbx protein levels (Waskiewicz, Rikhof et al. 2001).

Pbx and Meis function in pancreas development in vertebrates

Our results indicate that Meis3 and Pbx4 act in regulating *foxa2* and *shh* expression in the pharyngeal endoderm, but not in the CNS suggesting a

different regulation in transcription must occur. Since Meis and Pbx are known co-factors, we hypothesize that another transcription factor functions with Meis3 and Pbx4 to regulate transcription in the pharyngeal endoderm. Pbx and Meis do function in pancreas development by binding Pdx1, a ParaHox homeodomain transcription factor (Brooke, Garcia-Fernandez et al. 1998; Coulier, Burtey et al. 2000) which, is required for proper pancreas development and function (Jonsson, Carlsson et al. 1994; Offield, Jetton et al. 1996; Stoffers, Zinkin et al. 1997). Specifically, a mutation in Pbx1 mimics the defects seen in PDX1 null mutants; pancreas hypoplasia and defects in exocrine and endocrine cell differentiation with reduced *islet1* and *neurogenin3* expression and *Pbx1*$^{-/-}$ embryos die at embryonic (E) day 15 or E16 as a result. Notably, *Pbx1*$^{+/-}$ adults have pancreatic islet malformations, impaired glucose tolerance and hypoinsulinemia (Kim, Selleri et al. 2002). Thus, Pbx1 plays an important role in pancreatic development.

Pbx1$^{+/-}$*;Pdx1*$^{+/-}$ embryos develop age-dependent diabetes mellitus while single mutants do not, indicating a strong genetic interaction between the two proteins (Kim, Selleri et al. 2002). In fact, Pbx1 and Pdx1 bind DNA cooperatively in vitro (Peers, Sharma et al. 1995). Removing the Pbx interaction domain in PDX1 prevents PDX1 transgene from rescuing *pdx1*$^{-/-}$ mutant embryos (Dutta, Gannon et al. 2001). Notably, PDX/Pbx1 complexes are not necessary for glucose homeostasis and for differentiation of stem cells into ductal, endocrine and exocrine lineages, but they are essential for expansion of these populations (Dutta, Gannon et al. 2001; Wilson, Scheel et al. 2003). For

example, in mouse acinar cells, Pdx1:Pbx1b:Meis2 cooperates with PTF1 bHLH (exocrine pancreas) to activate the ELA1 (elastase 1 gene promoter) in exocrine pancreas development (Swift, Liu et al. 1998; Liu, MacDonald et al. 2001), but β-cells do not express Pbx1b or Meis2, therefore, PDX1 alone can activate transcription in β-cells (Swift, Liu et al. 1998). However, Pbx and Meis do function in other islet cell formation. Specifically, Pdx1:Pbx1:Prep1 bind the somatostatin promoter and activate transcription in δ-cells in the endocrine pancreas (Goudet, Delhalle et al. 1999). Also Pbx and Meis indirectly regulate, *glucagon, insulin* and *somatostatin* gene expression. Promoters in these genes contain Pax6 (a regulator of islet hormone gene expression) binding sites and Pax6 activates transcription in cell cultures (Sander, Neubuser et al. 1997; Andersen, Jensen et al. 1999; Ritz-Laser, Estreicher et al. 1999). A *pax6* enhancer contains binding sites for Meis and Pbx and in vitro studies confirm binding of Meis and Pbx to this site. Moreover, *Pbx1* and *Pbx2* knockout mice have downregulated *Pax6* expression indicating that *Pax6* is dependent on Pbx1 and Pbx2 function. Previously, Meis proteins were shown to regulate *Pax6* expression during lens development (Zhang, Rowan et al. 2006) suggesting that a conserved role of Meis regulating *Pax6* in pancreas formation.

Role of *shh* in endoderm development

Consistent with our results, exclusion of *shh* expression from the pancreas primordium is necessary for pancreas gene expression. This is conserved from

mouse to zebrafish. For example, in chick, activin-βB and FGF2 from the notochord represses SHH expression in the pancreatic primordium (Hebrok, Kim et al. 1998) and Shh secreted by mouse embryoid bodies blocks expression of pancreatic markers. Blocking Hedgehog signaling in the embryoid bodies results in the differentiation of Insulin-positive cells (Mfopou, De Groote et al. 2007) confirming that Hedgehog expression must be excluded for endocrine markers to be expressed.

The anterior border of the pancreatic domain is established by Hedgehog expression. Treating chick embryos with cyclopamine expands the *Pdx1* expression domain (Apelqvist, Ahlgren et al. 1997; Kim and Melton 1998) indicating that *Shh* acts to repress *Pdx1* expression and therefore the size of the pancreatic domain. In mice, *Shh* and *Ihh* have overlapping expression in the anterior endoderm and both act in pancreas development (Bitgood and McMahon 1995; Ramalho-Santos, Melton et al. 2000). Inactivation of *Ihh* allows branching of the ventral pancreatic tissue resulting in an annulus that encircles the duodenum (annular pancreas in humans). Also, $Shh^{-/-}$ and $Shh^{-/-}$;$Ihh^{-/-}$ mutant embryos have a threefold increase in pancreas mass and fourfold increase in pancreatic endocrine cell numbers (Hebrok, Kim et al. 2000) confirming that Hedgehog signaling establishes the size of the pancreatic domain. However, what regulates Hedgehog signaling upstream is not known.

Hedgehog signaling does not always play a repressive role in pancreas development. For example, Hedgehog signaling is required for maintenance of

adult endocrine cell functions (Hebrok 2003). *Ptc1* (Hedgehog receptor) is expressed during pancreas development and in mature islets and ducts (Hebrok, Kim et al. 2000; Thomas, Rastalsky et al. 2000) suggesting that Shh protein may act in pancreas development. Also, Hedgehog signaling activates insulin production and secretion in differentiated β-cells in cell culture. Specifically, treating INS-1 cells with cyclopamine inhibits *Pdx1* and *Ins* transcription (Thomas, Rastalsky et al. 2000; Thomas, Lee et al. 2001). Also, Hedgehog activates the *Pdx1* promoter suggesting that Hedgehog regulates *Pdx1* expression, but cannot be co-expressed. Overall, Hedgehog is expressed early to restrict pancreas growth (Apelqvist, Ahlgren et al. 1997; Kim and Melton 1998), but once the pancreas has formed, Hedgehog signaling (acting as a morphogen) is necessary for pancreatic function. Therefore, tight regulation of Hedghehog signaling is necessary for proper pancreas development and function.

In zebrafish, Hedgehog signaling plays a dual role. Early expression from the dorsal mesoderm (the organizer) is necessary to induce *ins* expression, but once gastrulation begins, *shh* expression in the anterior endoderm acts as a repressor, inhibiting *ins* expression and maintaining *ins* expression at s4 (diIorio, Moss et al. 2002; Hebrok 2003). Early Hedgehog signaling from the dorsal mesoderm acts directly on endodermal cells. These endodermal cells require Smoothened to function and lie laterally to the midline cells fated to become β-cells. These lateral cells gives rise to exocrine pancreas and intestine and in turn

will signal the medial cells to become β-cells. Therefore, there is a cell-nonautonomous requirement for Smoothened in endodermal cells during gastrulation which allows subsequent intra-endodermal interactions necessary to develop β-cells (Chung and Stainier 2008).

Like in other vertebrates, the regulation of *shh* is not know. Notably, we discovered that Meis3 and Pbx4 act to regulate *shh* expression in the pharyngeal endoderm (specifically the second role of Shh in zebrafish pancreas development), therefore patterning the anterior endoderm. Also, Meis proteins are involved in RA (Retinoic Acid)-dependent proximal cell identity during both regeneration and embryonic limb development (Mercader, Tanaka et al. 2005). Specifically, the *shh* promoter contains a RARE (Retinoic Acid Response Element) and Foxa2 binding sites that regulate its transcription in the floorplate (Chang, Blader et al. 1997) suggesting that Meis may act alongside retinoic acid to regulate *shh* expression in the endoderm. Also, it does not exclude the fact that other transcription factors might act with Meis3 and Pbx4 to regulate *foxa2* and *shh* expression.

Materials and Methods

Fish Maintenance

Wild type, gut GFP (Field, Ober et al. 2003), *lazarus* (*lzr*^{b557}; (Pöpperl, Rikhof et al. 2000)) and *monorail* (*mol*^{st20})(Norton, Mangoli et al. 2005) embryos were collected from natural matings and reared in 1/3 Ringer's. Embryos were staged using morphological criteria up to 24 hours post fertilization (hpf) and by time of development at 28.5°C thereafter (Kimmel, Ballard et al. 1995).

mRNA and morpholino injections

The dominant negative Meis construct (∆CPbx4) has been reported previously (Choe, Vlachakis et al. 2002) and ~1 nl of a 300µg/ml ∆CPbx4 m*RNA stock was injected at the 1-2 cell stage. Two antisense morpholino oligos designed to block translation of the *meis3* mRNA (tMO1 5'ATCCATGCGATACGGAAGCCGAGCT3' complementary to position -19 to +6 and tMO2 5'CACACACTCACTGACGGAGGACAAC 3' complementary to position -44 to -19, where +1 indicates the first nucleotide of the AUG codon) and one control morpholino (MOCO 5'ATCgATGCcATACcGAAcCCGAcCT3' that has 5 mismatches relative to tMO1) were obtained from Gene Tools. ~1nl of MO at various concentrations (see text; note that 100uM MO corresponds to ~0.84 ng/nl) was injected at the 1-2 cell stage. *Foxa2* morpholino (ATTTTGACAGCACCGAGCATCCTGG) was ordered from Open Biosystems and injected at 300 uM at the one cell stage along with a standard control MO.

For targeting of MOs to the endoderm, a stock containing 100 pg/nl *sox32* mRNA, 0.0125 pg/nl tetramethylrhodamine dextran (10,000 MW) and various concentrations of MO (see text) was injected into one of the four central blastomeres on the 8-cell side of the 8x4-cell array at the 32-cell stage. *sox32* encodes a transcription factor that is sufficient to drive cells to an endodermal fate (Figure 2.2G-I;(Alexander and Stainier 1999; Kikuchi, Agathon et al. 2001) and injecting *sox32* mRNA into a single cell at the 32-cell stage does not perturb endoderm development (Figure 2.3). In a control experiment 500uM fluorescein-tagged control MO was co-injected with *sox32* mRNA and rhodamine dextran at the 32-cell stage. We find that the rhodamine and fluorescein signals co-localize during cleavage and blastula stages (Figure 2.5) as well as at 24 hpf (Figure 2.2L-N) demonstrating that MOs are co-distributed with the rhodamine dextran lineage label in the endoderm. We also spiked the *sox32* mRNA with 200 pg GFP mRNA and co-injected with rhodamine dextran. Again, we find that the fluorescein and rhodamine signals co-localize at cleavage stages (Figure 2.4) and at 24hpf (Figure 2.2J, K), demonstrating that mRNAs also co-distribute with the lineage label. Taken together these experiments demonstrate that mRNA, MOs and rhodamine dextran co-localize in the endoderm following co-injection at the 32-cell stage. In a typical experiment (injecting *sox32* mRNA, MO and rhodamine dextran) ~50-60% of surviving embryos displayed rhodamine in 2 or 4 cells at the 64-cell stage and went on to express rhodamine primarily in the endoderm at 24 hpf (70-99% of embryos depending on the experiment; see

Figure 2.2G-N) and were morphologically normal. The remaining ~40-50% of embryos displayed broad rhodamine expression at the 64-cell stage and went on to develop with morphological abnormalities of the trunk and/or tail. These embryos were excluded from further analysis. mRNA and MO injected embryos were fixed in 4% paraformaldehyde at various developmental stages for in situ hybridization.

In situ hybridization

Antisense digoxigenin- and fluorescein-labeled probes were produced by standard methods. The *meis3, insulin, sid4, carboxypeptidase A*, *pdx1* and *shh* probes used were described previously (Sagerström, Kao et al. 2001; diIorio, Runko et al. 2005). In addition, probes to *hoxb6a* and *hoxb6b* (Davidson, Ernst et al. 2003), *foxa2* (Strähle, Blader et al. 1993) and *dlx2* (Akimenko, Ekker et al. 1994) were used. One- and two-color in situ hybridization was carried out as described previously (Sagerström, Grinblat et al. 1996; Sagerström, Kao et al. 2001).

CHAPTER III

ENU SCREEN FOR ENDOCRINE PANCREAS MUTANTS

Abstract

We performed two haploid ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis screens in search of new genes involved in early pancreas development. We used *islet1* (*isl1*) as a marker to screen for mutations. *isl1* is expressed in all endocrine lineages and will therefore detect mutations in endocrine development. In an initial pilot screen, we screened 200 genomes and recovered two mutations in diploid embryos; 71.39 (named *curvy*) and 88.21 (named *aldh1a2*^{um22}). *curvy* mutant embryos have reduced *isl1* expression in the neurons of the central nervous system (CNS) and have multiple anterior ectopic *isl1* spots within the endoderm. *aldh1a2*^{um22} mutants maintain *isl1* in the CNS but lack all endoderm expression of *isl1*. In our second screen, we screened 375 genomes and recovered 50 *isl1* mutant lines. Complementation analysis suggested that the majority of these lines carry the same mutation. Further analysis suggested that this mutation (835.4, named *kiribati* after a lost island) was not ENU induced, but rather arose spontaneously in the stock used for mutagenesis.

Background

Mutagenesis screens are used as an unbiased method for introducing alterations in the genome that result in the loss of function of individual genes. If the mutation lies within or adjacent to the affected gene, this gene can be identified and further studied. Mutagenesis methods include radiation, insertional mutagenesis and chemical treatment. X-rays and gamma-rays induce large lesions and rearrangements in the genome. An advantage of radiation mutagenesis is that it is relatively straight forward to induce large numbers of lesions. However, it is often difficult to clone the affected gene because more than one gene is often affected (Knapik 2000) and the nature of the lesion is often complicated. For instance, radiation treatment leads to chromosomal arm deletions, chromosomal breaks, and rearrangements (Streisinger, Walker et al. 1981; Chakrabarti, Streisinger et al. 1983; Streisinger, Singer et al. 1986).

Another option, insertional mutagenesis, involves injecting retroviruses or transposons, which are inserted into the genome and disrupt genes. The inserted DNA contains known sequences, which can be used as a starting point for sequencing genomic DNA flanking the insertion site. This provides a relatively straight forward method for locating the insertion and the gene affected. However, insertional mutagenesis may be ineffective in identifying the affected gene if the insert does not lie within an identifiable gene, the sequence identified adjacent to the virus is not unique in the genome, or if the sequence adjacent to the insertion cannot be obtained. Also, retrovirus insertional mutagenesis has a

seven to ten-fold lower success rate than chemical mutagenesis methods (Dahm 2005). Furthermore, transposons are not as efficient as pseudotyped retrovirus (Amsterdam and Hopkins 2006).

Two chemical mutagens, ethyl methanesulfonate (EMS) and *N*-ethyl-*N*-nitrosurea (ENU), induce mutations in zebrafish. In particular, ENU was found to be efficient at inducing mutations in pre-meiotic germ cells, as indicated by the identification of non-mosaic mutant progeny at four different pigmentation loci [*gol-1 (golden-1, slc24a5), gol-2 (brass), alb-1 (albino),* and *spa-1 (kit receptor a)*](Solnica-Krezel, Schier et al. 1994). ENU is a synthetic alkylating agent that acts by adding an ethyl group to oxygen or nitrogen radicals in individual base pairs of DNA. The ethylation of a base can lead to the modified base being misread during DNA replication, resulting in the integration of a non-complementary base in the second DNA strand. Specifically, ENU preferentially induces A/T to G/C conversions (Knapik 2000). Point mutations can cause a wide range of allelic variations including hypomorphs, gain-of-function alleles, dominant negative alleles and neomorphic alleles (Amsterdam and Hopkins 2006). Large-scale ENU mutagenesis screens in zebrafish have led to the isolation of several thousand mutations, which resulted in the identification of hundreds of genes and their function (Driever, Solnica-Krezel et al. 1996; Haffter, Granato et al. 1996; Haffter P 1996). Zebrafish are estimated to have 700-1600 essential developmental genes in which mutations would produce a visible phenotype (Driever, Solnica-Krezel et al. 1996; Haffter, Granato et al. 1996). .

In order to optimize an ENU screen, different parameters such as ENU concentration, time of single exposure, number of exposures, pH, and the temperature of water during treatment are taken into consideration to find ideal conditions that will saturate the mutagenesis rate but allow survival. Zebrafish males can survive a single exposure to 5 mM of ENU for 30 minutes or 0.5 mM for 6-8 hours (Knapik 2000). The optimal conditions for treatment are 3 mM for 1 hour. Once a mutation has been induced, the next step is to locate the mutation in the genome. Positional cloning aids in the location of the mutation by utilizing dense genetic maps containing numerous genetic markers thereby increasing the likelihood that the mutation will map near a marker, preferably within 1 centiMorgan (cM, approximately 625 kb). The zebrafish genome contains 25 chromosomes (Endo 1968) with an estimated genetic size of 2635 cM (Postlethwait, Johnson et al. 1994) and estimated 1.7 Gb (Dahm 2005). The established reference map for zebrafish is the MGH map (Knapik, Goodman et al. 1996; Knapik, Goodman et al. 1998; Shimoda, Knapik et al. 1999), which was generated with 3,881 microsatellite markers (all CA DNA repeats that occur in the genome) on a panel of 48 diploid F2 fish of an IN (India) X AB (derived from A and B lines) reference cross. It covers 2,295 cM at a resolution of 1.2 cM. The markers are highly polymorphic for zebrafish wildtype alleles including AB, EK (Ekkwill), TU (Tuebingen), TL (Tupfel long fin) and IN and provide the initial framework for positional cloning of 600 essential genes (Knapik, Goodman et al. 1998). In addition to CA repeats, over 50,000 ESTs (expressed sequence tags)

that define over 11,500 zebrafish transcripts have been identified accelerating the identification of candidate genes (Talbot and Hopkins 2000).

The CA mapping technique requires crossing mutant carriers against a polymorphic reference line. Genomic DNA is extracted from mutant and wildtype embryos to use in PCR of microsatellites. Comparing band intensities of various microsatellites in a pool of mutant F2 or F3 individuals with a pool of their wildtype siblings on a gel can determine linkage between a mutation and a microsatellite. Genetic distance between the mutant locus and a microsatellite can be determined by counting recombinations among siblings (Geisler, Rauch et al. 1999). The second phase of positional cloning is to isolate a series of overlapping genomic clones that connect markers on either side of the mutation. This ensures that the gene lies within a critical interval (Talbot and Hopkins 2000). Bacterial artificial chromosome (BAC), P1-derived artificial chromosome (PAC), and yeast artificial chromosome (YAC) vectors contain genomic libraries that can help locate the gene of interest (Amemiya, Zhong et al. 1999; Amemiya and Zon 1999; Zhong, Rosenberg et al. 2000). These libraries contain large pieces of genomic DNA (100-200 kb for BAC and PAC and 470 kb for YAC) reducing the number of clones needed to locate the gene of interest within an interval (Talbot and Hopkins 2000). Genes can then be identified either by screening cDNA libraries with genomic clones or by sequencing BAC and PAC clones. Microinjection of genomic clones containing the gene of interest can partially rescue the mutant phenotype (Yan, Talbot et al. 1998), providing another

method to identify the genomic clone containing the gene (Talbot and Hopkins 2000).

Results

Two haploid ENU screens for endocrine mutations

A haploid in situ hybridization screen of ENU (*N*-ethyl-*N*-nitrosurea) mutagenized Ekkwill (EK) males was carried out to identify mutations in endocrine pancreas development (Figure 3.1). EK males were treated with 3 mM ENU (Solnica-Krezel, Schier et al. 1994) and crossed to EK females. F1 progeny were then raised. F1 females were squeezed and In Vitro Fertilization (IVF) was performed using EK UV (Ultraviolet) irradiated sperm to produce haploid embryos. Haploid embryos were allowed to develop to approximately 30 hpf, fixed and assayed by in situ hybridization using an *islet1* (*isl1*) probe to detect endocrine pancreas defects (*isl1* is also expressed in the Central Nervous System [CNS] Figure 3.2 A,C,E). F1 females that produced progeny where 50% exhibited an endocrine pancreas defect were outcrossed to Tupfel long fin (TL) males. F2 progeny were raised, in-crossed and those progeny were screened for the phenotype in F3 diploid embryos. In our pilot screen of 200 genomes, we detected ten females exhibiting an endocrine phenotype. Six of the ten females died, developed tumors or did not produce progeny. Out of the remaining four females, we recovered the mutation in two, *aldh1a2*^{um22} and *curvy*. *curvy* embryos become deformed by 24 hpf and have decreased *isl1* expression in the CNS and exhibited anterior ectopic *isl1* clusters in the endoderm (Figure 3.2 A, B).

Figure 3.1 – ENU mutagenesis screen for endocrine mutations

EK males were mutagenized with 3mM of ENU for an hour once a week for 3 weeks (F0). In order to clean out post-meiotic mutations, the males were repeatedly crossed, allowing only pre-meiotic mutations to be passed on to the next generation. The males were crossed by natural matings to EK females and the progeny were raised (F1). The female progeny were squeezed and the eggs fertilized with UV irradiated sperm producing haploid embryos. Haploid embryos were fixed at 30 hpf and assayed for *isl1* expression (1). Females producing clutches that exhibit an endocrine phenotype in 50% of their embryos were then outcrossed to TL males (2) and the progeny raised (F2). F2s were then in-crossed to recover the mutation in diploid embryos (F3).

Figure 3.1

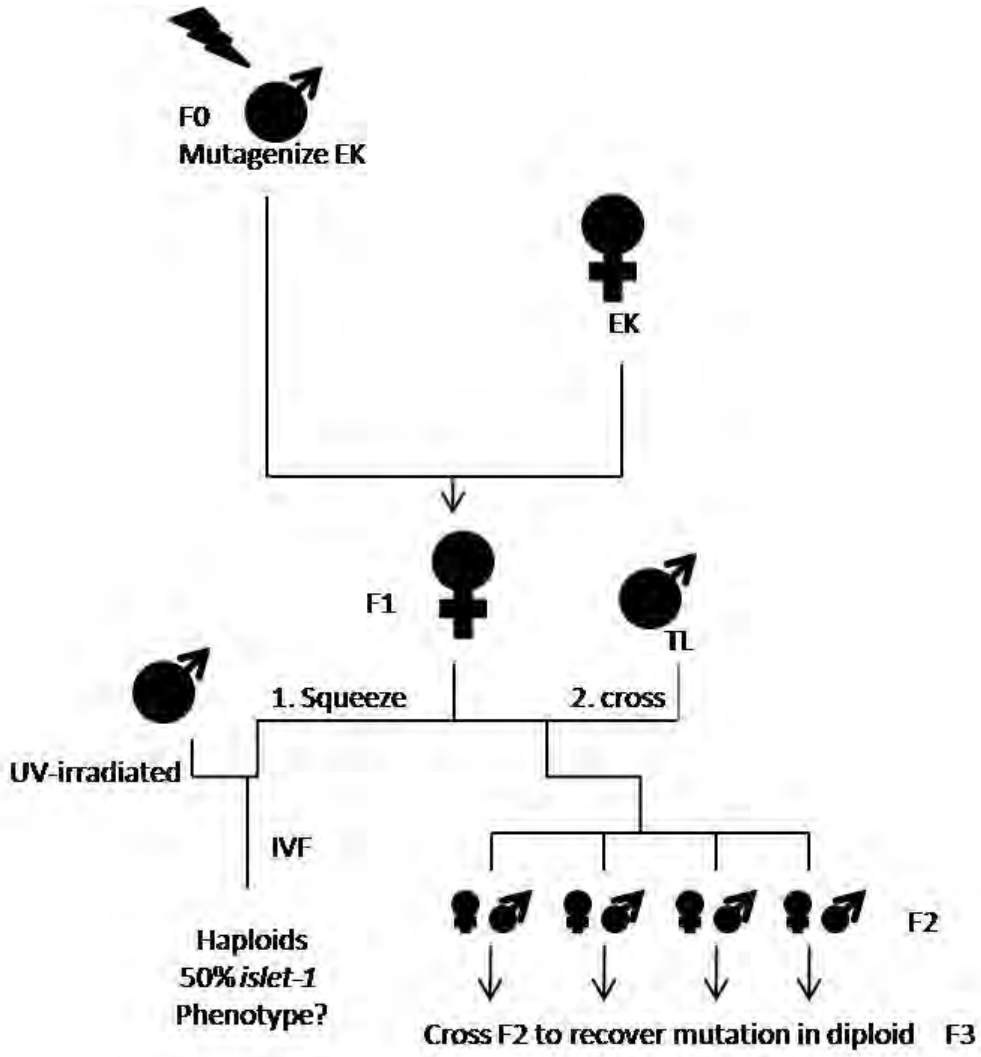
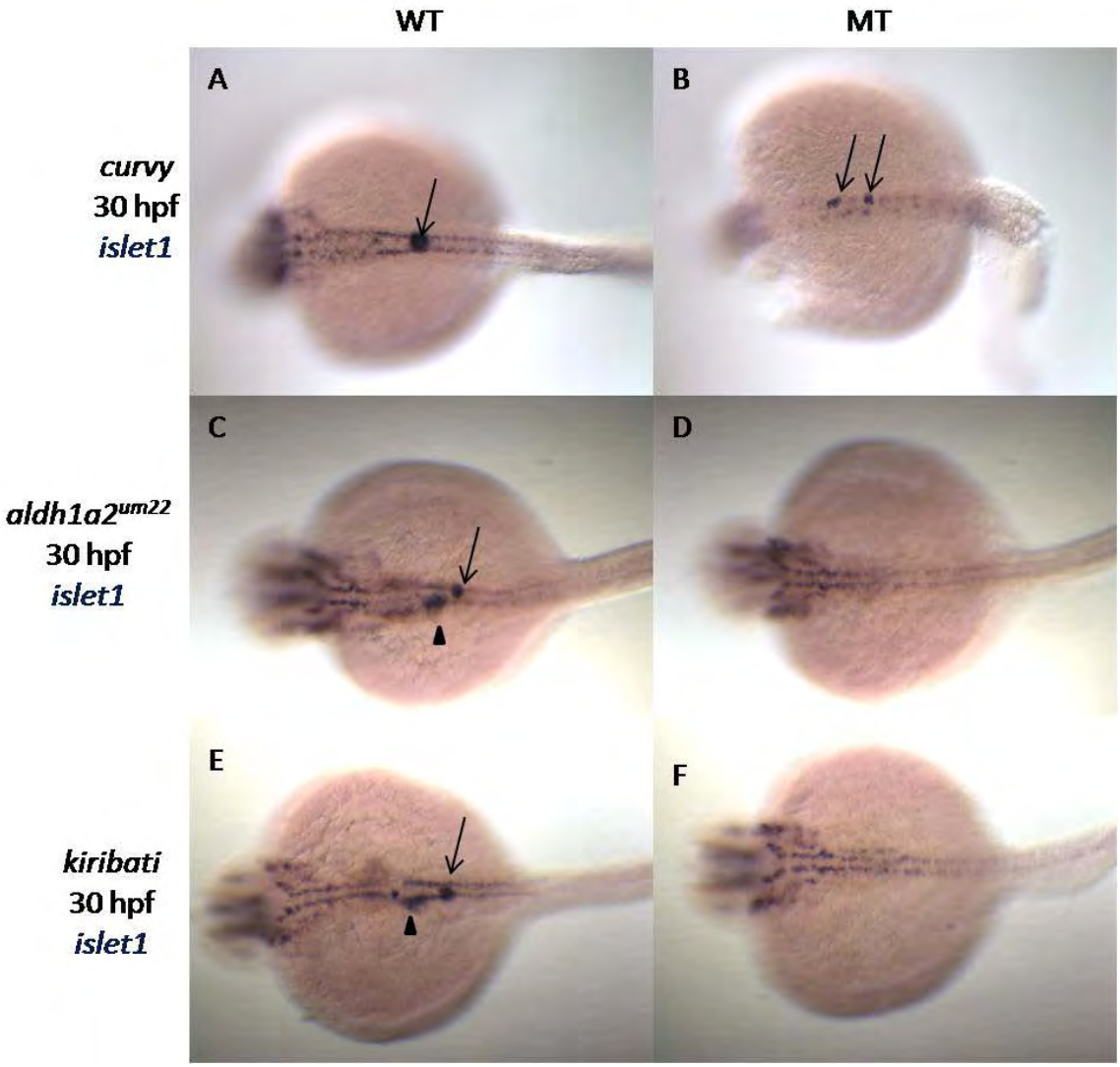

F0
Mutagenize EK
EK
F1
TL
1. Squeeze
2. cross
UV-irradiated
IVF
Haploids
50% islet-1
Phenotype?
F2
Cross F2 to recover mutation in diploid
F3

Figure 3.2 – *curvy*, *aldh1a2*^{um22} and *kiribati* mutants were isolated from two ENU mutagenesis screens

(A) 30 hpf wildtype embryos with *islet1* expression in the CNS and the endocrine pancreas (black arrow). (B) *curvy* mutant embryos have decreased CNS *isl1* expression and have anterior ectopic *isl1* spots in the endoderm (black arrows). (C, D) Wildtype embryo (C) with *isl1* in the CNS, endocrine pancreas (black arrow) and the hepatopancreatic duct (arrow head). The *aldh1a2*^{um22} mutant embryo has the CNS expression but no endoderm expression (D). (E, F) Wildtype *kiribati* embryo (E) with *isl1* expression in the CNS, endocrine pancreas (black arrow) and the hepatopancreatic duct (arrow head). *kiribati* mutant embryo (F) has no endoderm expression of *isl1* but maintains CNS expression.

Figure 3.2


WT
MT
A
B
curvy
30 hpf
islet1
C
D
aldh1a2um22
30 hpf
islet1
E
F
kiribati
30 hpf
islet1

aldh1a2^{um22} embryos maintain CNS expression of *isl1* but do not exhibit any endoderm expression of *isl1* (Figure 3.2 C, D).

In a second larger scale screen, we screened 375 genomes and identified 50 endocrine pancreas mutations. Complementation crosses among a subset of the 50 mutants indicated that they carried the same mutation. Furthermore, incrosses of the EK stock used for the mutagenesis recovered the same mutation. We conclude that this mutation arose spontaneously in the EK line that was utilized for the screen. As a result, the mutation is a background mutation and not an ENU induced mutation. The mutant embryos maintain *isl1* expression in the CNS, but not in the endoderm, similar to *aldh1a2*^{um22} (Figure 3.2E, F). We therefore performed complementation crosses between the new mutation and *aldh1a2*^{um22}. We do not observed complementation between the new mutation and *aldh1a2*^{um22}, suggesting that we have identified a novel mutation. Since this mutation lacks *isl1* expression, we named it *kiribati* after a lost island in the Pacific Ocean.

Positional cloning

A successful mutagenesis screen will produce mutations in essential genes affecting development in the embryo. However, this essential gene needs to be recovered (Knapik, Goodman et al. 1996). Systematic genetic mapping of the mutations is necessary to locate the mutated gene. Genome scanning by bulked segregant analysis with microsatellite markers allows a rough map

position to be obtained (Michelmore, Paran et al. 1991; Ruyter-Spira, Gu et al. 1997). We used a CA panel of 192 markers to map the location of the mutation (Knapik, Goodman et al. 1998; Roman, Pham et al. 2002; Lawson, Mugford et al. 2003). $(CA)_n$ repeat is a common example of a microsatellite, where n is variable between alleles such as EK and TL. CA repeats are amplified by PCR using primers targeting the unique sequences flanking the CA repeat (Figure 3.3). Phenotypically mutant and wildtype embryos are separated, digested and genomic DNA is isolated. Bulk segregant analysis is the first step in mapping the mutations where PCR is performed on pools of DNA, using the CA repeats to scan the genome. The PCR reactions are analyzed on an agarose gel and wildtype and mutant PCR bands are compared to determine linkage. If linkage occurs, then PCR on individual embryos is performed to determine how tightly linked the marker is to the gene. We used this method to successfully identify the gene affected in *aldh1a2*^{um22} embryos (Discussed in Chapter IV).

Figure 3.3 – Primers designed to amplify CA repeats within the genome

Approximately 20-25 bp primers designed to the unique flanking sequences outside the CA repeat is sufficient to amplify the region.

Figure 3.3

Discussion:

Mutagenizing zebrafish

ENU is the mutagen of choice for introducing mutations into the mouse germline (Russell, Kelly et al. 1979) and effectively mutagenizes the spermatogonia of medaka, a small teleost fish (Shima and Shimada 1991). Like medaka, zebrafish spermatozoa is efficiently mutagenized with ENU in vitro (Grunwald and Streisinger 1992) and in vivo. Unlike mouse males, where ENU is received by intraperitoneal injections (injections in body cavity), ENU is delivered to zebrafish by incubation in the mutagen solution (Russell, Kelly et al. 1979) ensuring that multiple fish are equally exposed to ENU, reducing variation in dosage and exposure time.

Zebrafish provide a good system for mutagenesis screens. They produce transparent embryos allowing details of embryogenesis and organogenesis to be visualized by microscope and embryonic development occurs outside of the mother (Streisinger, Walker et al. 1981; Kimmel 1989; Dahm 2005). Embryogenesis occurs quickly and by 24 hours, organs have appeared and numerous genes are expressed. Therefore, screening for possible phenotype can occur in a short amount of time. However, if a haploid screen is utilized, embryos start to develop abnormally after 24 hpf hampering the possibilities for screening for phenotypes later during embryogenesis (Streisinger, Walker et al. 1981). A benefit over mouse, ENU treated zebrafish males do not exhibit infertility after ENU treatment and continue to produce mutagenized sperm

derived from the mutagenized spermatogonia until the males either stop mating or die. Since females produce numerous eggs, many F1 founder animals from a single male can be produced. The mutagenized males crossed to wildtype females can produce multiple F2 lines where 50% will carry the mutation. Thus, only a small number of crosses are needed to recover a mutation. Also, recovery of a mutation several times during by screening the same male confirms the mutant phenotype and as a result several carriers can be identified at once (Solnica-Krezel, Schier et al. 1994). For these reasons, we were able to carry out two haploid ENU screens and recover three different mutations affecting endocrine pancreas formation in diploid embryos within 2 years.

Saturating the zebrafish genome

As of 2000, a total of 6194 (2337 in Boston and 3857 in Tübingen) mutagenized genomes were screened in zebrafish. Since then other labs have undertaken mutagenesis screens but are still far from saturating the genome, or mutagenizing all the essential developmental genes (estimated 700-1600 genes)(Knapik 2000). The screens revealed that ENU can introduce multiple mutations within a single allele. This brought up the questions: Why are all essential genes not isolated with an average of one mutation in each mutagenized genome and how many mutagenized genomes need to be screened to reach saturation? One answer is that the pigment locus test targets a mutational hotspot, where mutations can occur up to 100 times more frequently

than the normal mutation rate. Therefore, the pigment locus test might not be an actual representation of mutation rate for essential developmental genes (Knapik 2000) suggesting that our screen did not saturate the genome. Also another hindrance is that the zebrafish genome is duplicated and this could influence the mutation rate as well. If duplicated genes have preserved unique specific functions, then both will be recovered in a screen separately and the essential gene number will increase (even though it is the same gene that carries out different functions). However, if both genes act redundantly, then the screen will not find them since the second gene will compensate for the loss of the first gene. Overall, the zebrafish genome contains many essential developmental genes yet to be targeted by mutagenesis screens. Furthermore, finding them will give an understanding of the numerous developmental pathways that occur in zebrafish, which can lead to the understanding of conserved pathways in humans. This will yield insight into the diseases associated with these pathways and possible ways to treat them.

Materials and Methods

Fish and Handling

Adult Ekkwill (EK) and Tupfel long fin (*TL*) fish were maintained as previously described (Solnica-Krezel, Schier et al. 1994). Embryo*s* were collected from natural matings and reared in 1/3 Ringer's. Embryos were staged using morphological criteria up to 24 hours post fertilization (hpf) and then by time of development at 28.5°C (Kimmel, Ballard et al. 1995).

ENU Treatment

EK males were transferred to 1-liter beakers containing 3 mM ENU (N-ethyl-N-nitrosourea) for one hour followed by a series of recovery tanks once a week for three weeks. The males were then crossed repeatedly to clean out any post meiotic germ cells that were mutagenized. Mutagenized males were then crossed to EK females and the progeny (F1) were raised. Haploid embryos were produced by In Vitro Fertilization (IVF) of F1 female progeny with irradiated sperm. Haploid embryos were raised to approximately 30 hpf and fixed in 4% paraformaldehyde for in situ hybridization with an *islet1 (isl1)* probe. Embryos were screened based on *isl1* expression. F1 females that produced embryos with mutant phenotypes were out-crossed to TL males and the progeny (F2) were raised and in-crossed for recovery of mutation in diploid embryos.

Testing efficiency of mutagenesis

Specific locus test was carried out previously by crossing mutagenized males with tester females carrying the *golden* mutation. During the first 2-4 weeks after mutagenesis, treated males were crossed once per week with *gol/gol* females to determine frequency of newly induced mosaic and non-mosaic mutations at the *gol* locus. We mutagenized at rate of 1 mutant per 1,000 chromosomes tested. A spontaneous mutation rate of 3×10^{-5} has been reported at the *gol* locus per generation (Walker and Streisinger 1983) which is lower than what was previously obtained in ENU treatment (1.4×10^{-3})(Solnica-Krezel, Schier et al. 1994) and what we aimed for.

Mapping, DNA Extraction

Genomic DNA was extracted from phenotypically mutant and phenotypically wild type embryos and DNA pools were created. Bulk segregant analysis was performed using a 192 CA marker panel (Knapik, Goodman et al. 1998; Roman, Pham et al. 2002; Lawson, Mugford et al. 2003). Embryos used for meiotic mapping were digested overnight at 50 ^{o}C in 50 uL of buffer containing 10mM Tris, pH 8.0; 50 mM EDTA; 200 mM NaCL; 0.5 mg/mL proteinase K; and 0.5% SDS. Digests were spun through Sephacryl-S400 (Amersham Pharmacia) and the resulting genomic DNA was used for PCR. SSLP mapping was performed as previously described (Knapik, Goodman et al.

1998) except primers were not radiolabeled and PCR reactions were run through a 3% Certified Low Range Ultra Agarose gel (Bio Rad).

In situ hybridization

Antisense digoxigenin- and fluorescein-labeled probes were produced by standard methods. One- and two-color in situ hybridization was carried out as described previously (Sagerstrom, Grinbalt et al. 1996; Sagerstrom, Kao et al. 2001).

CHAPTER IV

MATERNAL AND ZYGOTIC *aldh1a2* ACTIVITY IS REQUIRED FOR PANCREAS DEVELOPMENT IN ZEBRAFISH

Abstract

We have isolated and characterized a novel zebrafish pancreas mutant. Mutant embryos lack expression of *isl1* and *sst* in the endocrine pancreas, but retain *isl1* expression in the CNS. Non-endocrine endodermal gene expression is less affected in the mutant, with varying degrees of residual expression observed for *pdx1, carbA, hhex, prox1, sid4, transferrin* and *ifabp*. In addition, mutant embryos display a swollen pericardium and lack fin buds. Genetic mapping revealed a mutation resulting in a glycine to arginine change in the catalytic domain of the *aldh1a2* gene, which is required for the production of retinoic acid from vitamin A. Comparison of our mutant (*aldh1a2*^{um22}) to *neckless* (*aldh1a2*^{i26}), a previously identified *aldh1a2* mutant, revealed similarities in residual endodermal gene expression. In contrast, treatment with DEAB (diethylaminobenzaldehyde), a competitive reversible inhibitor of Aldh enzymes, produces a more severe phenotype with complete loss of endodermal gene expression, indicating that a source of Aldh activity persists in both mutants. We find that mRNA from the *aldh1a2*^{um22} mutant allele is inactive, indicating that it represents a null allele. Instead, the residual Aldh activity is likely due to maternal *aldh1a2*, since we find that translation-blocking, but not splice-blocking, *aldh1a2* morpholinos produce a phenotype similar to DEAB treatment. We conclude that

Aldh1a2 is the primary Aldh acting during pancreas development and that maternal Aldh1a2 activity persists in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos.

Background

Similar to the pancreas of other vertebrates, the zebrafish pancreas consists of an endocrine and an exocrine portion. The zebrafish exocrine pancreas consists of acinar cells that release digestive enzymes into the intestine and the endocrine pancreas is composed of five cell types that secrete hormones directly into the blood stream; insulin producing β-cells, somatostatin producing δ-cells, glucagon producing α-cells, pancreatic polypeptide hormone secreting PP-cells and ghrelin producing ε-cells (Slack 1995; Molotkov, Molotkova et al. 2005). The zebrafish pancreas develops from a dorsal and a ventral bud associated with the gut tube, where the dorsal bud is located slightly posterior to the ventral bud (Field, Dong et al. 2003; Wallace and Pack 2003). The dorsal bud is the first to form at 24 hpf and eventually gives rise to endocrine pancreas. By 40 hpf, the ventral bud has formed and is composed of exocrine cells as well as a few endocrine cells. By 52 hpf, the two buds have merged to form one organ on the right side of the embryo, consisting of a single islet of endocrine cells surrounded by the exocrine pancreas (Field, Dong et al. 2003; Wallace and Pack 2003).

As in other vertebrates, expression of *pdx1* marks the future position of the pancreas in zebrafish embryos (Hebrok, Kim et al. 1998; Biemar, Argenton et al. 2001; Molotkov, Molotkova et al. 2005). Zebrafish *pdx1* expression is first observed at 14 hpf (Stafford, Hornbruch et al. 2004; Stafford, White et al. 2006); but by cell transplantation experiments it has been demonstrated endoderm

commitment as early as 5 hpf (David and Rosa 2001). At this early point, endoderm cells express *sox17*, a gene necessary for endoderm development (David and Rosa 2001; Reiter, Kikuchi et al. 2001; Aoki, David et al. 2002). Various intercellular signaling molecules act on these early endodermal cells to direct their differentiation into organs such as the pancreas. These factors include sonic hedgehog (Shh), bone morphogenetic protein (Bmp), transforming growth factor β (TGF-β), fibroblast growth factor (Fgf) and retinoic acid (RA) (Hebrok, Kim et al. 1998; Wells and Melton 2000; Stafford and Prince 2002; Kumar, Jordan et al. 2003; Norgaard, Jensen et al. 2003; Chen, Pan et al. 2004; Stafford, Hornbruch et al. 2004).

RA is involved in the formation of the central nervous system, lung, kidney, intestine, and pancreas (Plateroti, Sambuy et al. 1993; Mendelsohn, Lohnes et al. 1994; Malpel, Mendelsohn et al. 2000; Maden 2002; Stafford and Prince 2002; Chen, Pan et al. 2004). In particular, RA is needed at the end of gastrulation for pancreas development and blocking RA signaling in zebrafish embryos prevents pancreas formation (Stafford and Prince 2002). Accordingly, exogenously applied RA induces ectopic pancreatic gene expression in the anterior endoderm (Stafford and Prince 2002). Experiments in amphibian and avian models give similar results, indicating a requirement for RA in vertebrate pancreas development (Kumar, Jordan et al. 2003; Chen, Pan et al. 2004; Stafford, Hornbruch et al. 2004). RA is a small lipophilic molecule derived from dietary vitamin A (retinol). Retinol is converted to an aldehyde (retinaldehyde)

which is further converted to a carboxylic acid (retinoic acid). The first step, oxidation of retinol to retinaldehyde, is made possible by several retinol dehydrogenases (RDHs) that have widespread and overlapping expression patterns. The second step, oxidation of retinaldehyde to RA, is carried out by retinaldehyde dehydrogenases (Raldh or Aldh), which have more tissue specific expression patterns (Ang, Deltour et al. 1996; Mic, Haselbeck et al. 2002; Sandell, Sanderson et al. 2007; Wingert, Selleck et al. 2007; Duester 2008). In particular, *aldh1a2* (*raldh2*) is the major retinoic acid generating enzyme in the early mouse embryo and was thought until recently to be the only *raldh* expressed in zebrafish. Recently, *aldh1a3 (raldh3)* and *aldh8a1 (raldh4)* were identified in zebrafish (Liang, Zhang et al. 2008; Pittlik, Domingues et al. 2008) but *aldh1a1 (raldh1)* has not been found in zebrafish to date. *aldh1a3* is expressed in the developing eye and ear after gastrulation and *aldh8a1* is expressed later around 2 dpf in the liver and intestine (Liang, Zhang et al. 2008; Pittlik, Domingues et al. 2008) suggesting that these genes are not involved in early pancreas development. In contrast, *aldh1a2* is expressed at 30% epiboly in the mesendoderm and continues to be expressed in the posterior and lateral mesoderm during segmentation (Begemann, Schilling et al. 2001). At later stages, *aldh1a2* is expressed in the somites and the pronephric anlage (by 15hpf) as well as in pharyngeal arch and pectoral fin mesenchyme (32hpf) (Wang, Penzes et al. 1996; Zhao, McCaffery et al. 1996; Niederreither, McCaffery et al. 1997; Berggren, McCaffery et al. 1999; Swindell, Thaller et al.

1999; Begemann, Schilling et al. 2001). Expression of *aldh1a2* adjacent to, but not within, the pancreatic anlage is consistent with observations that the anterior paraxial mesoderm is a source of RA driving pancreas formation. Accordingly, three Retinoic Acid Receptors (two RARα and one RARγ) are expressed in the endoderm, indicating that the RA signal can be received directly in the endoderm (Stafford, White et al. 2006).

Results

An ENU screen for zebrafish pancreas mutants

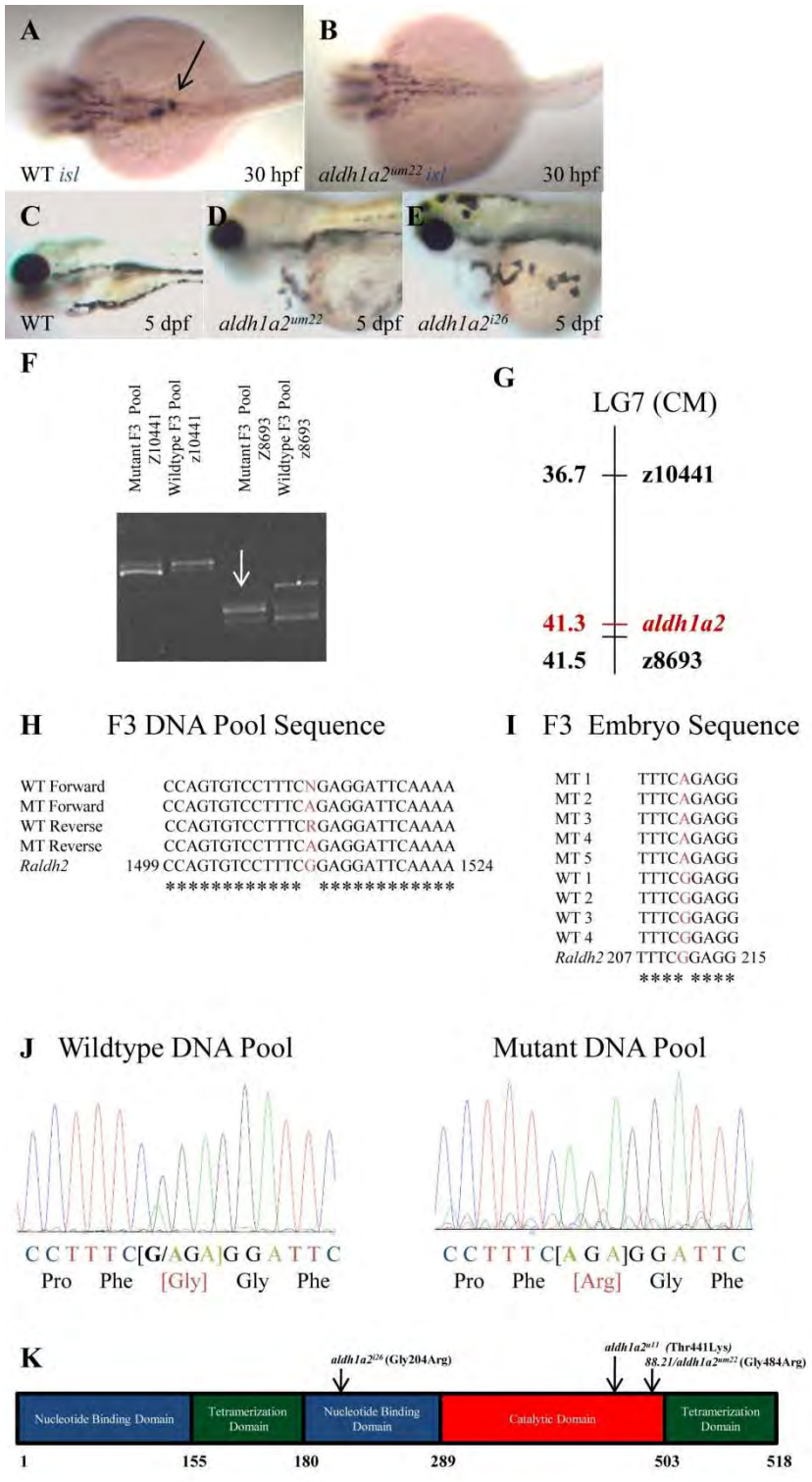
A haploid in situ hybridization screen of ENU (*N*-ethyl-*N*-nitrosourea) mutagenized zebrafish was carried out to identify mutations in endocrine pancreas development. Ekkwill (EK) males were treated with 3 mM ENU and crossed to EK females. F1 progeny were raised and eggs from F1 females were in vitro fertilized using irradiated sperm from EK males. The resulting haploid embryos were raised until 30 hpf and assayed by in situ hybridization for *islet1* (*isl1*) expression to detect defects in the endocrine pancreas. F1 females that produced clutches with 50% mutant embryos were outcrossed to Tupfel long fin (*TL*) males. F2 progeny were raised and screened for recovery of the mutation in the F3 generation. We screened 200 genomes and discovered ten females with defective endocrine pancreas formation. Six of the ten females died, developed tumors or did not produce progeny. Out of the remaining four females, we recovered diploid mutants for two.

Embryos from one of the recovered mutants (88.21) lack *isl1* expression in the endocrine pancreas, but maintain expression in the CNS (Figure 4.1B versus wild type in Figure 4.1A). 88.21 embryos first display a morphological phenotype approximately at day 4, as they do not develop fin buds and have a swollen pericardium (Figure 4.1D versus wildype in Figure 4.1C).

Figure 4.1 - 88.21 is a novel *aldh1a2* allele

A, B. *Islet1* (*isl1*) expression was used in a haploid ENU screen to identify mutants in endocrine pancreas development. Dorsal view of 30 hpf wild type embryo with *isl1* expression in the CNS and endocrine pancreas (A; black arrow indicates expression in pancreas) and 88.21 mutant embryo with *isl1* expression in the CNS, but not in the endoderm (B). C-E. Lateral view of live wild type (C), 88.21 (D), and *neckless aldh1a2*^{i26} (E) embryos at day 5. F. Linkage analysis using CA repeat markers on pooled genomic DNA from 88.21 mutants and pooled genomic wild type DNA. Marker z10441 amplifies a 450 bp band and a faint 500 bp band in the mutant pool compared to a faint 450 bp band and a 500 bp band in the wild type pool. Marker z8693 amplifies two bands at 250 bp and 300 bp in the mutant pool compared to 250 bp, 300 bp as well as a 400 bp band in the wild type pool. White arrow points to lack of 400bp band in mutant. G. Schematic drawing of part of linkage group 7 (LG7), showing the location of z10441 and z8693 and *aldh1a2* (in red) in reference to these markers. H-J. Sequence analysis of pooled 88.21 mutant (MT) genomic DNA and pooled wild type (WT) genomic DNA (H, J), as well as of individual mutant (MT) and wild type (WT) embryos (I). 88.21 fish carry a mutation that converts Gly^{484} to Arg (in red, and outlined in brackets in J) located in the catalytic domain. K. Schematic of Aldh1a2 protein and the location of the *aldh1a2* mutant alleles *aldh1a2*^{i26}, *aldh1a2*^{u11} and *88.21/aldh1a2*^{um22}.

Figure 4.1


A
WT isl
30 hpf
B
aldh1a2um22 isl
30 hpf
C
WT
5 dpf
D
aldh1a2um22
5 dpf
E
aldh1a2i26
5 dpf
F
Mutant F3 Pool Z10441
Wildtype F3 Pool z10441
Mutant F3 Pool Z8693
Wildtype F3 Pool z8693
G
LG7 (CM)
36.7 z10441
41.3 aldh1a2
41.5 z8693
H F3 DNA Pool Sequence
WT Forward CCAGTGTCCTTTCNGAGGATTCAAAA
MT Forward CCAGTGTCCTTTCAGAGGATTCAAAA
WT Reverse CCAGTGTCCTTTCRGAGGATTCAAAA
MT Reverse CCAGTGTCCTTTCAGAGGATTCAAAA
Raldh2 1499 CCAGTGTCCTTTCGGAGGATTCAAAA 1524
********** **********
I F3 Embryo Sequence
MT 1 TTTCAGAGG
MT 2 TTTCAGAGG
MT 3 TTTCAGAGG
MT 4 TTTCAGAGG
MT 5 TTTCAGAGG
WT 1 TTTCGGAGG
WT 2 TTTCGGAGG
WT 3 TTTCGGAGG
WT 4 TTTCGGAGG
Raldh2 207 TTTCGGAGG 215
**** ****
J Wildtype DNA Pool
Mutant DNA Pool
C C T T T C[G/AGA]G G A T T C
Pro Phe [Gly] Gly Phe
C C T T T C[A G A]G G A T T C
Pro Phe [Arg] Gly Phe
K
aldh1a2i26 (Gly204Arg)
aldh1a2m11 (Thr441Lys)
88.21/aldh1a2um22 (Gly484Arg)
Nucleotide Binding Domain
Tetramerization Domain
Nucleotide Binding Domain
Catalytic Domain
Tetramerization Domain
1
155
180
289
503
518

Since the EK and TL strains used in our screen are highly polymorphic with respect to their CA repeats, we used a PCR panel consisting of 192 primer pairs that amplify CA repeats in the zebrafish genome to map the position of the mutation (Knapik, Goodman et al. 1998; Roman, Pham et al. 2002; Lawson, Mugford et al. 2003). Specifically, genomic DNA pools from phenotypically wild type and phenotypically mutant embryos were amplified using primers from the CA marker panel. Based on the bulk segregant analysis of the DNA pools, two markers, z10441 and z8693, were found to be linked to the mutation (Figure 4.1F). Subsequent PCR of inividual embryos (not shown) confirmed the linkage. We detected three crossovers out of 44 meioses for the z10441 marker, which places the mutation approximately 7 cM away from this marker on linkage group 7 (Figure 4.1G).

The 88.21 mutant represents a novel *aldh1a2* allele

A closer examination revealed that the z10441 and z8693 markers are both located near the *aldh1a2* (*raldh2*) gene on chromosome 7. As noted, *aldh1a2* is a retinaldehyde dehydrogenase (Raldh) involved in RA synthesis and there are two previously reported *aldh1a2* mutants, *neckless* (*nls* or *aldh1a2*^{i26}*; Figure 4.1E*) and *no fin* (*nof* or *aldh1a2*^{u11}) (Begemann, Schilling et al. 2001; Grandel, Lun et al. 2002). Since the 88.21 mutant phenotype bears some resemblance to the *aldh1a2*^{i26} phenotype (Figure 4.1D, E) – lack of pectoral fins, swollen pericardium and embryonic lethality by day 6 - we tested if 88.21 might represent

a novel *aldh1a2* allele. To this end, we amplified full length *aldh1a2* from cDNA prepared from mutant and wild type embryo pools derived from an 88.21 incross. Sequencing of the PCR products identified a G to A change in the mutant pool that converts a glycine to an arginine at position 484 (Figure 4.1H, J) in the catalytic domain of Aldh1a2 (Figure 4.1K). Sequencing cDNA from individual embryos confirmed this change (Figure 4.1I).

To confirm that the 88.21 phenotype is caused by a mutation in the *aldh1a2* gene, we set out to rescue the mutant phenotype with wild type *aldh1a2* mRNA. We find that 26% of embryos from an incross of 88.21 heterozygotes fail to develop fin buds (Table 4.1), as assayed by *shh* expression in fin buds at 48 hpf (Figure 4.2A, B) or by visual inspection for fin bud formation at 72 hpf (not shown). However, following injection of wild type *aldh1a2* mRNA at the 1-2 cell stage, only 8.5% of embryos lack fin buds, demonstrating that *aldh1a2* mRNA rescues fin bud development (Table 4.1, Figure 4.2C). In contrast, injection of *aldh1a2* mRNA containing the 88.21 mutation does not rescue fin bud development (24% lack fin buds; Table 4.1, Figure 4.2D). Notably, the swollen pericardium phenotype was not rescued by injection of *aldh1a2* mRNA. This result is consistent with previous work showing that fin bud development in *aldh1a2*^{i26} and *aldh1a2*^{u11} can be rescued by injecting wild type *aldh1a2* mRNA, but the swollen pericardium cannot be rescued (Begemann, Schilling et al. 2001; Grandel, Lun et al. 2002).

Table 4.1: Rescue 88.21 fin bud development

Embryos from an incross of 88.21 heterozygotes were injected with 500 pg of wild type *aldh1a2* mRNA or *aldh1a2* mRNA containing the 88.21 mutation. Embryos were either fixed and assayed for fin bud expression by *shh* probe at 48 hpf or observed live at 72 hpf for fin bud development. Wild type *aldh1a2* rescued fin bud development (91.5% of embryos have fin buds) whereas *aldh1a2* carrying the 88.21 mutation did not rescue (76% of embryos have fin buds).

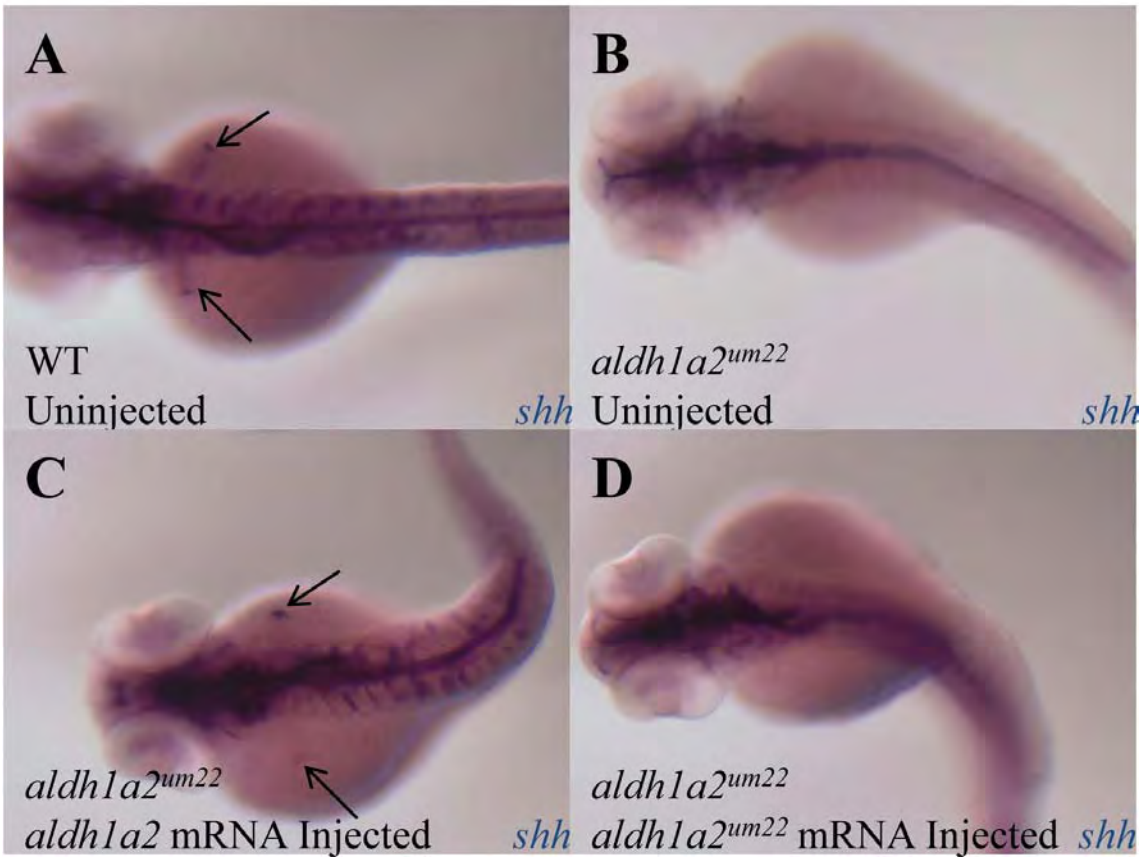
Table 4.1: Rescue *88.21* fin bud development

	Finbud	No Finbud
Uninjected	113/153 (74%)	40/153 (26%)
Injected aldh1a2 mRNA	107/117 (91.5%)	10/117 (8.5%)
Injected 88.21/aldh1a2^{um22} mRNA	61/80 (76%)	19/80 (24%)

Figure 4.2 - Wild type *aldh1a2* mRNA rescues 88.21 fin bud development

Dorsal views of 48 hpf embryos with *sonic hedgehog* (*shh*) expression in purple. A. Uninjected wild type embryo with *shh* expression in the CNS and fin buds (black arrows). B. *aldh1a2*^{um22} mutant embryos lack *shh* expression in the fin buds. C. *aldh1a2*^{um22} mutant embryo injected with *aldh1a2* wild type mRNA shows rescued fin bud expression (black arrows). D. *aldh1a2*^{um22} mutant embryo injected with *aldh1a2*^{um22} mutant mRNA is not rescued.

Figure 4.2


A
WT
Uninjected
shh
B
aldh1a2um22
Uninjected
shh
C
aldh1a2um22
aldh1a2 mRNA Injected
shh
D
aldh1a2um22
aldh1a2um22 mRNA Injected
shh

We conclude that the 88.21 mutation occurs in the *aldh1a2* catalytic domain and we refer to it as *aldh1a2*^{um22}. Since the mutant mRNA appears to be inactive even when overexpressed, the *aldh1a2*^{um22} allele is likely to represent a null allele. In particular, replacing a small conserved glycine residue with a large arginine in the catalytic domain may affect the function or folding of the Aldh1a2 protein.

Endoderm gene expression is variably affected in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutants

We observe variable effects on endoderm gene expression in *aldh1a2*^{um22} mutants and we therefore compared the *aldh1a2*^{um22} phenotype to the *aldh1a2*^{i26} phenotype. The *aldh1a2*^{i26} allele was previously analyzed with some endodermal markers (Stafford and Prince 2002) but we have expanded the analysis further. We find that endocrine-specific genes such as *isl1* (Table 4.2) and *sst1* (Table 4.2) are completely lost in both mutants at 24-30hpf, as is *p48* expression in the exocrine pancreas (Table 4.2). In contrast, *pdx1* expression remains in the majority of both *aldh1a2*^{i26} and *aldh1a2*^{um22} mutant embryos (Figure 4.3E versus 4.3G, H; Figure 4.4A versus 4.4C, D; Table 4.2), as does *carboxypeptidase A* (*carbA)* expression, although *carbA* expression is more pronounced in *aldh1a2*^{i26} (Figure 4.4I versus 4.4K, L; Table 4.2).

Table 4.2: DEAB, *aldh1a2*^{um22} and *aldh1a2*^{i26} in situ results

Summary of gene expression data reported in figures 4.3 and 4.4. DEAB treated embryos, as well as embryos from incrosses of *aldh1a2*^{um22} heterozygotes and *aldh1a2*^{i26} heterozygotes were assayed by in situ hybridization and their expression classified into one of three categories (no expression, wild type expression and weak expression). Embryos in each category is presented as a percent of the total number of embryos analyzed (actual numbers are given within parentheses).

Table 4.2: DEAB, *aldh1a2*^{um22}, and *aldh1a2*^{i26} in situ results.

Stage	Marker		DEAB Treated			aldh1a2^{um22} Cross			aldh1a2^{i26} Cross	
hpf		No Expression	Weak Expression	Wild type Expression	No Expression	Weak Expression	Wild type Expression	No Expression	Weak Expression	Wild type Expression
24	*ins*	100% (99/99)	0% (0/0)	0% (0/0)	22% (37/167)	7% (12/167)	71% (118/167)	16% (13/79)	13% (10/79)	71% (56/79)
24	*pdx1*	100% (40/40)	0% (0/0)	0% (0/0)	0% (0/115)	24% (28/115)	76% (87/115)	15% (16/105)	9% (9/105)	76% (80/105)
30	*hhex*	100% (88/88)	0% (0/0)	0% (0/0)	14% (32/228)	12% (28/228)	74% (168/228)	12% (14/116)	15% (17/116)	73% (85/116)
30	*isl1*	100% (26/26)	0% (0/0)	0% (0/0)	25% (26/104)	0% (0/104)	75% (78/104)	26% (13/50)	0% (0/50)	74% (37/50)
30	*p48*	100% (27/27)	0% (0/0)	0% (0/0)	26% (32/125)	0% (0/125)	74% (93/125)	25% (16/64)	0% (0/64)	75% (48/64)
30	*prox1*	100% (93/93)	0% (0/0)	0% (0/0)	0% (0/63)	24% (15/63)	76% (48/63)	0% (0/195)	26% (50/195)	74% (145/195)
30	*sst*	100% (23/23)	0% (0/0)	0% (0/0)	24% (22/92)	0% (0/92)	76% (70/92)	26% (21/80)	0% (0/80)	74% (59/80)
48	*ins*	100% (25/25)	0% (0/0)	0% (0/0)	16% (10/63)	8% (5/63)	76% (48/63)	15% (12/78)	10% (8/78)	74% (58/78)
48	*pdx1*	100% (95/95)	0% (0/0)	0% (0/0)	0% (0/167)	25% (41/167)	75% (126/167)	12% (9/76)	12% (9/76)	76% (58/76)
48	*shh*	100% (24/24)	0% (0/0)	0% (0/0)	24% (13/54)	0% (0/54)	76% (41/54)	24% (15/62)	0% (0/62)	76% (47/62)
48	*sid4*	100% (87/87)	0% (0/0)	0% (0/0)	6% (12/189)	20% (38/189)	74% (139/189)	12% (18/146)	12% (18/146)	75% (110/146)
72	*carbA*	100% (33/33)	0% (0/0)	0% (0/0)	23% (57/245)	1% (2/245)	76% (186/245)	11% (13/117)	15% (18/117)	74% (86/117)
72	*ifabp*	100% (15/15)	0% (0/0)	0% (0/0)	0% (0/63)	24% (15/63)	76% (48/63)	0% (0/54)	22% (12/54)	78% (42/54)
72	*ins*	100% (25/25)	0% (0/0)	0% (0/0)	10% (5/52)	12% (6/52)	79% (41/52)	0% (0/25)	12% (3/25)	88% (22/25)
72	*transf*	100% (27/27)	0% (0/0)	0% (0/0)	0% (0/115)	24% (28/115)	77% (87/115)	2% (2/100)	21% (21/100)	77% (77/100)

Figure 4.3 - *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos retain some endoderm gene expression at 24 and 30 hpf

DMSO treated wild type embryos (A, E, I, M), DEAB-treated wild type embryos (B, F, J, N), embryos from an incross of *aldh1a2*^{um22} heterozygotes (C, G, K, O) and embryos from an incross of *aldh1a2*^{i26} heterozygotes (D, H, L, P) were assayed for expression of *ins* at 24hpf (A-D; black arrows indicate residual expression), *pdx1* at 24hpf (E-H; black arrows indicate residual expression), *hhex* at 30hpf (I-L; residual expression is indicated in pancreas (arrow) and liver (arrowhead)) and *prox1* at 30hpf (M-P; residual expression is indicated in liver (arrowhead)). Embryos are in dorsal view with anterior to the left. See Table 4.2 for quantification.

Figure 4.3

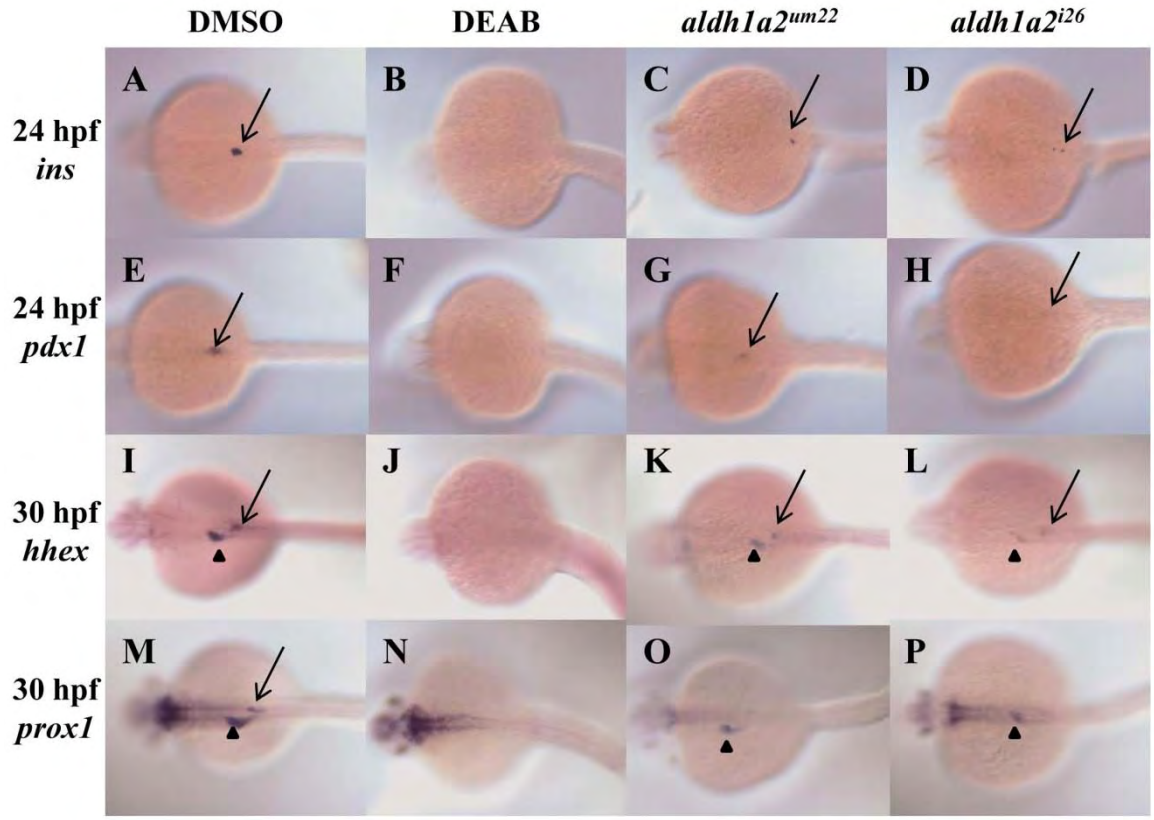
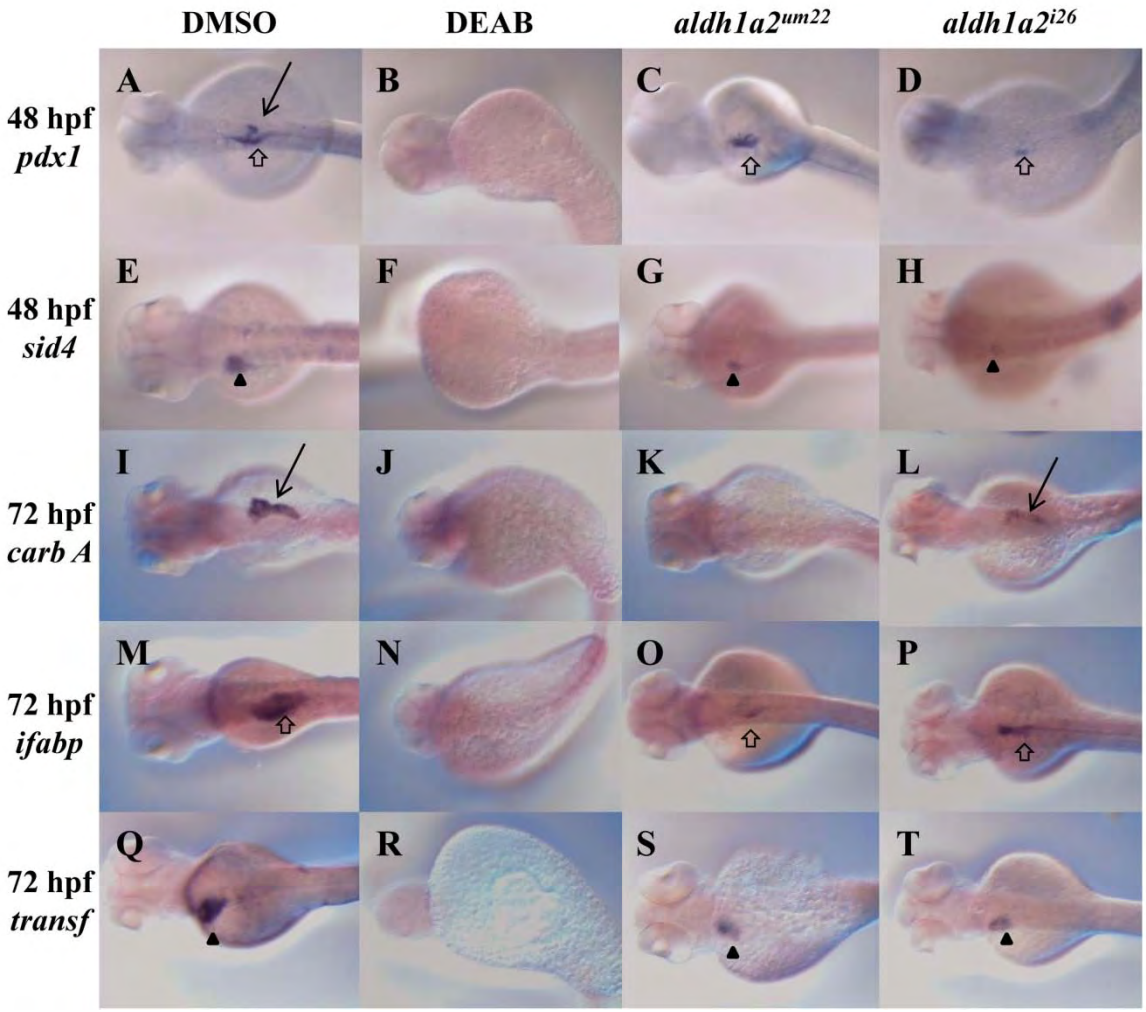

DMSO
DEAB
aldh1a2um22
aldh1a2i26
24 hpf ins
24 hpf pdx1
30 hpf hhex
30 hpf prox1
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 4.4 - *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos retain some endoderm gene expression at 48 and 72 hpf

DMSO treated wild type embryos (A, E, I, M, Q), DEAB-treated wild type embryos (B, F, J, N, R), embryos from an incross of *aldh1a2*^{um22} heterozygotes (C, G, K, O, S) and embryos from an incross of *aldh1a2*^{i26} heterozygotes (D, H, L, P, T) were assayed for expression of *pdx1* at 48hpf (A-D), *sid4* at 48hpf (E-H), *carbA* at 72hpf (I-L), *ifabp* at 72hpf (M-P) and *transf* at 72hpf (Q-T). Gene expression is observed in the intestine (open arrows), liver (black arrowheads) and pancreas (black arrows). Embryos are in dorsal view with anterior to the left. See Table 4.2 for quantification.

Figure 4.4

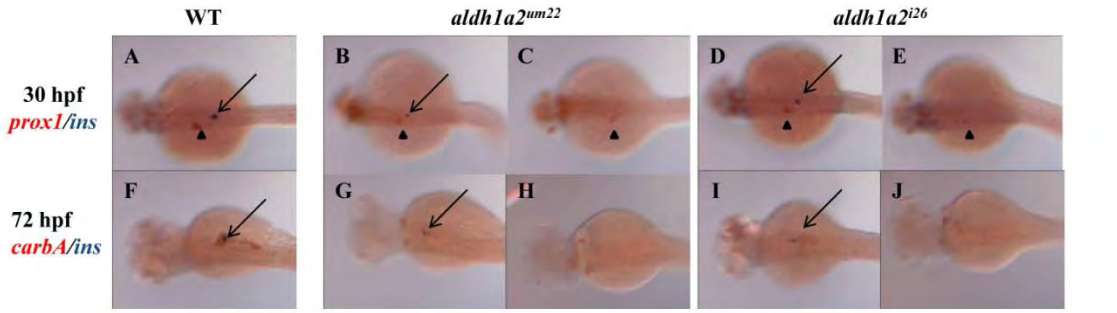

DMSO
DEAB
aldh1a2um22
aldh1a2i26
48 hpf pdx1
48 hpf sid4
72 hpf carb A
72 hpf ifabp
72 hpf transf
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

We also find that expression of *hhex* and *prox1* (that are expressed in both the ventral pancreatic bud and the liver) persists in both mutants (Figure 4.3I versus 4.3K, L and 4.3M versus 4.3O, P; Table 4.2). Analyzing other liver markers later in development revealed that expression of both *sid4* (at 48 hpf) and *transferrin* (*transf*, at 72 hpf) persists in both *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos (Figure 4.4E versus 4.4G, H; 4.4Q versus 4.4S, T; Table 4.2). *intestinal fatty acid binding protein* (*ifabp*) expression is decreased at 72 hpf (Figure 4.4M versus 4.4O, P; Table 4.2) in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos, suggesting that differentiation of the intestine takes place, although perhaps not to completion. Expression of the early endoderm marker *sox17* is maintained (data not shown). Also, while our data suggest that endocrine gene expression may be most sensitive to the loss of Aldh1a2 function, we find that *insulin* (*ins*) expression remains in some mutant embryos at 24, 48 and 72 hpf, suggesting that endocrine gene expression is not completely blocked in the mutants (Figure 4.3C, D; Table 4.2). Lastly, we tested whether embryos with residual expression of one endoderm gene had residual expression of other endoderm genes, but did not observe a correlation, suggesting that expression of each gene varies from embryo to embryo (Figure 4.5).

Figure 4.5 - Double in situ in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos

Wild type (A, F), *aldh1a2*^{um22} (B, C, G, H) and *aldh1a2*^{i26} (D, E, I, J) embryos were assayed for expression of *prox1/ins* at 30hpf (A-E) and *carbA/ins* at 72hpf (F-J). *Ins* expression is detected in purple, while *prox1* (A-E) and *carbA* (F-J) are detected in red. We do not observe any correlation in the extent of residual expression of these genes in individual embryos.

Figure 4.5


WT
aldh1a2um22
aldh1a2i26
30 hpf
prox1/ins
72 hpf
carbA/ins
A
B
C
D
E
F
G
H
I
J

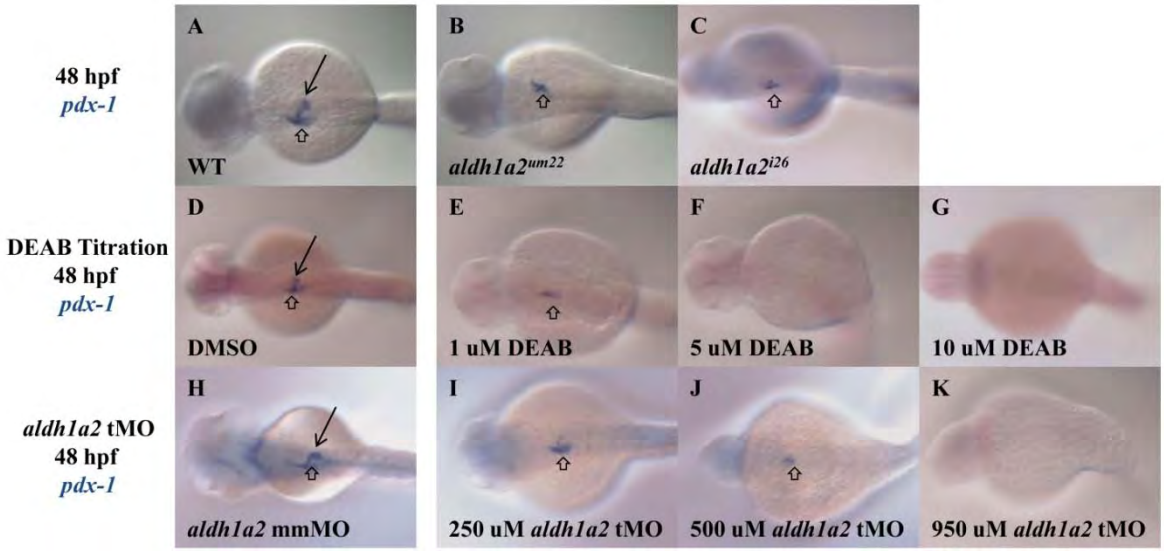
The Aldh inhibitor DEAB completely blocks expression of endoderm genes

We reasoned that the residual gene expression observed in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos could either indicate that RA signaling is not completely required for expression of all genes in the endoderm, or it might indicate residual Aldh activity in the mutants. To test this further, we made use of DEAB (diethylaminobenzaldehyde), a competitive reversible inhibitor of all Aldh enzymes. DEAB has previously been reported to block development of fin buds and otic vesicles (Berggren, McCaffery et al. 1999) and blocks expression of *hoxb1b, vhnf1, krx20* in rhombomere (r) 5, *val* in r5-6, *hoxd4a* and *efnb2a* in r7 of the hindbrain (Maves and Kimmel 2005). Zebrafish embryos treated with DEAB have been analyzed for a few endoderm markers (Stafford and Prince 2002; Kopinke, Sasine et al. 2006; Song, Kim et al. 2007; Wingert, Selleck et al. 2007; Kinkel, Sefton et al. 2009). In particular, *insulin::GFP* expression is lost in embryos treated with DEAB (Stafford and Prince 2002; Kopinke, Sasine et al. 2006; Song, Kim et al. 2007; Kinkel, Sefton et al. 2009). Also, *foxa3* expression in the pancreas and liver and *vhnf1* expression in the pancreas is lost in DEAB treated embryos (Song, Kim et al. 2007). Loss of pharyngeal arches 3-5 was also seen when DEAB was used (Kolm, Apekin et al. 1997). We find that treating zebrafish embryos with 10 uM DEAB starting at 8 hpf (see Figure 4.6 for DEAB titrations) blocks endoderm gene expression.

Figure 4.6 - Titration of DEAB and *aldh1a* tMO

Wild type (A), *aldh1a2*^{um22} mutant (B), *aldh1a2*^{i26} mutant (C), DEAB-treated (D-G) and *aldh1a2* tMO-injected (H-K) embryos were assayed for *pdx1* expression at 48hpf. DEAB and *aldh1a2* tMO were titrated as indicated (D-G and H-K, respectively). Black arrows indicate pancreas expression and open arrows indicate duodenum expression of *pdx1*. Note that intermediate concentrations of DEAB (1uM, panel E) and *aldh1a2* tMO (250-500uM, panels I, J) produce similar phenotypes to the *aldh1a2*^{um22} and *aldh1a2*^{i26} mutants. Embryos are in dorsal view with anterior to the left.

Figure 4.6


48 hpf
pdx-1
A
WT
B
aldh1a2um22
C
aldh1a2i26
DEAB Titration
48 hpf
pdx-1
D
DMSO
E
1 uM DEAB
F
5 uM DEAB
G
10 uM DEAB
aldh1a2 tMO
48 hpf
pdx-1
H
aldh1a2 mmMO
I
250 uM aldh1a2 tMO
J
500 uM aldh1a2 tMO
K
950 uM aldh1a2 tMO

Specifically, expression of *ins, pdx1*, *hhex, prox1, sid4*, *carbA, ifabp* and *transf* is completely lost in DEAB treated embryos (Figure 4.3B, F, J, N; Figure 4.4B, F, J, N, R; Table 4.2) while *sox17* expression is unaffected (not shown). Notably, treatment with lower concentrations (1-5 uM) of DEAB closely mimics the phenotypes observed in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos (Figure 4.6). We conclude that Aldh activity is absolutely required for endoderm gene expression and that there is residual Aldh activity in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos.

Maternal *aldh1a2* activity persists in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos

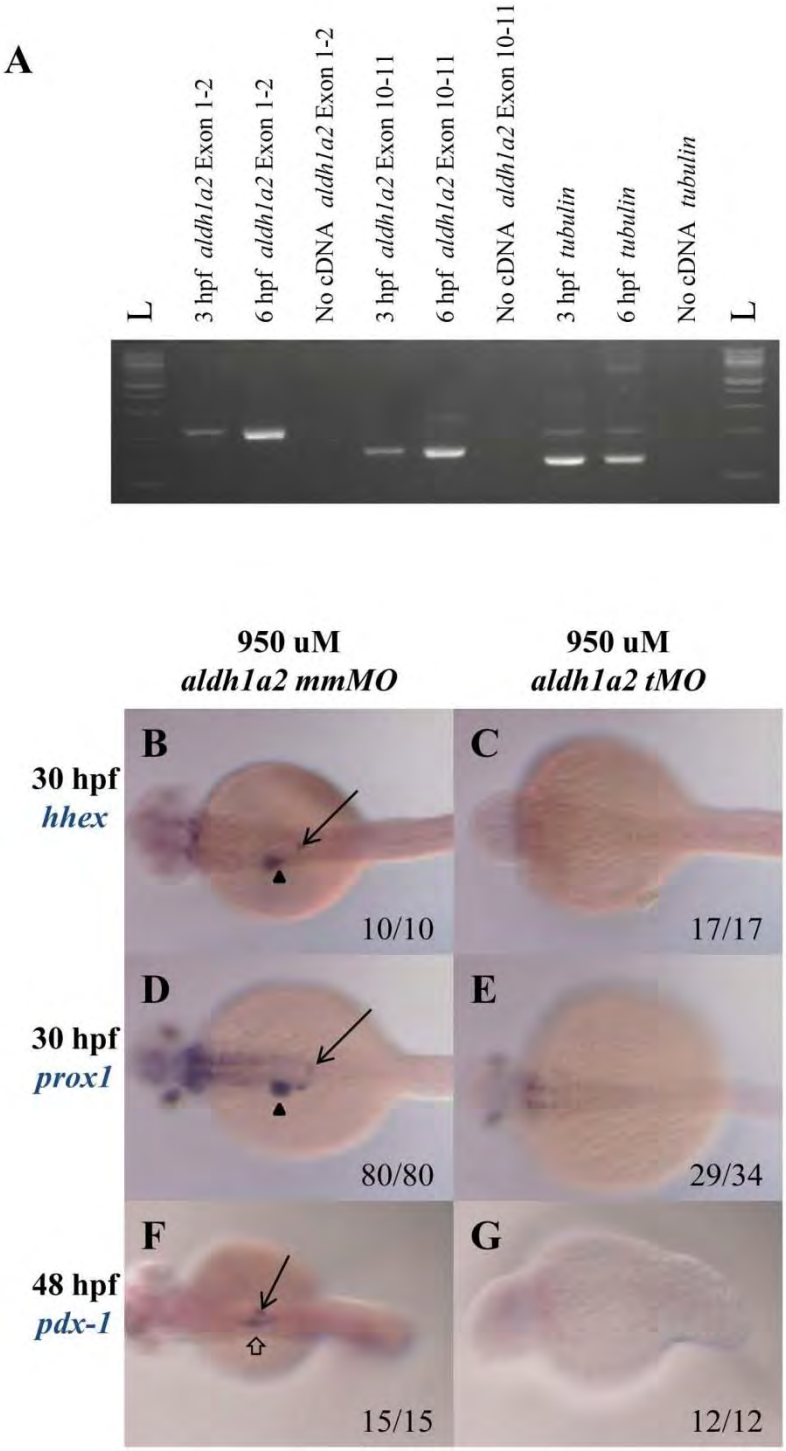
We next considered the likeliest source of residual Aldh activity in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos. The expression patterns of *aldh1a3* (*raldh3*; observed primarily in developing eye, inner ear, pituitary gland and swim bladder) and *aldh8a1* (*raldh4;* found in liver and intestine, but not until day 2)(Liang, Zhang et al. 2008; Pittlik, Domingues et al. 2008) make them unlikely candidates for providing Aldh activity in early pancreas development. In addition, *raldh1* is expressed in the dorsal retina and mesencephalic flexure in mice (Liang, Zhang et al. 2008), but has not been found in zebrafish. Instead, we reasoned that there may be residual *aldh1a2* activity in the mutants. Since the *aldh1a2*^{um22} and *aldh1a2*^{i26} mutations are likely to be null mutations, we considered the most likely source of residual *aldh1a2* activity to be maternally deposited mRNA.

To test this possibility, we first carried out RT-PCR on 3hpf (before the onset of zygotic transcription) and 6hpf (after the onset of zygotic transcription) zebrafish embryos. We find that *aldh1a2* mRNA is present already at 3hpf (Figure 4.7A), consistent with a role for maternal *aldh1a2* mRNA. We reasoned that if the residual *aldh1a2* activity observed in the mutants is due to maternal mRNA, then blocking *aldh1a2* translation with antisense morpholino oligonucleotides (*aldh1a2* tMO) should produce the same phenotype as DEAB treatment. Indeed, we find that injecting *aldh1a2* tMO, completely blocks expression of *hhex* (Figure 4.7C), *prox1* (Figure 4.7E) and *pdx1* (Figure 4.7G), producing a phenotype indistinguishable from the DEAB phenotype and more severe than the *aldh1a2* mutant phenotype, while embryos injected with a mismatch MO control show wild type expression of all endoderm markers (Figure 4.7B, D, F). In contrast, we find that a MO targeting the *aldh1a2* exon 1/intron 1 splice junction (which should not affect already spliced maternal *aldh1a2* mRNAs) cannot fully block endoderm gene expression even at the highest concentration that could be tested (750uM, not shown). We conclude that *aldh1a2* is the predominant *aldh* required for RA signaling during endoderm development and that *aldh1a2* has a significant maternal component.

Figure 4.7 - *aldh1a2* is maternally expressed and *aldh1a2* translational morpholino knocks down endoderm expression

A. PCR of 3 and 6 hpf wild type embryos using primers targeting exon1-2 and exon10-11 of *aldh1a2* reveals *aldh1a2* expression already at 3hpf. A no DNA sample and amplification of tubulin is used as negative and positive controls. B-G. Wild type embryos were injected with either 950 uM *aldh1a2* mismatch (mm) morpholino (MO; B, D, F) or 950 uM of *aldh1a2* translational (tMO; C, E, G) and assayed for expression of *hhex* (B, C), *prox1* (D, E) or *pdx1* (F, G). Embryos are in dorsal view with anterior to the left.

Figure 4.7


A
L
3 hpf aldh1a2 Exon 1-2
6 hpf aldh1a2 Exon 1-2
No cDNA aldh1a2 Exon 1-2
3 hpf aldh1a2 Exon 10-11
6 hpf aldh1a2 Exon 10-11
No cDNA aldh1a2 Exon 10-11
3 hpf tubulin
6 hpf tubulin
No cDNA tubulin
L
950 uM
aldh1a2 mmMO
950 uM
aldh1a2 tMO
30 hpf
hhex
B
10/10
C
17/17
30 hpf
prox1
D
80/80
E
29/34
48 hpf
pdx-1
F
15/15
G
12/12

Discussion

We report results from an ENU (*N*-ethyl-*N*-nitrosourea) screen for genes involved in endocrine pancreas development. We characterize the *aldh1a2*^{um22} allele, which corresponds to a glycine to arginine mutation in the catalytic domain of the Aldh1a2 protein. *aldh1a2*^{um22} mutant embryos show similarities to embryos of two previously identified *aldh1a2* mutants*, neckless* (*nls* or *aldh1a2*$^{i26/i26}$) and *no fin* (*nof, aldh1a2*$^{u11/u11}$)(Begemann, Schilling et al. 2001; Grandel, Lun et al. 2002) in that all three mutants do not develop fin buds and have a swollen pericardium. We compare the endoderm phenotype of *aldh1a2*^{i26} and *aldh1a2*^{um22} mutant embryos to that of embryos treated with DEAB (a pan-Aldh inhibitor). Interestingly, endoderm markers are not uniformly lost in *aldh1a2* mutant embryos, but are lost in DEAB-treated embryos, suggesting residual Aldh activity in the mutants. We detect the presence of maternal *aldh1a2* transcripts and demonstrate that a morpholino targeting the *aldh1a2* translation start site copies the DEAB phenotype. We conclude that Aldh1a2 is the predominant Aldh enzyme acting in early pancreas development and that there is a significant role for maternally derived Aldh in this process.

Aldh activity is required for pancreas development

Disrupted RA signaling has broad effects such as shorter body length, curved body axis, lighter pigmentation, immobility, and a swollen pericardium. As a result, many developmental defects are observed, including neural crest cell

death, the absence of limb buds and posterior branchial arches, small somites, and hindbrain segmentation defects, which have been known in general as VAD (vitamin A-deficiency syndrome) (Maden, Gale et al. 1996; Morriss-Kay and Sokolova 1996; Dickman, Thaller et al. 1997). In mouse, a null mutation in the *Aldh1a2* gene mimics the hindbrain phenotypes associated with full VAD, establishing *Aldh1a2* as the main RA producing enzyme required in hindbrain development (Niederreither, McCaffery et al. 1997; Berggren, McCaffery et al. 1999; Niederreither, Subbarayan et al. 1999; Niederreither, Vermot et al. 2000). As a result of losing RA, rhombomeric and gene expression boundaries posterior to rhombomere (r) 3 are lost (Blumberg, Bolado et al. 1997; Dickman, Thaller et al. 1997; Kolm, Apekin et al. 1997; van der Wees, Schilthuis et al. 1998; White, Shankar et al. 1998; Dupe, Ghyselinck et al. 1999; White, Highland et al. 2000). In zebrafish embryos that are treated with DEAB to block Aldh activity, defects in anterior-posterior patterning of the neural tube also resemble severe VAD cases. The neural tube is strongly anteriorized and hindbrain development posterior to r4 is stopped. Also, loss of fin buds and reduction of branchial arches are observed (Costaridis, Horton et al. 1996; Begemann, Schilling et al. 2001; Grandel, Lun et al. 2002). This indicates a conserved role for Aldh enzymes in the production of RA required for hindbrain development in both zebrafish and mice.

RA is also involved in endoderm development in vertebrates. In mice, *Aldh1a2* is expressed in the dorsal pancreatic mesenchyme during pancreas

specification and RA-responding cells reside in both pancreatic endoderm and mesenchyme (Martin, Gallego-Llamas et al. 2005). As a result, defects in the endoderm are observed in the absence of RA. In particular, *Aldh1a2*$^{-/-}$ mice lack *Pdx1* and *Prox1* expression in the dorsal pancreatic bud but the ventral bud appears normal (Martin, Gallego-Llamas et al. 2005; Molotkov, Molotkova et al. 2005). Accordingly, *Insulin* and *Glucagon*-expressing cells do not develop and *Isl1* expression is severely decreased (Martin, Gallego-Llamas et al. 2005). *Hlxb9*, expressed in the dorsal foregut endoderm, is also reduced (Martin, Gallego-Llamas et al. 2005). Expression of *Foxa2* in the dorsoventral axis of the endoderm is not affected, indicating that early endoderm development is unaltered (Molotkov, Molotkova et al. 2005). *Hhex* expression is not affected in the liver, suggesting that RA is not involved in liver development – similar to observations in *Xenopus* and avian embryos (Kumar, Jordan et al. 2003; Chen, Pan et al. 2004; Molotkov, Molotkova et al. 2005). Treating *Xenopus* embryos with a RA receptor antagonist (BMS493) blocks dorsal pancreatic development, but does not affect ventral pancreatic development or the liver (Chen, Pan et al. 2004). Similarly, in RA-deficient avian embryos or VAD (obtained from birds fed on a retinoid-deficient defined diet (Gale, Zile et al. 1999)), dorsal pancreas is lost but not ventral pancreas or liver (Maden, Gale et al. 1996; Gale, Zile et al. 1999; Stafford, Hornbruch et al. 2004). Since *Xenopus* embryos treated with BMS493, VAD quail embryos and *Aldh1a2*$^{-/-}$ mutant mice display a similar phenotype - loss of dorsal pancreas but not ventral pancreas or liver – it appears

that Aldh1a2 is the only Aldh acting in endoderm and that it is only necessary for dorsal pancreas development in these species. In contrast, blocking RA completely in zebrafish embryos eliminates all pancreas and liver gene expression. Embryos treated with DEAB lose *vhnf1* expression in the pancreas, *insulin*:GFP expression in the endocrine pancreas, *foxa3* expression in the pancreas and liver, and pharyngeal arches 3-5 are lost as well (Stafford and Prince 2002; Kopinke, Sasine et al. 2006; Song, Kim et al. 2007; Wingert, Selleck et al. 2007; Kinkel, Sefton et al. 2009). We treated embryos with 10 uM DEAB at 8 hpf and found that various endoderm markers expressed in the pancreas, liver, and intestine are lost, similar to embryos treated with BMS493 (pan-RAR antagonist) (Stafford and Prince 2002). Also, injecting *aldh1a2* translational MO (tMO) knocks down *insulin* expression (Stafford, White et al. 2006) and we find that *aldh1a2* tMO knocks down expression of genes such as *hhex* (liver and pancreas), *prox1* (liver and pancreas), and *pdx1* (pancreas and duodenum) as well (Figure 4.7).

Thus, there appears to be a conserved role for RA in pancreatic development among vertebrates, but mouse, *Xenopus* and avian embryos have restricted RA's role to the dorsal pancreas. The liver and ventral pancreas emerge adjacent to one another from the ventral endoderm in a default state as pancreas, but the liver receives signals from the cardiac mesoderm (FGF) to express liver markers (Deutsch, Jung et al. 2001). Interestingly, the markers that continue to be expressed in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant zebrafish

embryos are those expressed in the ventral pancreas and liver (*hhex, prox1*, *sid4*, *carbA,* and *transf*), indicating that less RA is needed to turn on expression of these genes, possibly consistent with an evolutionary phasing out of RA's involvement in these regions. Therefore, RA's role in ventral pancreas and liver development does not appear evolutionarily conserved among vertebrates. Other signaling factors may have taken precedence over RA in development of these regions in mouse. For instance, BMP and FGF signaling is necessary for liver development in mouse embryos, but inhibiting FGF and BMP signaling in zebrafish embryos leads to a decrease, not a loss, of *hhex* and *prox1* expression (Gualdi, Bossard et al. 1996; Rossi, Dunn et al. 2001; Calmont, Wandzioch et al. 2006; Shin, Shin et al. 2007).

Lastly, treatment with DEAB does not affect early endoderm gene expression in zebrafish embryos (*sox17*) or mutant mouse embryos (*FoxA2*) (Molotkov, Molotkova et al. 2005), indicating a conserved role that RA is not necessary for early endoderm development in vertebrates.

The *aldh1a2*^{i26}, *aldh1a2*^{u11} and *aldh1a2*^{um22} alleles likely represent null mutations

The zebrafish *aldh1a2* mutant alleles exhibit defects in patterning of the neural tube and the endoderm, although the phenotype is not as severe as in DEAB-treated zebrafish embryos (Figure 4.3, 4.4)(Begemann, Schilling et al. 2001; Grandel, Lun et al. 2002), *Aldh1a2*$^{-/-}$ mutant mice or VAD quail and rat embryos

(Begemann, Schilling et al. 2001; Grandel, Lun et al. 2002). Instead, it is similar to the phenotype we observe upon treatment with a low concentration of DEAB (Figure 4.6), as well as to a mild version of VAD seen in rat embryos and to partial rescue of *Aldh1a2*$^{-/-}$ mouse embryos by maternal application of RA (Niederreither, Vermot et al. 2000; White, Highland et al. 2000; Begemann, Marx et al. 2004). Since Aldh activity appears absolutely required for pancreas formation (because DEAB-treated embryos lack endoderm gene expression, see above), the weaker phenotype of *aldh1a2* mutant zebrafish embryos could be explained if the *aldh1a2*^{i26}, *aldh1a2*^{u11} and *aldh1a2*^{um22} alleles represent hypomorphic mutations that maintain some residual Aldh activity.

However, the mutations occurring in the *aldh1a2*^{i26}, *aldh1a2*^{u11} and *aldh1a2*^{um22} alleles appear likely to be null mutations. In each case, the mutated residue is conserved across human, mouse, rat, *Xenopus*, and zebrafish (Begemann, Schilling et al. 2001; Grandel, Lun et al. 2002), indicating that amino acid sequence is important for the overall function of the Aldh1a2 protein and changing it will most likely affect the protein function. Furthermore, in each case, the mutation introduces a large charged residue (Gly -> Arg in *aldh1a2*^{i26}, Thr -> Lys in *aldh1a2*^{u11}, Gly -> Arg in *aldh1a2*^{um22}). Such replacements are likely to affect the proper folding of the protein and therefore affect the catalytic function of Aldh1a2.

Further support for the idea that *aldh1a2*^{i26}, *aldh1a2*^{um22} and *aldh1a2*^{u11} represent null mutations comes from rescue experiments, which indicate that the

mutant proteins are not functional. When we injected *aldh1a2*^{um22} embryos with mRNA containing the *aldh1a2*^{um22} mutation, it could not rescue fin bud development (Figure 4.2 and Table 4.1). However, when we injected wild type *aldh1a2* mRNA, we were able to rescue fin bud development. The same was seen in rescue experiments using both *aldh1a2*^{i26} and *aldh1a2*^{u11} (Begemann, Schilling et al. 2001; Grandel, Lun et al. 2002). Furthermore, overexpression of the *aldh1a2*^{um22} mutant mRNA in zebrafish embryos did not affect development (not shown), further demonstrating that the *aldh1a2*^{um22} allele is inactive. Together, this indicates that the *aldh1a2*^{i26}, *aldh1a2*^{um22} and *aldh1a2*^{u11} mutations do not result in hypomorphic proteins, but represent null mutations.

A role for maternal *aldh1a2* mRNA

If the *aldh1a2*^{i26}, *aldh1a2*^{u11} and *aldh1a2*^{um22} alleles encode inactive Aldh1a2, the fact that *aldh1a2* mutant zebrafish embryos do not display a severe VAD phenotype suggest that Aldh activity must be coming from another source. The expression pattern of other *aldhs* rules them out as likely candidates and we therefore focused on maternal *aldh1a2* mRNA. We find that *aldh1a2* is expressed already at 3hpf, albeit at somewhat lower levels – this lower level may explain the weak phenotype observed in the mutants. We also find that a MO targeting the exon 1/intron 1 splice site of *aldh1a2* (sMO, which should target only zygotic transcripts) produces a milder phenotype (not shown) and that lower doses of *aldh1a2* tMO (500uM) permit some expression of *pdx1*, similar to the

phenotype observed in *aldh1a2*^{um22} and *aldh1a2*^{i26} mutant embryos). We also note that after treating with DEAB, it became clear that *aldh1a2*^{i26} embryos still have some RA activity since DEAB treated embryos display a severe phenotype similar to VAD (Costaridis, Horton et al. 1996; Grandel, Lun et al. 2002; Begemann, Marx et al. 2004). This residual *aldh1a2* is most likely due to maternally supplied mRNA.

In contrast, Aldh enzymes do not appear to be deposited maternally in other vertebrates. In particular, the fact that *aldh1a2* mutations in mice mimic the VAD phenotype (Maden, Gale et al. 1996; Morriss-Kay and Sokolova 1996; Dickman, Thaller et al. 1997), suggests that there are no maternally contributed Aldhs in the mouse. In *Xenopus*, both retinol and retinaldehyde are present in embryos before gastrulation, indicating that RDHs may be present (possibly maternally deposited) (Creech Kraft, Schuh et al. 1994; Costaridis, Horton et al. 1996). Furthermore, microinjection of *Aldh1* or *Aldh1a* induces premature RA signaling in *Xenopus* (Ang and Duester 1999) by acting on this retinaldehyde pool, suggesting that maternally deposited Aldhs are not present in the *Xenopus* embryo (Ang and Duester 1999; Niederreither, Subbarayan et al. 1999; Begemann, Schilling et al. 2001; Chen, Pollet et al. 2001). It is not clear why *aldh1a2* is maternally deposited in zebrafish, but our observation that treatment with DEAB before gastrulation results in death or severely deformed embryos (not shown) suggests that there may be an early role for *aldh1a2* in zebrafish embryos.

Materials and Methods

Fish Maintenance

Ekkwill (EK), Tupfel long fin (*TL*) and *neckless (aldh1a2*^{i26}) (Gift from Prince Lab) embryo*s* were collected from natural matings and reared in 1/3 Ringer's. Embryos were staged using morphological criteria up to 24 hours post fertilization (hpf) and then by time of development at 28.5°C (Kimmel, Ballard et al. 1995).

ENU Screen

EK males were treated with 3 mM ENU (*N*-ethyl-*N*-nitrosourea) once a week for 3 weeks. The males were then crossed repeatedly to clean out any post meiotic germ cells that were mutagenized. Mutagenized males were then crossed to EK females and the progeny (F1) were raised. Haploid embryos were produced by In Vitro Fertilization (IVF) of F1 female progeny with irradiated sperm. Haploid embryos were raised to approximately 30 hpf and fixed in 4% paraformaldehyde for in situ hybridization with *islet1 (isl1)* probe. Embryos were screened based on *isl1* expression. F1 females that produced embryos with mutant phenotypes were out-crossed to TL males and the progeny (F2) were raised and in-crossed for recovery of mutation in diploid embryos.

Mapping, DNA extraction, RNA extraction and cDNA synthesis

Mutant carriers were in-crossed and progeny raised to 4 dpf. Embryos were sorted based on their phenotype; mutants develop a swollen pericardium and lack fin buds. Genomic DNA was extracted from phenotypically mutant and phenotypically wild type embryos at day 4. DNA pools were created from phenotypically mutant and wild type embryos. Bulk segregant analysis was performed on the DNA pools using a 192 CA marker panel (Knapik, Goodman et al. 1998; Roman, Pham et al. 2002; Lawson, Mugford et al. 2003). Two markers were found to be linked to the mutation: z10441 (FW:GCATTCAGATTCTGGGGTGT, RV: CGGATGAACCCATCAATCTC) and z8693 (FW: GCTTTTTGAGCAGATGAGGC, RV: CATGTACGCGTTGACTTTGC). PCR was performed on individual embryos using the same primers. cDNA was synthesized from RNA extracted from pools of 10 phenotypically mutant and 10 phenotypically wild type embryos using Invitrogen Superscript III Reverse Transcriptase Kit. PCR primers, FW: CCAAAGTTGTAATCGCACATC, RV:TTTTTTTTTTTTTTTCAGAGGTAAAAC, were used to clone full-length *aldh1a2* cDNA. Stratagene Hi Fi taq polymerase was used in the PCR and the product was sequenced. Primers FW: AGCGGCCGTCTTCCCAGAGATATC and RV: GGAATGGGTGTAGGCAGTTAATGGTGG were used to sequence *aldh1a2* from individual embryos.

mRNA and morpholino injections

An antisense morpholino oligo (MO) designed to block translation of the *aldh1a2* mRNA (tMO) 5'GCAGTTCAACTTCACTGGAGGTCAT3' (Begemann, Schilling et al. 2001) and one control mismatch morpholino (mmMO 5'GCAcTTgAACTTCAgTGGAcGTgAT3' that has five mismatches relative to tMO) were obtained from Gene Tools. 1nl of 100 uM, 250 uM, 500 uM, 750 uM and 950 uM of tMO was injected at the 1-2 cell stage. A splice MO (sMO) designed to exon1/intron1 splice junctions: 5'TTGAAAAAGTCCGACAAACCTTGGT3' and one control morpholino (mmMO: 5'TTcAAAAAcTCgGACAAtCCTTcGT3' with five mismatches relative to sMO) was obtained from Gene tools. 1 nl of 500 uM, 750 uM, and 950 uM of sMO was injected at the 1-2 cell stage.

For rescue experiments, the *aldh1a2* ORF was amplified from TL embryos or *aldh1a2*^{um22} mutant embryos using FW: ATGACCTCCAGTGAAGTTGAACTGCCA and RV:TTAAGACGTCTTGCTTCATCGTAATGGTTTTCA. Both ORFs were cloned with Invitrogen Topo TA Cloning kit, digested using EcoR1 and cloned into PCS2+. Constructs were linearized with Not1 and Ambion Kit Sp6 was used to make mRNA. 500 pg of mRNA was injected into an *aldh1a2*^{um22} in-cross at 1-2 cell stage. mRNA and MO injected embryos were fixed in 4% paraformaldehyde at various developmental stages for in situ hybridization.

DEAB Treatment

A 1 mM stock of DEAB was dissolved in DMSO. Embryos were treated in the dark with 1uM, 5 uM and 10 uM of DEAB dissolved in 1X PTU at 6, 8, 10, or 12 hpf. Embryos were fixed at various stages and assayed by in situ hybridization. Control embryos were treated in DMSO under similar conditions.

In situ hybridization

Antisense digoxigenin- and fluorescein-labeled probes were produced by standard methods. The *krx20*, *myosin heavy chain* (*mhc*), *insulin, sid4, carbA*, *pdx1, isl1, transferrin, p48, somatostatin, ifabp (intestinal fatty acid binding protein)* and *shh* probes used were described previously (Sagerstrom, Grinbalt et al. 1996; Sagerstrom, Kao et al. 2001; diIorio, Runko et al. 2005). Full-length *prox1* was obtained from Open Biosystems, One- and two-color in situ hybridization was carried out as described previously (Sagerstrom, Grinbalt et al. 1996; Sagerstrom, Kao et al. 2001).

RT-PCR

RT-PCR was performed using a Qiagen PCR Kit (Cat. No 204054) and cDNA synthesized from wild type embryos at 3 and 6 hpf. RNA was extracted from 10 wild type embryos at 3 hpf or 6 hpf and cDNA was synthesized using Invitrogen Superscript III Reverse Transcriptase Kit. The following primers were used to obtain PCR product: BActin FW: ATACACAGCCATGGATGAGGAATTCC and

RV: GGTCGTCCAACAATGGAGGGGAAAA, Tubulin 1 FW:

AAGAGATGACGCAGTCTGTCGTAGTC and RV:

AGAAGCTCGTCAGCGCGTCATCATAA, Odc-1 FW:

TTTGACTTCGCCTTCCTGGAGGAGGG and RV:

CCCCAGATCCGCCACATAGAAGGCAT, *aldh1a2*$^{1\text{-}2}$ FW:

ATGACCTCCAGTGAAGTTGAACTGC and RV:

CTTGTCGGATTCCTGGACATCACAG, and *aldh1a2*$^{10\text{-}11}$ FW:

GCAAAGCTCCTCCTACTAAAGGCTTCTTC and RV:

TTCTGTGTTGTTGGCTCTCTCAATCACT.

CHAPTER V

EARLY ENDODERM MUTANT IN ZEBRAFISH

Abstract

From an ENU screen, we recovered a mutation, *kiribati*, which spontaneously arose in the genome background of the Ekwill line we were mutagenizing. Mutant embryos exhibit a phenotype by 48hpf, where they become severely curved and shortened. Upon closer examination, mutant embryos have reduced *sox32* expression and lack *sox17* expression at 6 hours post fertilization (hpf). Furthermore, they do not express pancreas, liver, or intestinal markers at 24, 48 and 72 hpf and are embryonic lethal by 96 hpf. In addition to the endoderm defects observed, embryos display cardiac bifida at 24 hpf and other mesoderm and ectoderm markers are affected by 48 hpf, in response not endoderm formation. From rescue experiments, we determined that *sox32* mRNA is unable to rescue the endoderm phenotype but injecting *sox17* mRNA does rescue some endoderm markers. Also, injecting *pou5f1* mRNA, which acts with Sox32 to regulate *sox32* and *sox17* expression, partially rescues endoderm markers suggesting the mutation lies between *sox32* and *sox17*. Currently, we are mapping the mutation using CA repeats (discussed in Chapter III).

Background

During gastrulation, the three primary germ layers, endoderm, mesoderm, and ectoderm are established. Gastrulation movements internalize the mesoderm and endoderm cells, bringing them to their appropriate location within the embryo. Endodermal precursors have the ability to differentiate into a wide variety of cell types that will develop into tissues and organs of the digestive system. First, the endoderm is regionalized into specific areas and then further differentiation and organogenesis occurs, completing the two-step process. For example, initial regionalization of the foregut occurs then the liver and pancreas develop as buds from the gut and further differentiate into functional organs (Edlund 2002; Field, Dong et al. 2003; Kinkel and Prince 2009). Overall, an appropriate combination of regionalization, cell differentiation, and morphogenesis must happen to produce organs that function properly in metabolism and digestion (Kinkel and Prince 2009).

Many factors are necessary for endoderm formation and epistasis experiments have established the order in which they function (Alexander and Stainier 1999; Kikuchi, Trinh et al. 2000; Dickmeis, Mourrain et al. 2001; Reiter, Kikuchi et al. 2001; Aoki, David et al. 2002; Poulain and Lepage 2002). The endoderm specification pathway is initiated by Nodal signaling. In particular, *nodal related protein 1* and *nodal related protein 2* (*ndr1* and *ndr2* also called *squint* and *cyclops*) encode two Nodal ligands and are expressed in the vegetal marginal region of the blastoderm where the endoderm precursors are located

(Erter, Solnica-Krezel et al. 1998; Feldman, Gates et al. 1998; Rebagliati, Toyama et al. 1998; Sampath, Rubinstein et al. 1998). *ndr1* and *ndr2* single mutants still develop endoderm, but *ndr1;ndr2* double mutants lack all endoderm and develop very little mesoderm indicating a functional redundancy between the two genes. Ndr1 and Ndr2 bind TARAM-A, a zebrafish type I TGF-β receptor (Renucci, Lemarchandel et al. 1996; Peyrieras, Strahle et al. 1998; Aoki, Mathieu et al. 2002). Another factor necessary for Nodal signaling is One-eyed pinhead (Oep), a transmembrane protein that acts as a co-receptor for Nodal ligands (Zhang, Talbot et al. 1998). Embryos mutant for maternal and zygotic *oep* (MZ*oep*) lack all endoderm (Gritsman, Zhang et al. 1999) confirming Nodal signaling importance in initiating endoderm formation.

Zygotic endoderm specific transcription factors are induced by Nodal signaling and act as key regulators in endoderm development. These include Mix-like homeobox protein, Bon (Bonnie and Clyde); the zinc finger-containing factor, Gata5; the paired-like homeobox protein, Mezzo (Og9x, *mez*), which is related to the Mix-like/Mixer homeoproteins; the Sox-related protein Casanova (Sox32); the Sox-related protein Sox17; and the POU class 5 protein, Pou5f1(Alexander, Rothenberg et al. 1999; Alexander and Stainier 1999; Kikuchi, Trinh et al. 2000; Dickmeis, Mourrain et al. 2001; Kikuchi, Agathon et al. 2001; Schier 2003; Reim, Mizoguchi et al. 2004). A mutation in any of these genes results in endodermal defects (Poulain and Lepage 2002). Overall, *sox32* expression depends on Gata5, Bon, Eomesodermin (Eomes) and Mez (Reiter,

Kikuchi et al. 2001) and is upstream of *sox17*. *gata5* is expressed in the endodermal progenitors from the late blastula stages on and mutant embryos express lower levels of *sox17.* Both Gata5 and Bon work with Eomes and are required for *sox32* expression (Reiter, Kikuchi et al. 2001). Eomes is a maternal T-box protein that interacts with both Gata5 and Bon to form a trimeric complex that regulates *sox32* expression by binding the *sox32* promoter (Bjornson, Griffin et al. 2005). Lastly, Mez function in parallel to Bon, Eomes and Gata5, but does not directly interact with them, to regulate *sox32* expression. Then, Sox32 works synergistically with Pou5f1 to bind the *sox17* promoter and activate expression as well as regulate *sox32* expression (Chan, Chao et al. 2009). Overall, a complex transcriptional network operates during endoderm formation.

Results

An ENU screen for endocrine pancreas mutants

A haploid in situ hybridization screen of ENU (*N*-ethyl-*N*-nitrosourea) mutagenized zebrafish was carried out to identify mutations in endocrine pancreas development. Ekkwill (EK) males were treated with 3 mM ENU for 1 hour for three weeks and crossed to EK females. F1 progeny were raised and eggs from F1 females were in vitro fertilized using irradiated sperm from EK males. The resulting haploid embryos were raised until 30 hpf and assayed by in situ hybridization for *islet1* (*isl1*) expression to detect defects in the endocrine pancreas. F1 females that produced clutches with 50% embryos exhibiting a mutant phenotype were outcrossed to Tupfel long fin (*TL*) males. F2 progeny were raised and screened for recovery of the mutation in the F3 generation. We screened 375 genomes and discovered fifty females with defective endocrine pancreas formation. Upon re-examination, a spontaneous mutation (*kiribati*) in the original EK (wildtype line) line treated with ENU was discovered to affect endocrine pancreas formation. Specifically, *kiribati* embryos do not express *isl1* in the endoderm but maintain expression in the central nervous system (CNS)(Figure 5.1B). *kiribati* embryos develop a phenotype by 48 hpf; the embryos become severly curved and a swollen pericardum results (Figure 5.2B) and this phenotype worsens by 72 hpf (Figure 5.2D). Also, the mutation is embryonic lethal by 96 hpf.

Figure 5.1 – A spontaneous mutation affects *isl1* expression in the endoderm

A haploid ENU screen resulted in embryos that lack *isl1* expression in the endoderm. (A,B) (A) Wildtype *isl1* expression in the central nervous system (CNS) and endoderm (endocrine pancreas-black arrow and hepatopancreatic duct). Mutant embryos (B) lack *isl1* expression in the endoderm but maintain expression in the CNS. The numbers on bottom right of panels represents the number of mutant embryos per total count.

Figure 5.1

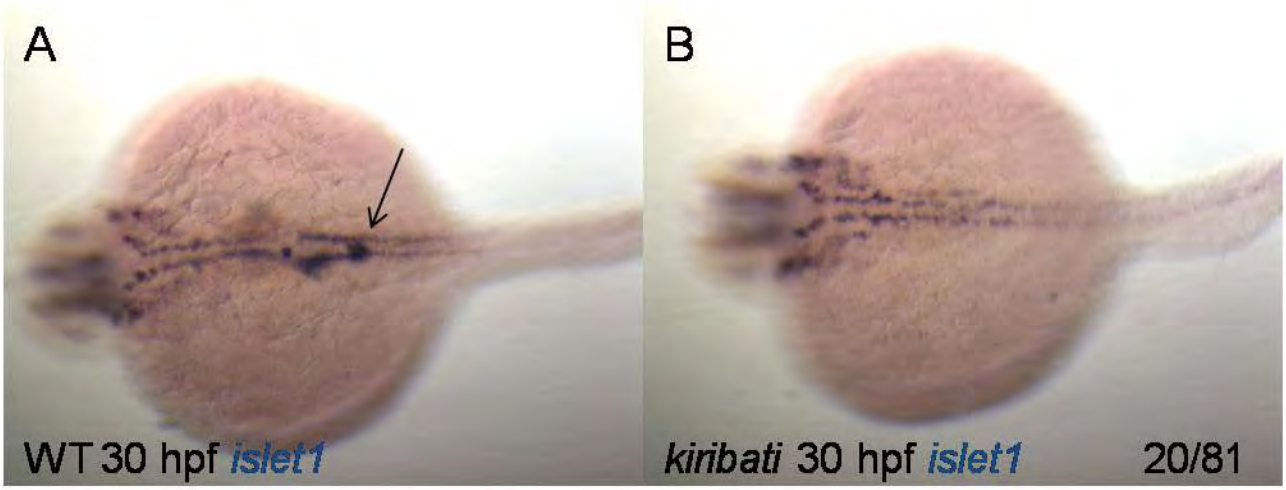
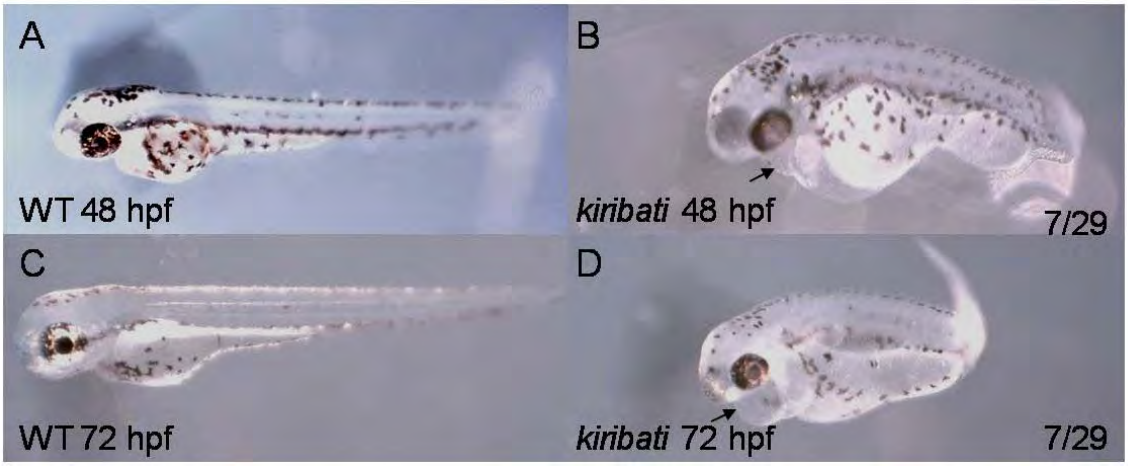


Figure 5.2 – *kiribati* embryos exhibit a phenotype by 48 hpf

Live images of wildtype and *kiribati* mutant embryos at 48 hpf (A,B) and 72 hpf (C,D). Embryos become severely curved and a swollen pericardium (black arrow) develops when compared to wildtype. The numbers on bottom right of panels represents the number of mutant embryos per total count.

Figure 5.2


A
WT 48 hpf
B
kiribati 48 hpf
7/29
C
WT 72 hpf
D
kiribati 72 hpf
7/29

kiribati mutant embryos do not express endodermal markers

Since *kiribati* mutants do not express *isl1* in the pancreas, we analyzed other pancreatic markers to determine if they were expressed. *pdx1* (expressed in the endocrine and exocrine pancreas and the duodenum) is the first pancreatic marker to be expressed during embryogenesis. At both 24 and 48 hpf, *kiribati* mutant embryos do not express *pdx1* (Figure 5.3D, 5.4F). We next analyzed endocrine and exocrine pancreas genes. *insulin* (*ins*) is not expressed at 24, 48 or 72 hpf (Figure 5.3D, 5.4D, 5.5D) nor is *somatostatin* (*sst*) at 30 hpf (Figure 5.3L) in the endocrine pancreas and *p48* (30 hpf, Figure 5.3J), *carboxypeptidase a* (*carb a)* or *amylase* (72 hpf, Figure 5.5B,H) are not expressed in the exocrine pancreas. Since *pdx1* is also expressed in the duodenum, we assayed *intestinal fatty acid binding protein* (*ifabp*) in mutant embryos at 72 hpf and was unable to detect expression (Figure 5.5J). Thus, *kiribati* mutant embryos appear to lack both a pancreas and intestine.

We next analyzed endodermal gene expression for other organs such as the liver and pharyngeal endoderm. Interestingly, *hhex* (Figure 5.3F) and *prox1* (Figure 5.3H) are not expressed in liver or the ventral pancreas. Similarly, *sid4* (Figure 5.4H) and *transferrin* (*transf*, Figure 5.5F) are not expressed in the liver at 48 and 72 hpf.

Figure 5.3 –Endodermal gene expression is lost in *kiribati* mutant embryos at 24 hpf and 30 hpf

kiribati mutant embryos do not express *insulin* (*ins,* endocrine pancreas) or *pdx1* (endocrine, exocrine pancreas and duodenum) at 24 hpf (B, D) compared to wildtype (A,C – black arrows). Mutant embryos lack *hhex* and *prox1* expression in the ventral pancreas and liver (black arrow)(F and H vs. E, G). Both *p48* (I, exocrine pancreas) and *somatostatin* (K, *sst*, endocrine pancreas) expression is abolished at 30 hpf but *p48* expression in the CNS remains (J). The numbers on bottom right of panels represents the number of mutant embryos per total count.

Figure 5.3

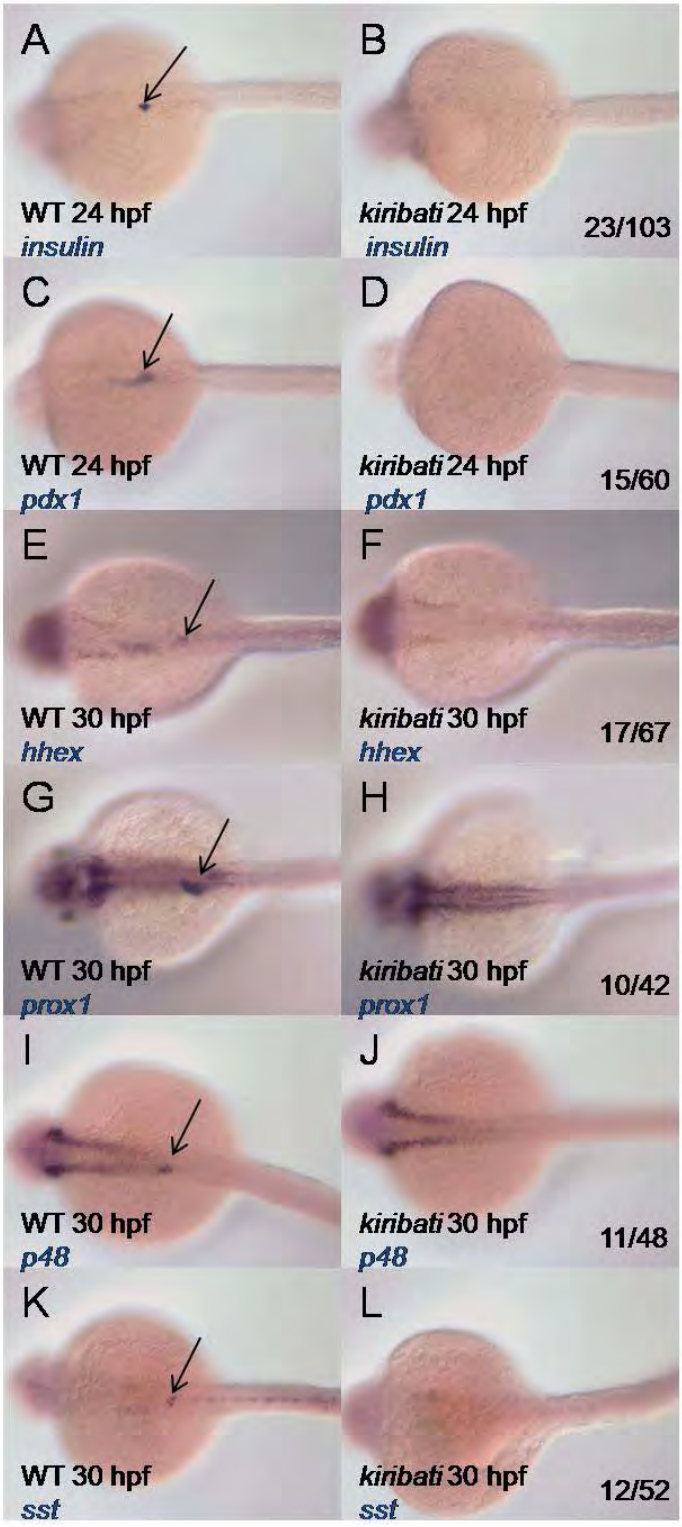

A
WT 24 hpf
insulin
B
kiribati 24 hpf
insulin
23/103
C
WT 24 hpf
pdx1
D
kiribati 24 hpf
pdx1
15/60
E
WT 30 hpf
hhex
F
kiribati 30 hpf
hhex
17/67
G
WT 30 hpf
prox1
H
kiribati 30 hpf
prox1
10/42
I
WT 30 hpf
p48
J
kiribati 30 hpf
p48
11/48
K
WT 30 hpf
sst
L
kiribati 30 hpf
sst
12/52

Figure 5.4 –Endodermal gene expression is lost in *kiribati* mutant embryos at 48 hpf

pdx1 (endocrine and exocrine pancreas, duodenum), *insulin* (*ins,* endocrine pancreas) and *sid4* (liver) expression are absent in *kiribati* mutant embryos (D, F, and H vs. C, E, G –black arrows) at 48 hpf. Mutant embryos lack *foxa2* and *shh* expression in the pharyngeal endoderm (B, J vs. A, I – black arrows) but maintained in the floor plate, notochord and fin buds. The numbers on bottom right of panels represents the number of mutant embryos per total count.

Figure 5.4

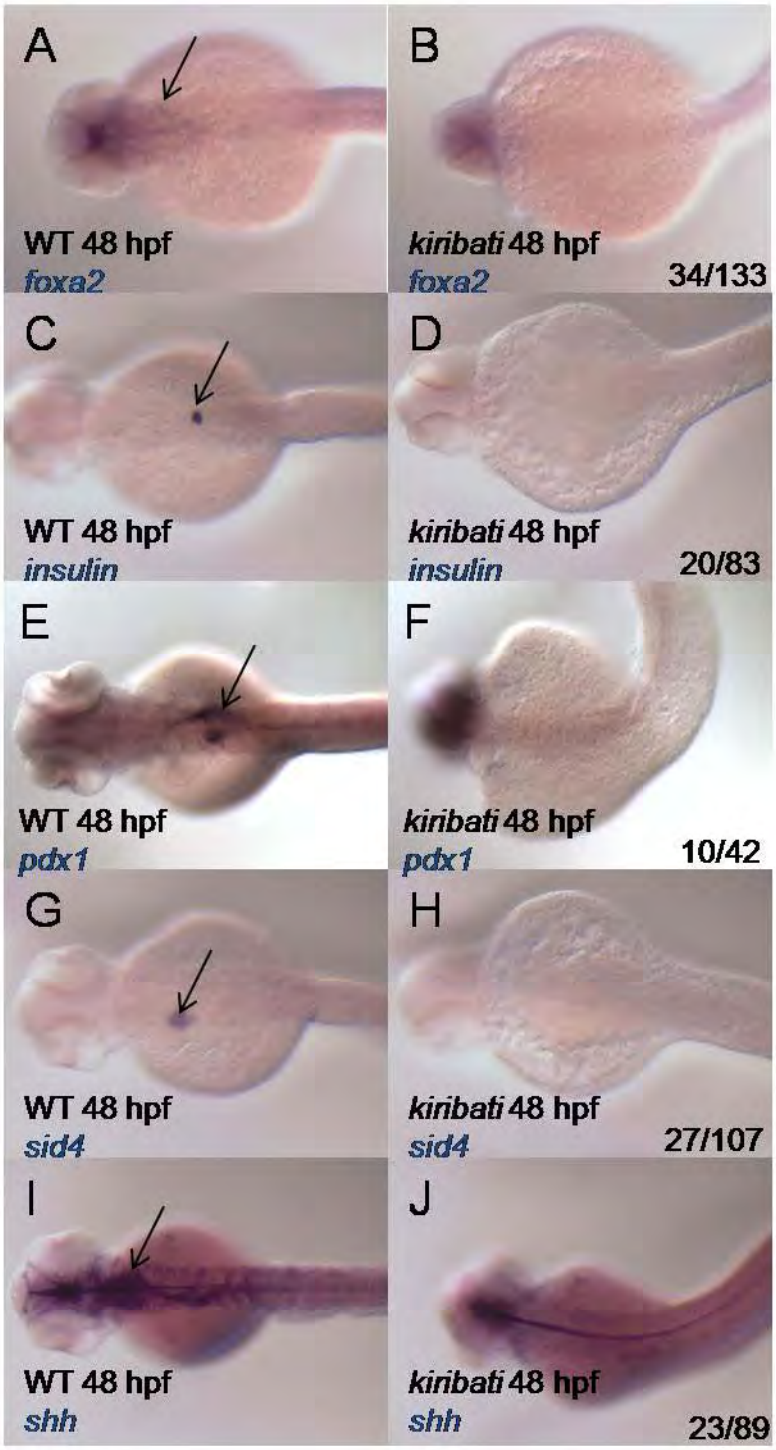
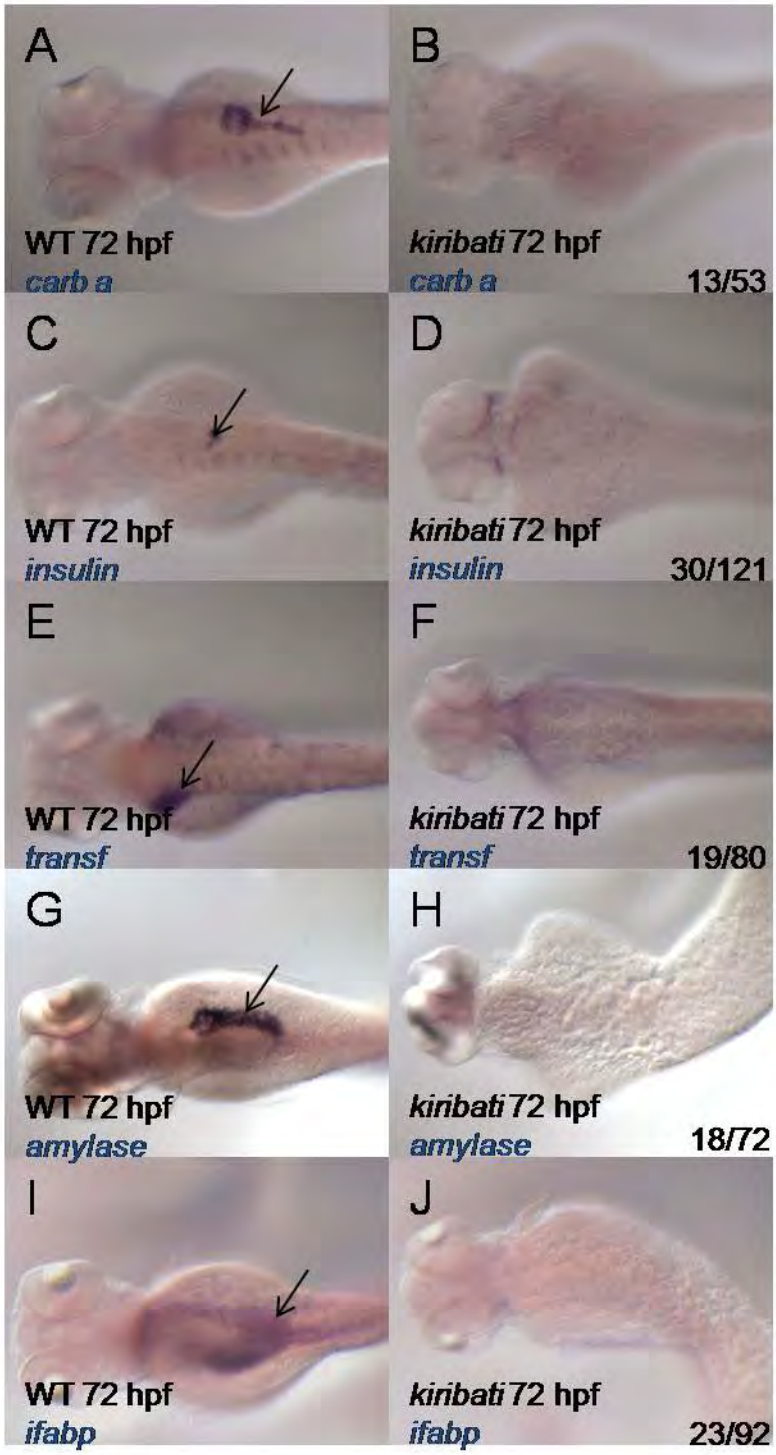

A
WT 48 hpf
foxa2
B
kiribati 48 hpf
foxa2
34/133
C
WT 48 hpf
insulin
D
kiribati 48 hpf
insulin
20/83
E
WT 48 hpf
pdx1
F
kiribati 48 hpf
pdx1
10/42
G
WT 48 hpf
sid4
H
kiribati 48 hpf
sid4
27/107
I
WT 48 hpf
shh
J
kiribati 48 hpf
shh
23/89

Figure 5.5 – Endodermal gene expression is lost in *kiribati* mutant embryos at 72 hpf

Exocrine pancreas markers, *carboxypeptidase a* (*carb a*) and *amylase* are not expressed in mutant embryos (B, H vs A, G – black arrows) at 72 hpf. Neither is *insulin* in the endocrine pancreas (D vs. C- black arrow). The liver marker *transferrin* (*transf*) is absent (F vs. E- black arrow), as well as *intestinal fatty acid binding protein* (*ifabp*) at 72 hpf (J vs. I - black arrow).

The numbers on bottom right of panels represents the number of mutant embryos per total count.

Figure 5.5


A
WT 72 hpf
carb a
B
kiribati 72 hpf
carb a
13/53
C
WT 72 hpf
insulin
D
kiribati 72 hpf
insulin
30/121
E
WT 72 hpf
transf
F
kiribati 72 hpf
transf
19/80
G
WT 72 hpf
amylase
H
kiribati 72 hpf
amylase
18/72
I
WT 72 hpf
ifabp
J
kiribati 72 hpf
ifabp
23/92

Both *foxa2* and *shh* are expressed in the pharyngeal endoderm as well as the notochord and floorplate and *shh* is also expressed in the fin bud primordium. *kiribati* mutant embryos maintain expression in the the notochord, floorplate and fin buds but lack pharyngeal endoderm expression, indicating that only the endoderm is lacking in these embryos (Figure 5.4J).

Hindbrain and somite markers are affected at 48 hpf in *kiribati* mutant embryos

kiribati mutant embryos do not develop differentiated endodermal organs. Since the embryos become deformed by 48 hpf, we next tested hindbrain and muscle markers to see if they are also affected in mutant embryos. At 24hpf, *krox20* (*krx20*) is expressed in rhombomeres (r) 3 and 5 (Figure 5.6A) and in *kiribati* mutant embryos, *krx20* expression persists but the distance between the two rhombomeres is shortened (Figure 5.6B, arrows). *hoxb4a* is expressed in the posterior hindbrain and notochord (Figure 5.7A) and *hoxb6b* is expressed in the notochord, CNS and mesoderm (Figure 5.7C). At 24 hpf expression of either gene is not affected in *kiribati* embryos but at 48 hpf, both gene expression patterns have shifted (Figure 5.7F, H vs. 5.7E, G). Specifically, *hoxb4a* expression has shifted posteriorly and *hoxb6b* expression has shifted anteriorly. At 48 hpf, *hoxc6a* is expressed in the notochord, mesoderm, and pancreas primordium (Figure 5.7I). Expression in the pancreas is abolished in mutant embryos (Figure 5.7J) while mesoderm expression is upregulated.

Figure 5.6 –*krx20* and *mhc* expression is no affected at 24 hpf but is at 48 hpf

The distance in *krox20* (*krx20*) expression in rhombomeres (r) 3 and 5 is shortened in mutant embryos (B vs A arrows). *mhc* expression in the somites is unaffected at 24 hpf (D vs. C) but gaps appear at 48 hpf (F, arrow). The numbers on bottom right of panels represents the number of mutant embryos per total count.

Figure 5.6

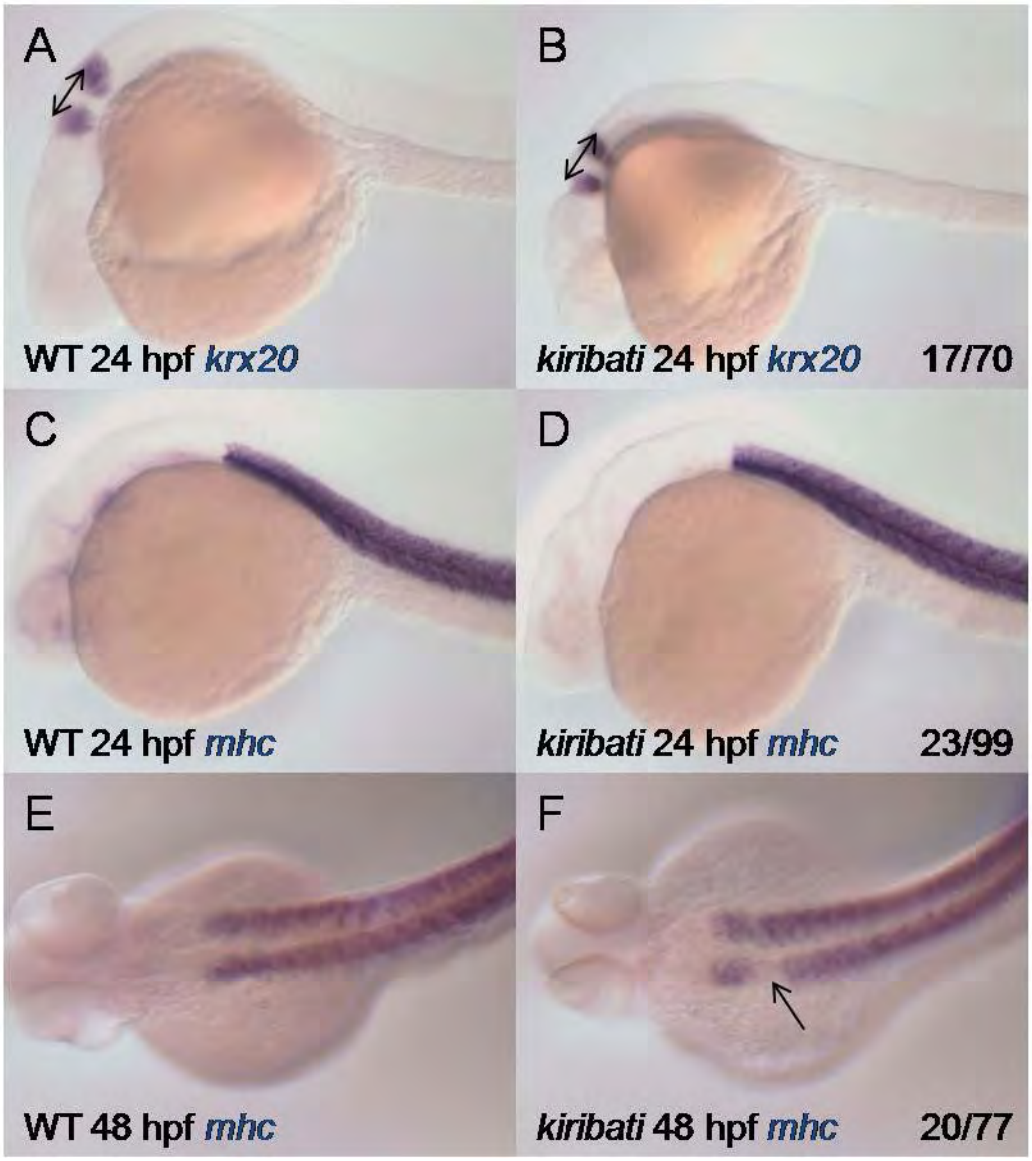
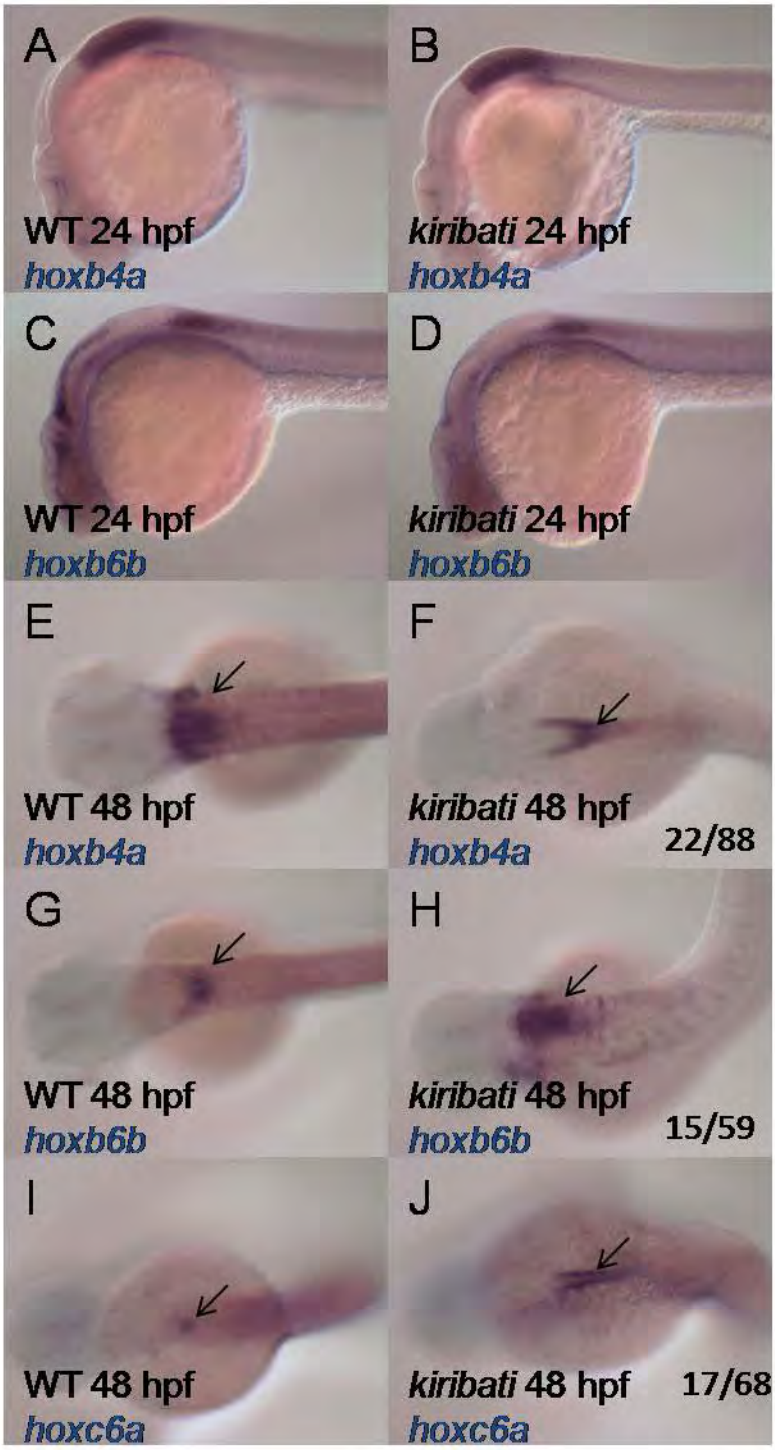

A
WT 24 hpf krx20
B
kiribati 24 hpf krx20
17/70
C
WT 24 hpf mhc
D
kiribati 24 hpf mhc
23/99
E
WT 48 hpf mhc
F
kiribati 48 hpf mhc
20/77

Figure 5.7 – *hox* gene expression is affected by 48 hpf

hoxb4a and *hoxb6b* expression is unaffected at 24 hpf (B,D vs. A,C). At 48 hpf, expression of both *hoxb4a* and *hoxb6b* has changed significantly (F, H) when compared to wildtype (E,G). *hoxb4a* expression has shifted posteriorly (E) and *hoxb6b* (G) has shifted anteriorly in mutant embryos (black arrow). *hoxc6a* expression in the pancreas is abolished in mutant embryos (J vs. I – black arrow) where mesoderm expression is upregulated (J). The numbers on bottom right of panels represents the number of mutant embryos per total count.

Figure 5.7


A
WT 24 hpf
hoxb4a
B
kiribati 24 hpf
hoxb4a
C
WT 24 hpf
hoxb6b
D
kiribati 24 hpf
hoxb6b
E
WT 48 hpf
hoxb4a
F
kiribati 48 hpf
hoxb4a
22/88
G
WT 48 hpf
hoxb6b
H
kiribati 48 hpf
hoxb6b
15/59
I
WT 48 hpf
hoxc6a
J
kiribati 48 hpf
hoxc6a
17/68

We also assayed *myosin heavy chain* (*mhc*) expression in the somites at both 24 and 48 hpf (Figure 5.6 C,E). Expression is unaffected at 24 hpf but gaps appear by 48 hpf which coincides with the embryo becoming severely curved (Figure 5.6 D, F). These results suggest that the endoderm defect occurs before the mesoderm and ectoderm defects since hindbrain and somite markers are not affected at 24 hpf (Figure 5.6 and 5.7).

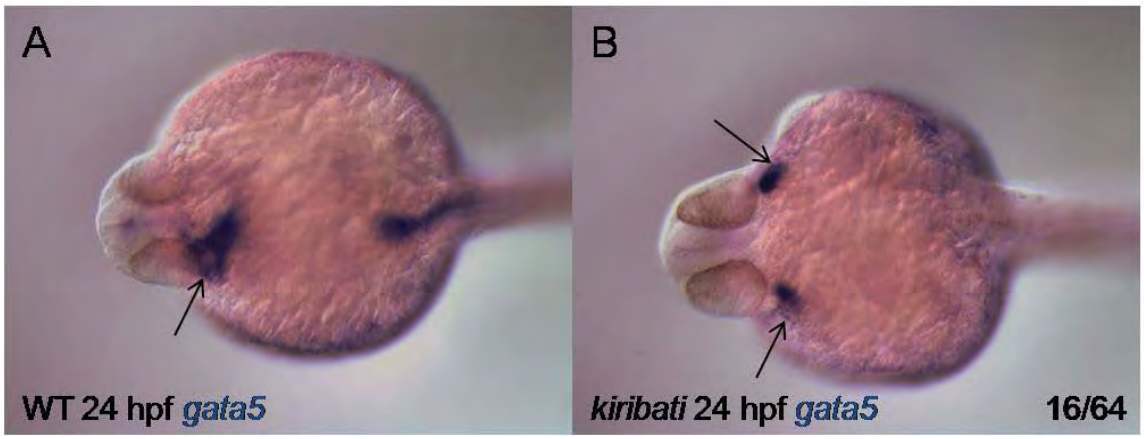
kiribati mutant embryos exhibit a cardia bifida phenotype at 24 hpf

hox gene expression appears normal at 24 hpf, however, early endoderm is necessary for proper heart migration and development in the mesoderm (Alexander, Rothenberg et al. 1999). Embryos that lack early endodermal precursors exhibit cardiac bifida, where the two heart primordia do not migrate properly to the left side of the embryo. As a result, two hearts develop on the right and left side of the embryo. Since *kiribati* embryos do not express endoderm markers at 24 hpf, we assayed *gata5* expression (expressed in the endoderm and heart) to detect if a cardiac bifida phenotype arises. Notably, *gata5* expression in the endoderm is lost and cardiac bifidia is evident by dual expression of *gata5* on either side of the mutant embryo (Figure 5.8B, black arrows).

Figure 5.8 – *kiribati* mutant embryos display a cardiac bifida phenotype

gata5 is expressed in both the endoderm and heart precursors (black arrow) at 24 hpf (A). Mutant embryos lack *gata5* expression in the endoderm and display a cardiac bifida phenotype (B, black arrows). The numbers on bottom right of panels represents the number of mutant embryos per total count.

Figure 5.8



sox32 expression is downregulated and *sox17* expression is abolished in *kiribati* mutant embryos

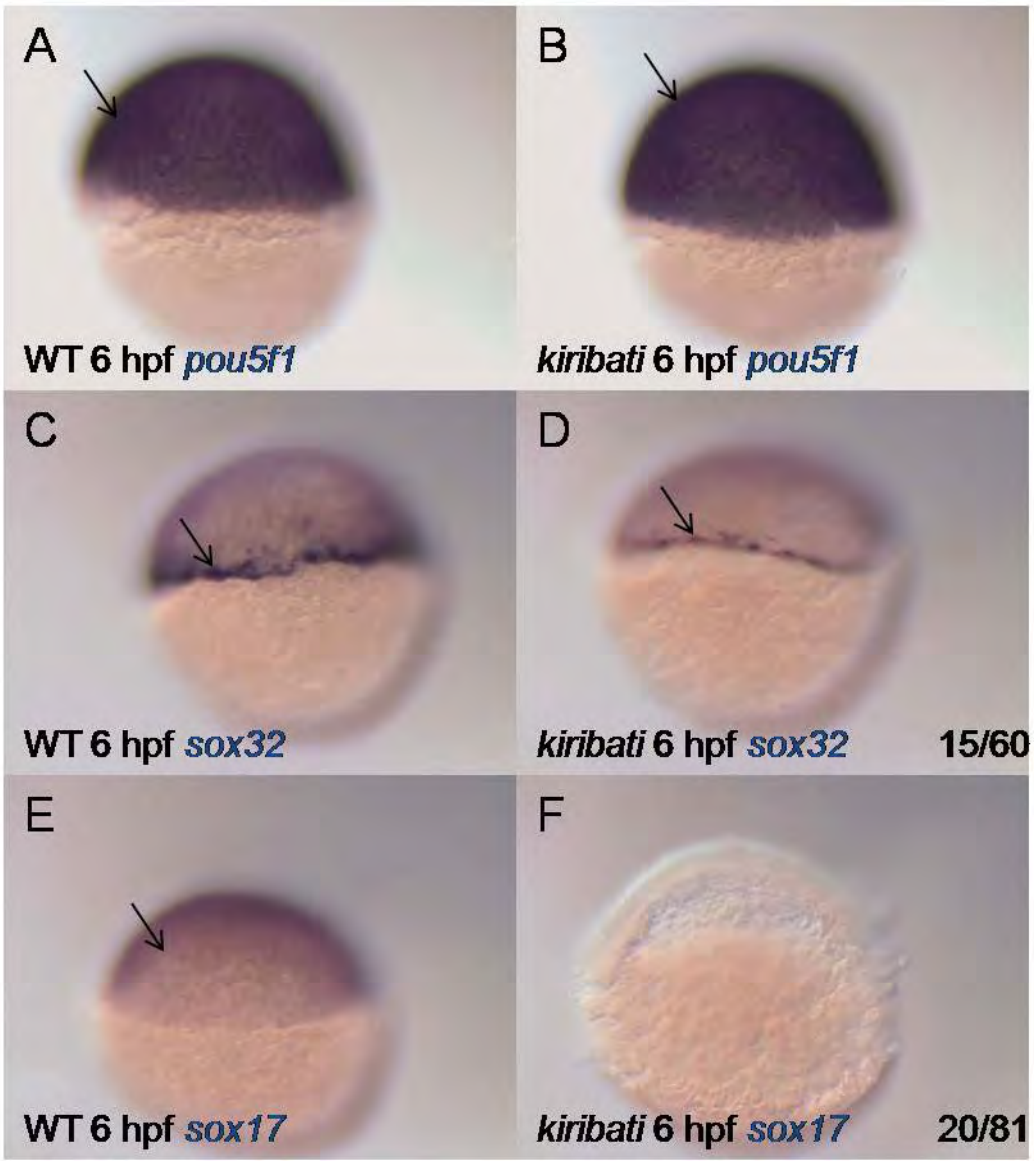
kiribati embryos do not develop endodermal organs, display cardiac bifida (similar to *sox32* mutants) and have a curved phenotype by 48 hpf, which affects mesoderm markers such as *mhc* and *hox* gene expression. Since, the cardiac and endoderm phenotype mimics *sox32* mutants; we analyzed early endodermal markers in mutant embryos to determine if early endoderm forms in mutant embryos. We discovered that *sox32* expression is downregulated in endodermal precursors in mutant embryos (Figure 5.9D vs. 5.9C) and *sox17* expression is completely abolished at 6 hpf (Figure 5.9F vs. 5.9E). Interestingly, even though some *sox32* expression is detected, endodermal organs do not form suggesting that *sox32* expression is not maintained. Next we assayed *pou5f1* expression since it acts in parallel to Sox32 to maintain *sox32* expression and induce *sox17* expression (Lunde, Belting et al. 2004; Chan, Chao et al. 2009). At 6 hpf, *pou5f1* expression is normal in *kiribati* mutant embryos (Figure 5.9B vs. 5.9A).

Figure 5.9 – *sox32* expression is downregulated and *sox17* expression is lost in *kiribati* mutant embryos

pou5f1 expression is normal in *kiribati* embryos at 6 hpf (A,B black arrow). However, *sox32* expression is downregulated in the endodermal precursors (D vs. C - black arrow) and embryos lack *sox17* expression at 6 hpf (F vs. E – black arrow).

The numbers on bottom right of panels represents the number of mutant embryos per total count.

Figure 5.9


A
WT 6 hpf pou5f1
B
kiribati 6 hpf pou5f1
C
WT 6 hpf sox32
D
kiribati 6 hpf sox32
15/60
E
WT 6 hpf sox17
F
kiribati 6 hpf sox17
20/81

sox17 mRNA but not _sox32_ mRna rescues early endodermal gene expression

Based on the expression patterns, we hypothesize that the mutation lies between *sox32* and *sox17*. First, we set out to determine if we can rescue the phenotype using either *sox32* or *sox17* mRNA and assayed for early endoderm markers. We injected either 100 pg or 200 pg of *sox17* mRNA at the one cell stage and analyzed *foxa2* expression at 6 hpf. Injecting 100 pg of *sox17* partially rescues *foxa2* expression in mutant embryos (only 7.6% embryos lack *foxa2* expression, Figure 5.10C-D), whereas injecting 200 pg resulted in 6% of embryos lacking *foxa2* expression (Figure 5.10F-H) when compared to the uninjected controls (24% embryos lack *foxa2*, 5.10A,B).

Next, we injected *sox32* mRNA or *sox32* and *sox17* mRNA into one cell at the 32 cell stage to target the mRNA to the endoderm and assayed for *pdx1* expression (discussed in Chapter II). *sox32* mRNA alone did not rescue *pdx1* expression (24% embryos lack *pdx1* similar to uninjected controls 25% Figure 5.11C,D vs. 5.11A,B). However, targeting *sox17* mRNA to the endoderm rescues *pdx1* expression in 100% of the embryos (Figure 5.11E,F).

Figure 5.10 – Injecting *sox17* at the one cell stage partially rescue *foxa2* expression

A heterozygous incross of *kiribati* fish results in 25% homozygous mutants that lack *foxa2* expression at 6 hpf represented in the uninjected controls (A,B). Injecting 100 pg of *sox17* mRNA at the one cell stage partially rescues *foxa2* expression at 6 hpf (7.6% lack *foxa2*, E). Injecting 200 pg of *sox17* mRNA resulted in a higher rescue rate (6.1% lack *foxa2*, H).

Figure 5.10

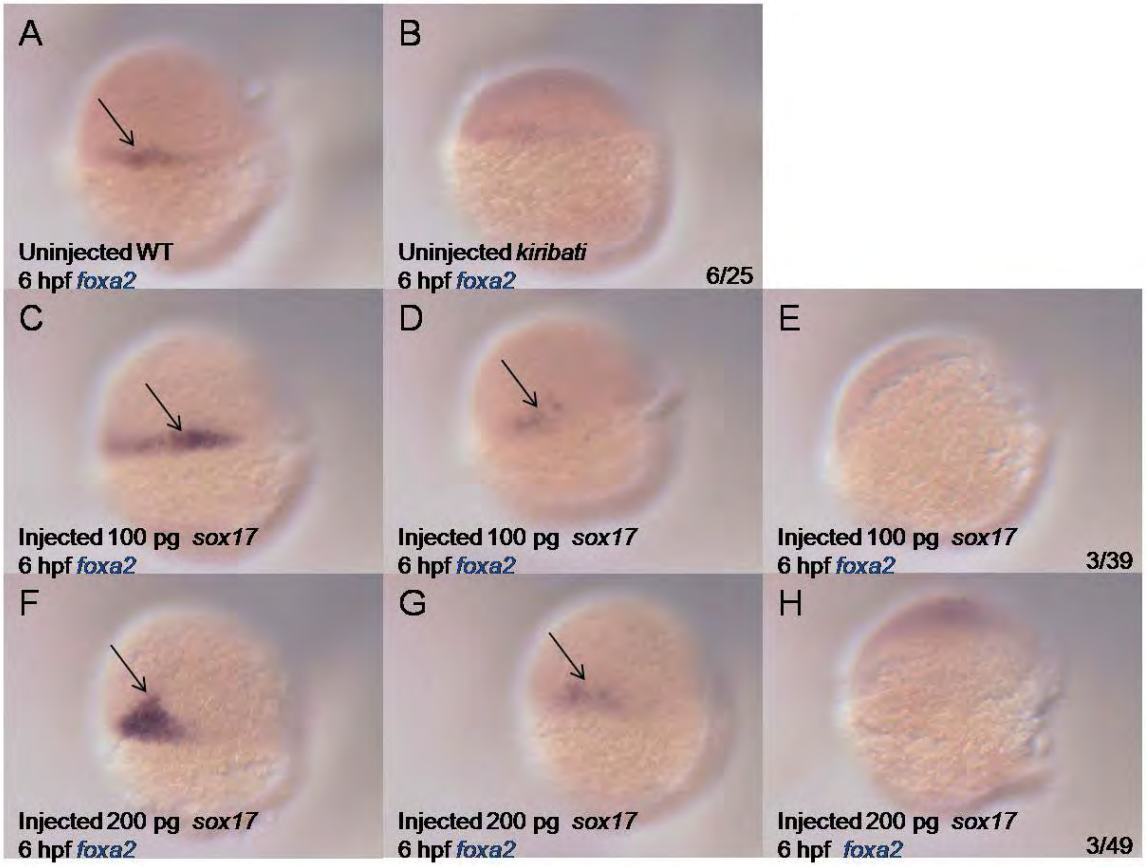
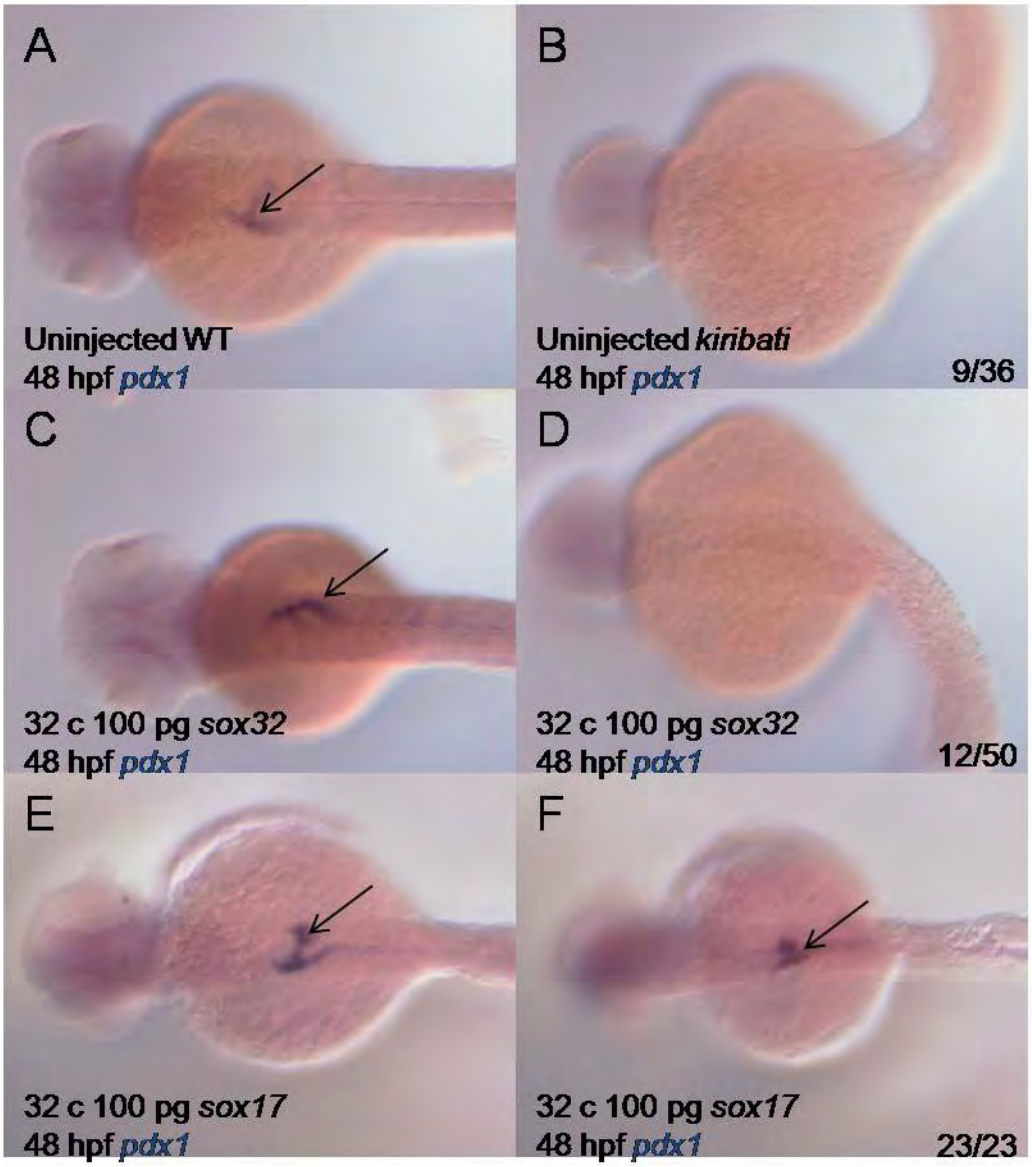

A
Uninjected WT
6 hpf foxa2
B
Uninjected kiribati
6 hpf foxa2
6/25
C
Injected 100 pg sox17
6 hpf foxa2
D
Injected 100 pg sox17
6 hpf foxa2
E
Injected 100 pg sox17
6 hpf foxa2
3/39
F
Injected 200 pg sox17
6 hpf foxa2
G
Injected 200 pg sox17
6 hpf foxa2
H
Injected 200 pg sox17
6 hpf foxa2
3/49

Figure 5.11 – *sox17* but not *sox32* rescues *pdx1*

sox32 and *sox17* mRNA was injected into one cell at the 32 cell stage and *pdx1* expression was assayed at 48 hpf. *pdx1* expression is lost in mutant embryos (B) and *sox32* mRNA was unable to rescue *pdx1* expression (A,B, 24% C,D vs. 25%) but *sox17* resulted in 100% rescue (E,F, 23/23 embryos express *pdx1*).

Figure 5.11


A
Uninjected WT
48 hpf pdx1
B
Uninjected kiribati
48 hpf pdx1
9/36
C
32 c 100 pg sox32
48 hpf pdx1
D
32 c 100 pg sox32
48 hpf pdx1
12/50
E
32 c 100 pg sox17
48 hpf pdx1
F
32 c 100 pg sox17
48 hpf pdx1
23/23

We repeated the 32 cell injections using *sox17* mRNA and assayed for endocrine and exocrine pancreas markers. In contrast to *pdx1* expression, neither *isl1* nor *p48* expression is rescued (Figure 5.12 C,D vs. 5.12A,B and 5.12G,H vs. 5.12E,F). Therefore, targeting *sox17* mRNA to the endoderm can rescue *pdx1,* which is expressed in both endocrine and exocrine pancreatic lineages, but differentiated endocrine and exocrine lineage markers cannot be rescued. Thus, early pancreatic precursors can form but are unable to differentiate into endocrine and exocrine pancreas cells.

pou5f1 partially rescues *pdx1* and *sox17* expression

Our results indicate that *sox17* mRNA, but not *sox32* mRNA, is capable of rescuing *foxa2* and *pdx1* expression in *kiribati* mutant embryos. We repeated the rescue injections using *pou5f1* mRNA since it acts with Sox32 to regulate *sox17* expression. Unlike *sox32, pou5f1* expression is not limited to the endoderm, but is expressed ubiquitously early and restricted to the midhindbrain boundary later in development (Lunde, Belting et al. 2004). Injecting 200 pg of *pou5f1* mRNA at the one cell stage resulted in partial rescue of *pdx1* expression (13% lack *pdx1* expression when compared to 25% in uninjected control, Figure 5.13C-E vs. 5.13A,B). Injecting a higher dose at 300 pg resulted in 11% of the embryos lacking *pdx1* expression (Figure 5.13F-H).

Figure 5.12 – *sox17* injections are unable to rescue differentiated pancreas markers

sox17 mRNA was injected into one cell at the 32 cell stage and embryos were assayed for *isl1* and *p48* expression at 30 hpf. *isl1* and *p48* expression is lost in the endoderm but maintained in the CNS in mutant embryos (B,F vs. A,C black arrow). Injecting *sox17* mRNA at the 32 cell stage was unable to rescue either *isl1* (D, 25% lack expression) or *p48* (H, 25% lack expression).

Figure 5.12

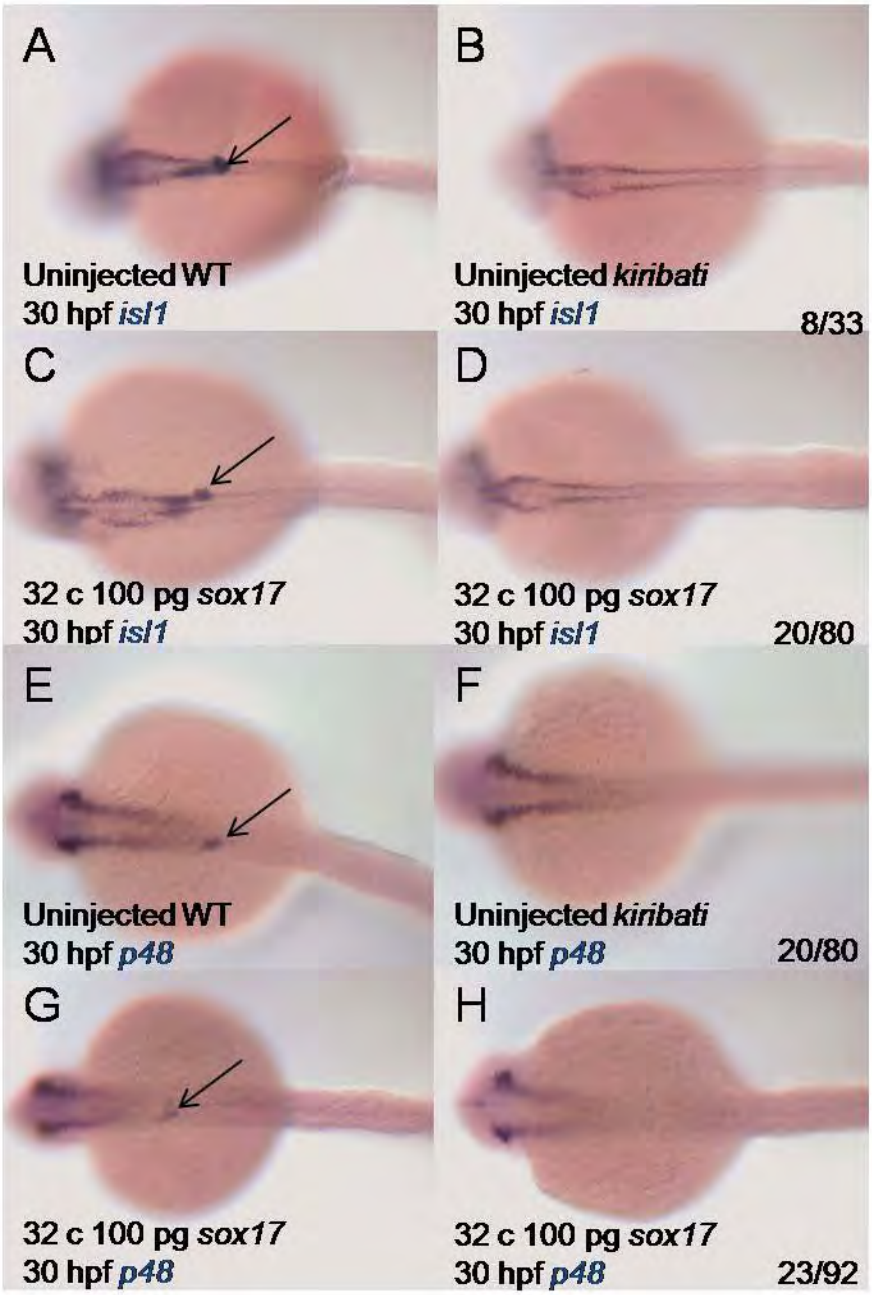
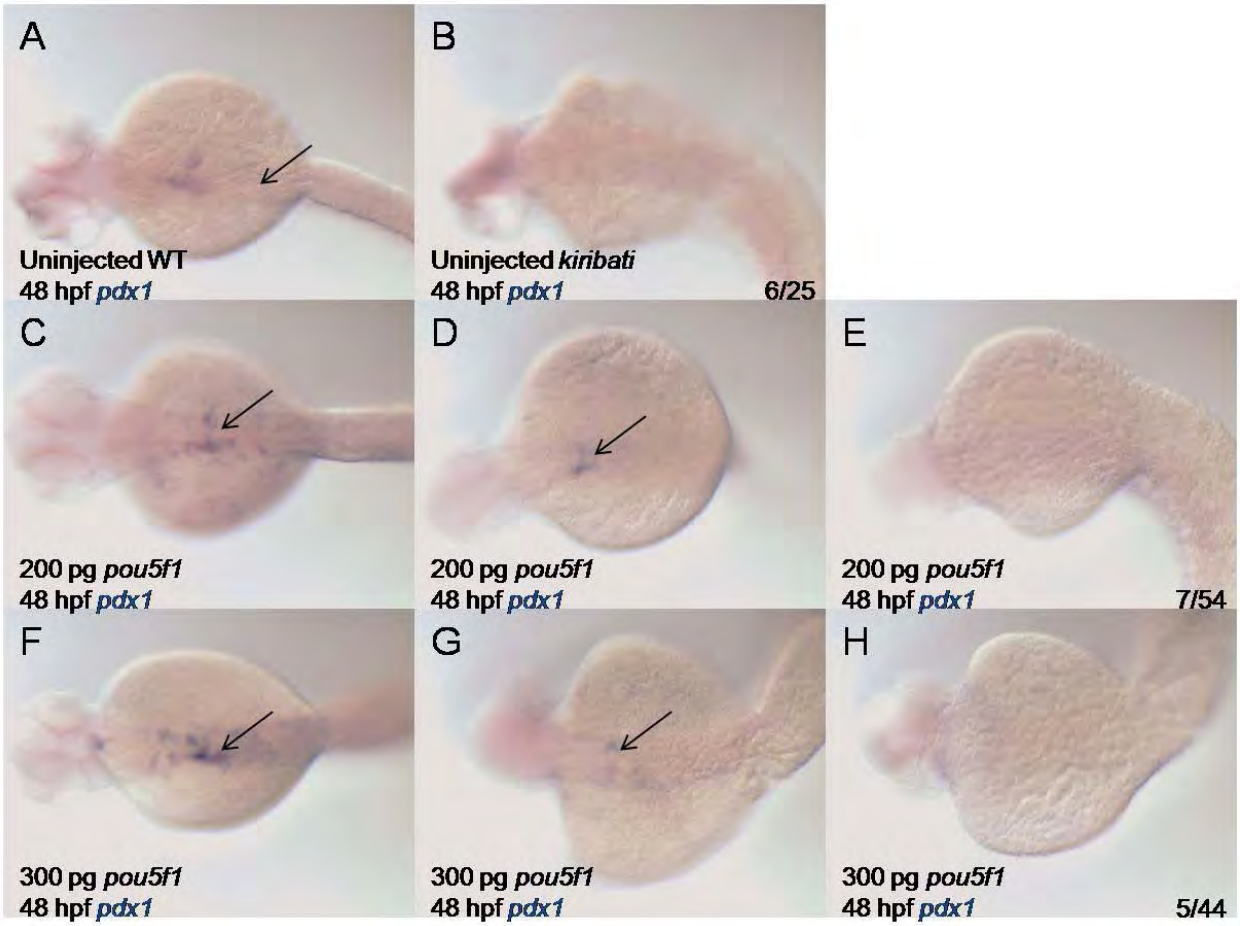

A
Uninjected WT
30 hpf isl1
B
Uninjected kiribati
30 hpf isl1
8/33
C
32 c 100 pg sox17
30 hpf isl1
D
32 c 100 pg sox17
30 hpf isl1
20/80
E
Uninjected WT
30 hpf p48
F
Uninjected kiribati
30 hpf p48
20/80
G
32 c 100 pg sox17
30 hpf p48
H
32 c 100 pg sox17
30 hpf p48
23/92

Figure 5.13 – *pou5f1* partially rescues *pdx1* expression

pou5f1 mRNA was injected at the one cell stage and *pdx1* expression was detected at 48 hpf. Injecting 200 pg (C-E) and 300 pg (F-H) of *pou5f1* mRNA partially rescued *pdx1* expression at 48 hpf (13% and 11% lack *pdx1* compared 24% of uninjected control embryos (B).

Figure 5.13

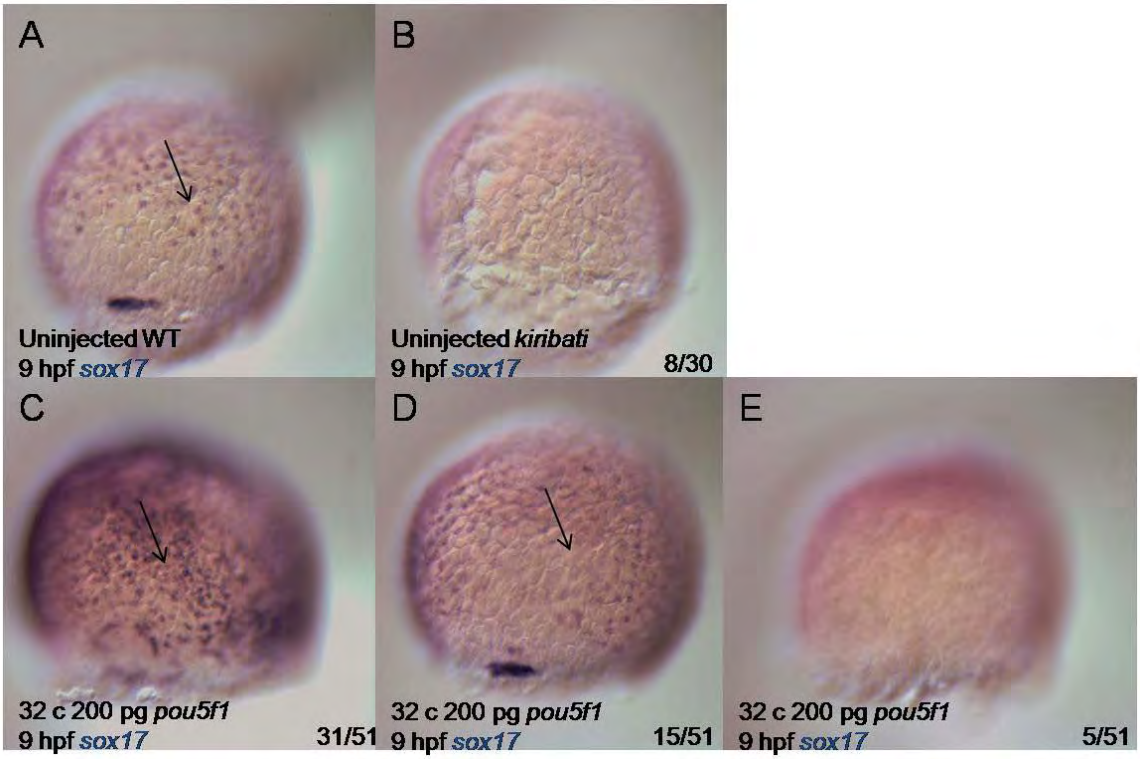

A
Uninjected WT
48 hpf pdx1
B
Uninjected kiribati
48 hpf pdx1
6/25
C
200 pg pou5f1
48 hpf pdx1
D
200 pg pou5f1
48 hpf pdx1
E
200 pg pou5f1
48 hpf pdx1
7/54
F
300 pg pou5f1
48 hpf pdx1
G
300 pg pou5f1
48 hpf pdx1
H
300 pg pou5f1
48 hpf pdx1
5/44

We then targeted *pou5f1* mRNA to the endoderm using *sox32* mRNA. We assayed for *sox17* expression in endodermal precursor cells and forerunner cells at 9 hpf and observed partial rescue (10% lack *sox17* expression vs. 26.7% uninjected control, Figure 5.14C-D vs. 5.14A,B). Our results indicate that *pou5f1* is capable of partially rescuing the early endoderm phenotype exhibited in mutant embryos.

Figure 5.14 – Targeting *pou5f1* to the endoderm partially rescues *sox17* expression

pou5f1 mRNA was targeted to the endoderm using *sox32* and *sox17* expression was detected at 9 hpf. *sox17* is expressed in endodermal precursor cells (A – black arrow) as well as the forerunner cells. *kiribati* mutant embryos (B) lack *sox17* expression in the endodermal precursor cells and forerunner cells. Targeting *pou5f1* to the endoderm partially rescues *sox17* expression (10% of embryos lack *sox17* vs. 26.7% uninjected control embryos in B).

Figure 5.14


A
Uninjected WT
9 hpf sox17
B
Uninjected kiribati
9 hpf sox17
8/30
C
32 c 200 pg pou5f1
9 hpf sox17
31/51
D
32 c 200 pg pou5f1
9 hpf sox17
15/51
E
32 c 200 pg pou5f1
9 hpf sox17
5/51

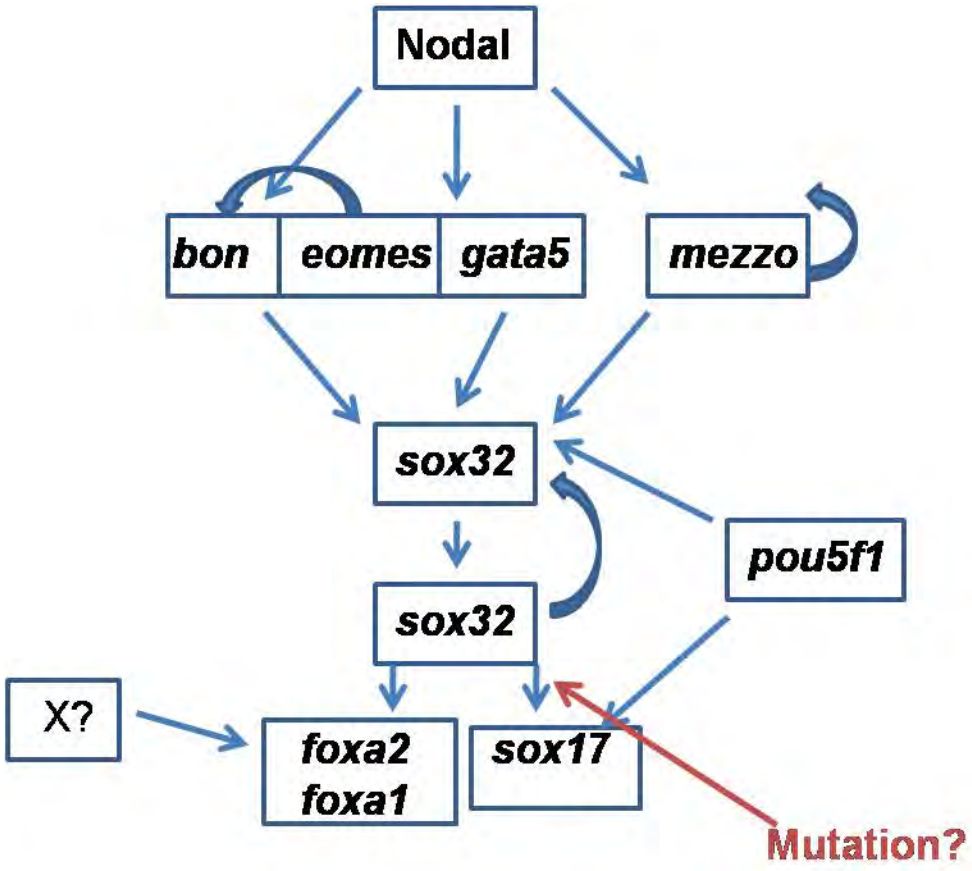
Discussion

Based on our data we predict that our mutation lies between *sox32* and *sox17* in the early endoderm pathway (Figure 5.15).

Figure 5.15 – Early endoderm pathway in zebrafish

Nodal signaling lies upstream in the endoderm pathway in zebrafish and directly activates *bon* (*bonnie and clyde,* mixer), *gata5* (*faust*), and *mezzo* (mix-type gene). Bon and Gata5 bind to Eomes (Eomesodermin), a protein that increases the efficiency of these two proteins binding the *sox32* promoter. Another factor, *mezzo*¸ does not interact with *bon, eomes,* or *gata5*, but has proven to be important in activating *sox32* gene expression. *sox32* is the earliest gene specific to the endoderm. It is necessary and sufficient to initiate endoderm formation. Sox32 and Pou5f1 act together to regulate *sox32* expression and both bind conserved regulatory regions that lie upstream in the *sox17* promoter. *foxa2* and *foxa1* expression are dependent on *sox17* as well as another unknown factor, X.

Figure 5.15


Nodal
bon
eomes
gata5
mezzo
sox32
sox32
pou5f1
X?
foxa2
foxa1
sox17
Mutation?

kiribati mutant embryos do not resemble *sox32* or *pou5f1* mutants

Early endoderm expression, such as *sox17, foxa2* and *foxa1,* are not expressed in *sox32* mutants and as a result a gut tube does not form, indicating that the endoderm expression and formation is defective from the onset of gastrulation. Interestingly, *sox32* mutant embryos exhibit upregulation of *sox32* expression is up-regulated in the YSL (Yolk Syncytial Layer) but lack *sox32* expression in endoderm progenitors (Kikuchi, Agathon et al. 2001). Also, the forerunner cells (noninvoluting dorsal mesendodermal cells that express *sox17*) do not form in *sox32* mutants. Mesodermal defects in the heart (develop cardia bifida), vasculature, blood, and kidney are also apparent in mutant embryos (Alexander, Rothenberg et al. 1999). Furthermore, in the absence of *sox32,* endoderm progenitors differentiate into mesodermal derivatives (Dickmeis, Mourrain et al. 2001). We find that injecting *sox32* into *kiribati* mutant embryos does not rescue *pdx1* expression suggesting that despite the similarities between phenotypes, we do not have another *sox32* mutant.

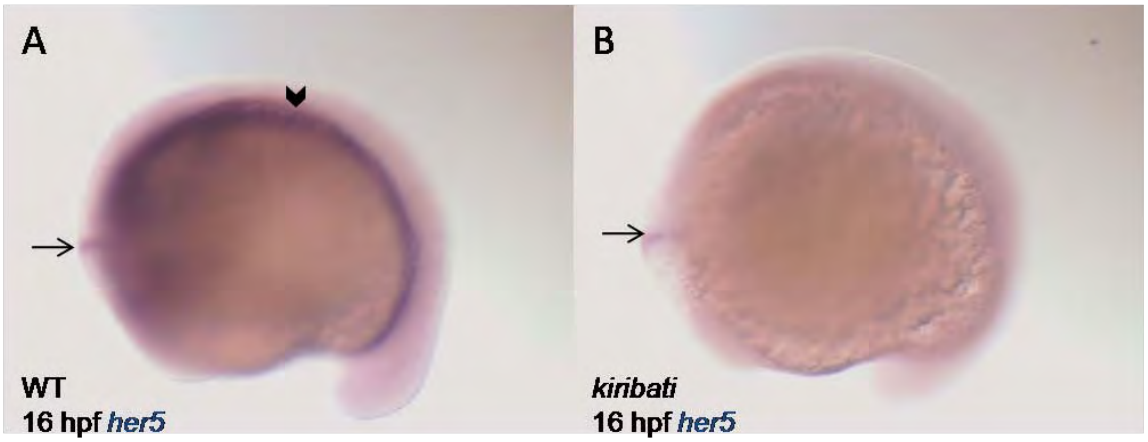
MZ*spg* (maternal and zygotic *spiel ohne grenzen* mutant, *pou5f1*) mutants do not maintain expression of *sox32*, express little or no *sox17*, and fail to develop endodermal tissue. Constitutively active *Nodal* receptor TARAM-A or *sox32* overexpression induces ubiquitous *sox17* expression in wild-type embryos (Alexander and Stainier 1999; David and Rosa 2001; Kikuchi, Agathon et al. 2001) but not in MZ*spg* mutants. Also, overexpression of a Pou5f1-VP16 activator fusion protein can rescue gastrulation and endodermal tissues in MZ*spg*

mutants. Therefore, Pou5f1 is necessary to maintain *sox32* expression during gastrulation and acts with Sox32 to induce *sox17* expression in endodermal precursor cells. (Lunde, Belting et al. 2004). Also, Pou5f1 functions in midhindbrain boundary (MHB) development and MHB expression is either completely absent or severly reduced in *spg* mutants. We discovered that *kiribati* mutant embryos have normal *her5* expression in the MHB at 16 hpf but lack expression in the endoderm indicating that we do not have a *spg* mutant (Figure 5.16B vs. 5.16A).

Figure 5.16 – Midhindbrain boundary (MHB) develops normally in *kiribati* mutant embryos

her5 is expressed in the MHB and endodermal precursor cells. (A) Wildtype embryos with *her5* expression in the MHB (black arrow) and endoderm (arrowhead) at 16hpf. (B) Mutant embryo with normal MHB expression (black arrow) and lack endoderm expression at 16 hpf.

Figure 5.16



Is *kiribati* a *sox17* mutant?

Sox17 plays a conserved role in endodermal differentiation in *Xenopus* (Hudson, Clements et al. 1997), zebrafish (Alexander and Stainier 1999), and mouse (Kanai-Azuma, Kanai et al. 2002). Targeted deletion of Sox17 in mouse embryos results in deficient gut endoderm, in particular an overall reduction in definitive endoderm and results in embryonic death. In mouse stem cells, overexpression of *Sox17* leads to the upregulation of endodermal markers (Qu, Pan et al. 2008). Therefore, *Sox17* plays an important role in endoderm development in mouse (Kanai-Azuma, Kanai et al. 2002).

In *Xenopus,* blocking endogenous Sox17 with Sox17:EnR (Hudson, Clements et al. 1997) or morpholinos (MOs)(Clements and Woodland 2003) disrupts endoderm development and reduces endoderm gene expression. Three Sox17 genes in *Xenopus, Xsox17β, Xsox17α$_1$* and *Xsox17α$_2$*, function redundantly in endoderm formation. Ectopic expression of these genes induces endoderm markers in animal caps and plays an important role in normal endoderm differentiation in embryos (Hudson, Clements et al. 1997). Injecting MOs targeting *Xsox17α$_1$*, *Xsox17α$_2$*, and *Xsox17β* individually results in defects in late midgut development. Moreover, loss of both Xsox17α proteins inhibits hindgut formation and inhibiting *Xsox17α$_1$* individually disrupts foregut development in a dose dependent manner. Blocking the function of all three *Xsox17* proteins halts cell movements during late gastrulation and the transcription of several endodermal genes is reduced. Thus, *Xsox17* genes play

redundant roles in early endoderm formation and distinct roles later in organogenesis (Clements and Woodland 2003).

In zebrafish, the role of *sox17* has not as extensively studied. *sox17* gain and loss of function studies have not been performed and therefore we cannot compare the *kiribati* phenotype to a *sox17* morphant. However, we did sequence the full-length *sox17* gene but no mutation was discovered. However, this does not conclude that upstream or downstream regulatory regions are unaffected in *kiribati*.

A new factor necessary for endoderm development?

An unknown factor regulatin *sox17* expression has not been ruled at as a possible candidate. Sox factors sometimes require binding partners to activate or repress transcription of their target genes. For example, Pou5f1 together with Sox32 is required for mesendodermal precursors to form endoderm in zebrafish. Wildtype mesendodermal cells express both *sox32* and *pou5f1,* and without *pou5f1,* the cells take on a mesodermal fate. This is confirmed by transplanting MZ*spg* cells into wildtype embryos and as a result these cells give rise to mesoderm even when placed in the endodermal primordium (Reim, Mizoguchi et al. 2004). Therefore, another Sox protein may act with Sox32 and Pou5f1 or just Pou5f1 to regulate *sox17* gene expression.

In support of this, other Sox proteins function in endoderm formation in mice and *Xenopus*, for example, Sox7. In *Xenopus, Sox7* is maternally

expressed and functions in endoderm formation(Fawcett and Klymkowsky 2004) and in mouse, *Sox7* is expressed in the parietal endoderm (Kanai-Azuma, Kanai et al. 2002; Murakami, Shen et al. 2004). Also, expressing constitutively active SOX7 in human embryonic stem cells produces extraembryonic endoderm and definitive endoderm progenitors (Seguin, Draper et al. 2008). In zebrafish, *sox7* is not expressed in the endoderm. However, Pou5f1 has shown to interact with other Sox factors in midhindbrain formation suggesting that other Sox partners could exist for Pou5f1 in endoderm formation.

Materials and Methods

Fish Maintenance

Ekkwill (EK) and Tupfel long fin (*TL*) embryo*s* were collected from natural matings and reared in 1/3 Ringer's. Embryos were staged using morphological criteria up to 24 hours post fertilization (hpf) and then by time of development at 28.5°C (Kimmel, Ballard et al. 1995).

ENU Screen

EK males were treated with 3 mM ENU (*N*-ethyl-*N*-nitrosourea) once a week for 3 weeks. The males were then crossed repeatedly to clean out any post meiotic germ cells that were mutagenized. Mutagenized males were then crossed to EK females and the progeny (F1) were raised. Haploid embryos were produced by In Vitro Fertilization (IVF) of F1 female progeny with irradiated sperm. Haploid embryos were raised to approximately 30 hpf and fixed in 4% paraformaldehyde for in situ hybridization with *islet1 (isl1)* probe. Embryos were screened based on *isl1* expression. F1 females that produced embryos with mutant phenotypes were out-crossed to TL males and the progeny (F2) were raised and in-crossed for recovery of mutation in diploid embryos.

Mapping, DNA extraction, and Sequencing

Mutant carriers were in-crossed and progeny were raised to 72 hpf. Embryos were sorted based on their phenotype; mutants have curved bodies. Genomic

DNA was extracted from phenotypically mutant and phenotypically wild type embryos at day 4. DNA pools were created from phenotypically mutant and wild type embryos. Bulk segregant analysis was performed on the DNA pools using a 192 CA marker panel (Knapik, Goodman et al. 1998; Roman, Pham et al. 2002; Lawson, Mugford et al. 2003).

Sox17 full length was amplified using stratagene hi fi taq using

FW:TTTTCTGACCTTTCATGTTGAATG

RV:TATTTTTTCTTCTGTTTTATAAAAAGAGAAAGA

mRNA injections

For rescue experiments, the *sox17* ORF and *pou5f1* ORF were amplified from TL embryos sox17 FWEcoR1:GAATTCATGAGCAGTCCCGATGC and sox17 RVXho1:CTCGAGTCAAGAATTATTATAGCCGCAGTAG and pou5f1 FWEcoR1:GAATTCATGACGGAGAGAGCGCAG and pou5f1 RVXho1:CTCGAGTTAGCTGGTGAGATGACCCAC using stratagene hi fi taq. Both ORFs were amplified, digested using EcoR1 and Xho1 and cloned into PCS2+. Constructs were linearized with Not1 and Ambion Kit Sp6 was used to make mRNA. 100 pg and 200 pg of *sox17* mRNA and 200pg and 3000 pg of *pou5f1* mRNA was injected into an 835.5 in-cross at 1-2 cell stage.

To target mRNA to the endoderm *sox32* mRNA was utilized and injected were performed as previously described in chapter 2. mRNA injected embryos were

fixed in 4% paraformaldehyde at various developmental stages for in situ hybridization.

In situ hybridization

Antisense digoxigenin- and fluorescein-labeled probes were produced by standard methods. The *krx20*, *myosin heavy chain* (*mhc*), *insulin, sid4, carb a*, *pdx1, isl1, transferrin, p48, somatostatin, ifabp (intestinal fatty acid binding protein), foxa2* and *shh* probes used were described previously (Sagerstrom, Grinbalt et al. 1996; Sagerstrom, Kao et al. 2001; diIorio, Runko et al. 2005). Full-length *gata5*, *prox1, sox17* and *sox32* probes were a gift from Philip diIorio. *hoxb4a, hoxb6b* and *hoxc6a* probes were used (Davidson, Ernst et al. 2003). *pou5f1* ORF was amplified and cloned into PCS2+ (see mRNA), digested with stu1 and T7 used for probe . *her5* pME18SFL3 was ordered from openbiosystems, extracted using Xho1, cloned into PCS2+, digested with EcoR1 and T7 used to make probe. One- and two-color in situ hybridization was carried out as described previously (Sagerstrom, Grinbalt et al. 1996; Sagerstrom, Kao et al. 2001).

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

Early endoderm formation in zebrafish

The early endoderm pathway is evolutionarily conserved and Nodal signaling initiates the pathway. In zebrafish, two nodal related proteins *ndr1* and *ndr2,* and their co-receptor, *oep* (*one eyed pinhead*), signal through TARAM-A, a TGF-β receptor. Signals are translocated to the nucleus through Smads and downstream transcription factors are activated including Bonnie and Clyde (Bon, a Mix-like protein), Gata5, and Mezzo (Og9x, a Mix-like protein unique to zebrafish). Eomesodermin (Eomes) is not regulated by Nodal signaling but binds Bon and Gata5 and this trimeric complex acts in parallel to Mezzo to activate *sox32* expression, the first specific endoderm marker in zebrafish. *sox32* is necessary and sufficient to induce endoderm. Sox32 regulates its own expression with the help of Pou5f1 and these two proteins then initiate *sox17* expression, which is evolutionarily conserved in endoderm development. From here, other downstream genes such as *foxa2* and *foxa1* are activated and the endoderm forms and differentiates to produce various organs and glands. Understanding how endodermal organs are formed may be helpful in designing therapies to target various disorders and diseases associated with the organs.

Signaling pathways that pattern the pancreas

Sonic hedgehog signaling

Inhibition of *Shh* (*sonic hedgehog*) expression in the pancreas primordium is critical in zebrafish pancreas development. In contrast, Shh signaling from the mesoderm is required for the induction of pancreas markers. Consistent with this, cyclopamine (Hedgehog receptor inhibitor) treated embryos or *smo* (mutant for Hedgehog receptor Smoothened) fail to develop a pancreas (Roy, Qiao et al. 2001; dilorio, Moss et al. 2002). The regulatory factors upstream of *shh* signaling in the pharyngeal endoderm that ultimately patterns the pancreas are not known. We found that TALE (Three Amino Loop Extension) homeodomain transcription co-factors Meis and Pbx act upstream of *shh* expression in the endoderm.

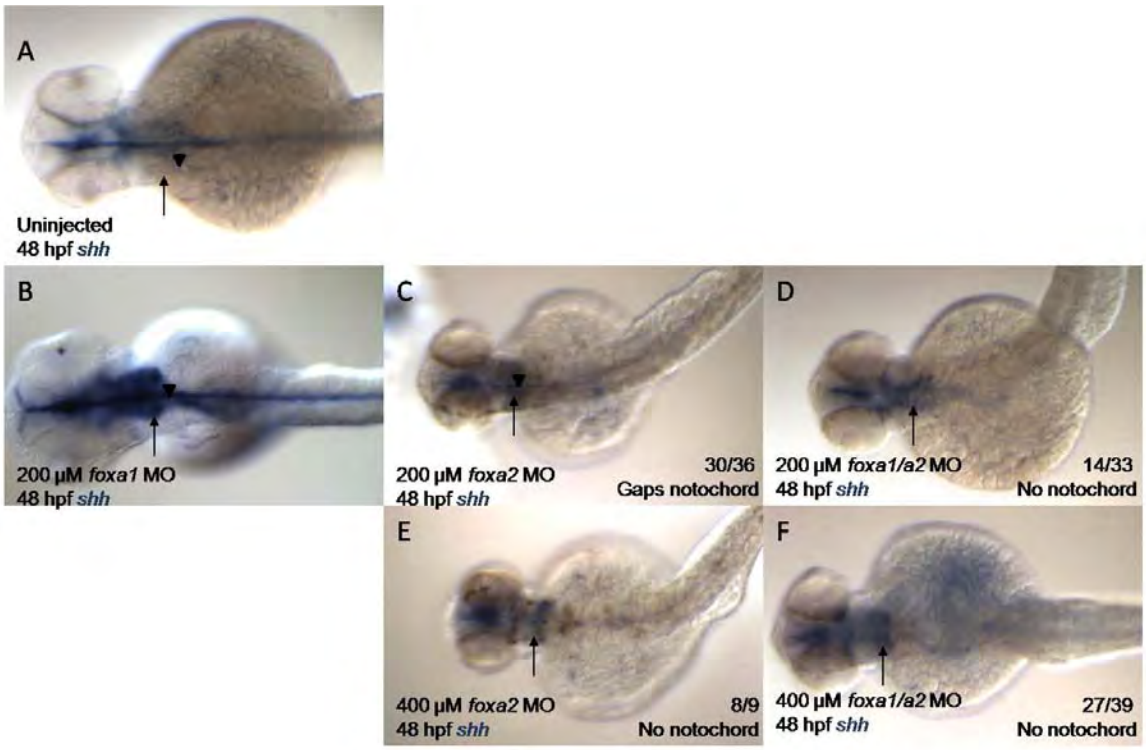
Since Pbx and Meis are co-factors that function with other partners to regulate transcription in hindbrain development, we were interested in finding potential binding partners that work with Meis3 and Pbx4 to regulate *shh* expression in the endoderm. We observed that *foxa2* expression in the pharyngeal endoderm is decreased when *meis3* and *pbx4* are disrupted. *foxa2* acts upstream of *shh* in the notochord, floorplate and the neuroectoderm (Chang, Blader et al. 1997; Strahle, Lam et al. 2004). We analyzed *shh* and *ins* expression in *foxa2* morphants and *monorail* (*mol, foxa2*) mutant embryos. In these embryos, we detected a similar phenotype that we observed in *meis3* and *pbx4* deficient embryos. *shh* expression is lost in the notochord and floorplate and reduced in the pharyngeal endoderm in both *foxa2* morphants and *mol*

mutant embryos. This indicates that *foxa2* may regulate *shh* expression in the pharyngeal endoderm although it does not appear to be the key regulator. When *meis3* is knocked down, *shh* expression in the endoderm is affected but not in the CNS, suggesting that *shh* expression in each region is regulated by different factors. In *mol* mutants, *shh* expression in the endoderm is still present while notochord and floorplate is absent. This implies that some other factor may work with *foxa2* to regulate endodermal *shh* expression. We considered *foxa1* as a possible candidate since it is expressed in a similar pattern to *foxa2* in the pharyngeal endoderm (Odenthal and Nusslein-Volhard 1998; Piotrowski and Nusslein-Volhard 2000). We co-injected *foxa1* MO with *foxa2* MO and completely abolished *shh* expression in the notochord and floorplate but still detected endoderm expression of *shh*, although it is reduced (Figure 6.1). We also evaluated *ins* expression and found anterior ectopic *ins* spots but did not appear to worsen the phenotype when compared to *foxa2* MO (Not shown). This leads us to believe that a missing factor that acts with Meis and Pbx to regulate *shh* is still yet to be discovered.

Figure 6.1 – *foxa1* does not act with *foxa2* to regulate *shh* in the pharyngeal endoderm

(A) Uninjected control embryo with *shh* expression in the pharyngeal endoderm (black arrow) and notochord (arrowhead). Injecting 200 μM *foxa1* MO does not affect *shh* expression in either the notochord (arrowhead) or endoderm (black arrow). (C) 200 μM *foxa2* MO introduces gaps in *shh* expression in the notochord (arrowhead) and reduces endoderm expression (black arrow). (D) Injecting a combination of *foxa1/foxa2* equaling 200 μM results in 14/33 embryos lacking *shh* in the notochord and reduced endoderm expression (black arrow). (E) Injecting 400 μM *foxa2* MO produces 8/9 embryos that lack notochord expression but have some endoderm expression (black arrow). (F) 400 μM combination of *foxa1/foxa2* results in 27/39 embryos that lack notochord but still have some endoderm expression (black arrow).

Figure 6.1


A
Uninjected
48 hpf shh
B
200 µM foxa1 MO
48 hpf shh
C
200 µM foxa2 MO
48 hpf shh
30/36
Gaps notochord
D
200 µM foxa1/a2 MO
48 hpf shh
14/33
No notochord
E
400 µM foxa2 MO
48 hpf shh
8/9
No notochord
F
400 µM foxa1/a2 MO
48 hpf shh
27/39
No notochord

Meis and Pbx in pancreatic cancer

Shh signaling is linked to several human cancers such as small cell lung carcinomas, medulloblastomas, basal cell carcinomas and digestive tract tumors (Morton and Lewis 2007). Researchers have determined that Shh expression, in conjunction with the loss of tumor suppressor loci, can induce pancreatic tumorigenesis in pancreatic duct epithelial cells (Morton and Lewis 2007). Since Meis and Pbx regulate *shh* expression in zebrafish, they could also regulate *Shh* expression in other organisms. As a result, Meis and Pbx may play a role in cancer by misregulating *Shh* expression. Also, Meis proteins regulate K19 gene transcription in pancreatic ductal epithelial cells (von Burstin). Specifically, Meis and Pbx regulate *elastase* (exocrine) and *pax6* (endocrine)(von Burstin ; Zhang, Rowan et al. 2006) suggesting that Meis and Pbx play a role in pancreatic development and that misregulation of these genes could result in defects such as pancreatic cancer.

Human disorders linked to disrupted *Shh* signaling

Two conditions that occur within the human pancreas are linked to improper Hedgehog signaling. Annular pancreas is a rare condition in which part of the duodenum is surrounded by a ring of pancreatic tissue that continuous with the head of the pancreas. This portion of the pancreas constricts the duodenum and decreases or blocks the flow of food to the rest of the intestines. Homozygous *Indian hedgehog* mutant mice develop annular pancreas (Hebrok,

Kim et al. 2000) and homozygous Shh null mutant mice also have foregut (Litingtung, Lei et al. 1998) and gastrointestinal defects including annular pancreas (Ramalho-Santos, Melton et al. 2000). Understanding the role Hedgehog signaling in pancreas development will help understand how this condition can occur.

Another disease, Nesidioblastosis, is a condition where the islets of Langerhans develop from pancreatic ductal epithelium outside of the pancreas domain resulting in ectopic islets (Laidlaw 1938). Hyper-insulin production occurs resulting in hypoglycemia. The disorder later was called persistent hyperinsulinemic hypoglycemia of infancy (PHHI). If left untreated, PHHI can lead to brain damage or death caused by hypoglycemia (Gillespie 2008). Nesidioblastosis is linked to disrupted embryonic *Shh* signaling (McKeever and Young 1990; Lachman, Wright et al. 1991; Porter, Young et al. 1996) indicating a possible role for Hedgehog signaling in human islet development and glucose regulation (Kim and Melton 1998).

Retinoic acid (RA) signaling in pancreas development

Another signaling factor involved in pancreas patterning is Retinoic Acid (RA). RA acts to specify the pancreas domain between 9 -13 hpf, at the end of gastrulation. Treatment of exogenous RA expands the pancreatic domain throughout the anterior endoderm acting in a dose-responsive manner to expand foregut fates at the expense of pharyngeal structures (Stafford and Prince 2002).

From an ENU (*N*-ethyl-*N*-nitrosourea) screen for endocrine pancreas mutants we isolated an *aldh1a2* mutant, *aldh1a2*^{um22}. We performed an ENU screen to search for possible candidates that act in pancreas development. We discovered a silent mutation and a missense mutation that resulted in a glycine to arginine change in the Aldh1a2 catalytic domain resulting in a null mutation. Interestingly, we discovered two mutations in the *ald1a2* gene where two alleles have already been discovered *aldh1a2*^{i26} and *aldh1a2*^{u11}, both ENU induced. *aldh1a2*^{i26} has a mutated NAD domain and *aldh1a2*^{u11} has a mutation in the catalytic domain like *aldh1a3*^{um22}. DNA is known to have "hotspots" where mutations can occur up to 100 times more frequently than the normal mutation rate. The goal of a mutagenesis screen is to saturate the genome or in other words target all the essential genes to determine their function. Hotspots prevent the genome from being saturated because they are targeted at higher rate than other regions. Currently, the accepted parameters for ENU treatment are established but it is clear that hotspots exist and are targeted therefore, not saturating the genome.

Hotspots are present in every genome and may not always be a hindrance. It would be beneficial to find hotspots and understand why these might be hotspots. They could bring insight into mutations that occur spontaneously resulting in diseases. For example, DNA hotspots found in humans may reveal how genetic differences between individuals result in psoriasis, an autoimmune disease of the skin and genes linked to it are DNA

hotspots in the human genome. New genes discovered from this study may become potential targets in treatments (Staff 2009).

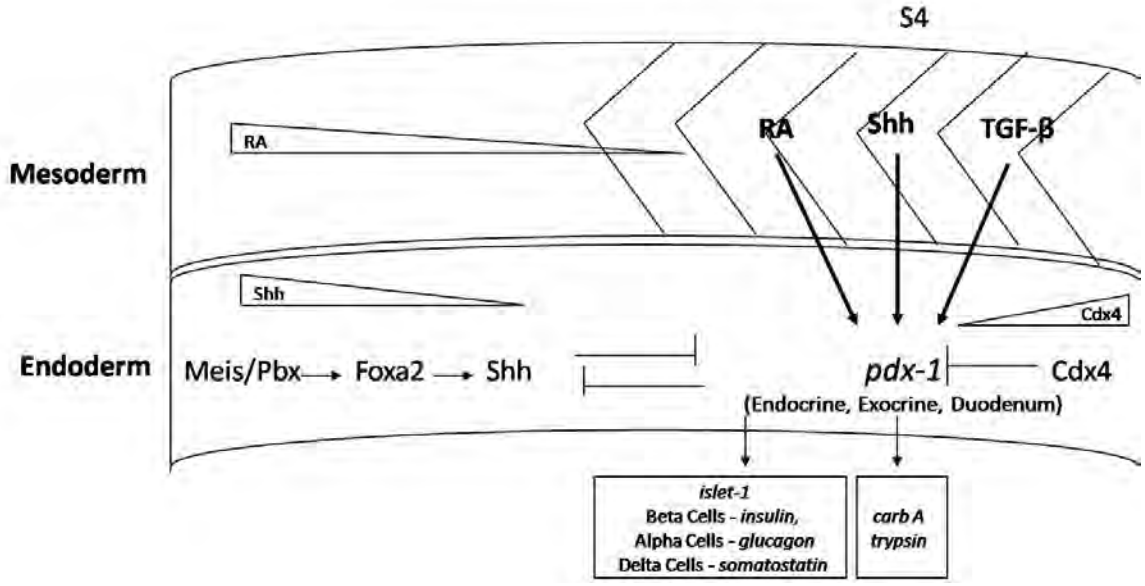
Incorporating our results in the pancreas signaling pathway

In zebrafish the various signaling factors and transcription factors involved in pancreas patterning have been established but what regulates these is still unclear (Figure 6.2). One possibility is that RA acts upstream of *meis3* and *pbx4* in the pharyngeal endoderm which then regulates *shh* expression. Overall, this relationship patterns the anterior endoderm.

Figure 6.2 – Early pathway in pancreas patterning

Signals from the mesoderm (upper layer represented by somites), Retinoic Acid (RA), Sonic Hedgehog (Shh), and TGF-β, are necessary to activate *pdx1* expression which is necessary for pancreas formation. Furthermore, *pdx1* can then activate downstream pancreas markers specific to endocrine and exocrine cells. *Shh* expression in the anterior endoderm establishes the anterior border for the pancreas primordium. *Pdx1* and *shh* cannot be co-expressed but a gradient of Shh is established in the anterior endoderm and is further involved in patterning the pancreas, endocrine versus exocrine fate. A RA gradient is formed in the anterior mesoderm which signals to the endoderm and pancreas. Posteriorly, *cdx4* is expressed and establishes the posterior border for the pancreas primordium. RA induces pancreas markers and Cdx4 represses them. These factors act together to maintain the pancreas primordium at somite 4 during early embryogenesis.

Figure 6.2


S4
RA
Shh
TGF-β
Mesoderm
RA
Shh
Cdx4
Endoderm
Meis/Pbx → Foxa2 → Shh
pdx-1
Cdx4
(Endocrine, Exocrine, Duodenum)
islet-1
Beta Cells - insulin,
Alpha Cells - glucagon
Delta Cells - somatostatin
carb A
trypsin

New technique to target reagents to the endoderm

Cell transplantation is the method of moving labeled cells from a donor embryo to a host embryo (Sambrook 1989) successfully incorporating the labeled cells into a specific germ layer. This technique does efficiently produce mosaic embryos that can be used to test autonomous and non-autonomous effects of gene activity (Jessen, Meng et al. 1998). In this case, cells from one genotype are moved to another genotype to determine which cells express the gene of interest and what effect these cells have on other cells that do not express this gene.

While cell transplantations studies have proven to be very useful they do have limitations. First, blastomeres are removed from one embryo by pipette and then inserted into the host embryo. Not only do these cells need to be deposited in the correct location within the embryo but also at the correct time. Due to the time constraint, a limited number of cell transplantations can be performed and only a certain percent of embryos will have cells incorporated in the correct location.

Another method to target reagents to the endoderm is injecting one cell at the 32 cell stage. Injections at the 32 cell stage circumvent the need for transplantations by injecting reagents directly into one cell which will then contribute to the endoderm. Notably, reagents can then be targeted to the endoderm using this technique. The embryos injected with *sox32* at the 32 cell stage develop normal endoderm allowing the direct affect of a reagent on the

endoderm to be tested. One set of injections at the 32 cell stage yields approximately 50 embryos with reagents specifically targeted to the endoderm. However, injections at the 32 cell stage have a time constraint and must be performed at the 32 cell stage. This allows only a 15 minute window at 28^{o}C or about a half hour at room temperature before the embryos become 64 cell stage. Also, injections at the 32 cell stage is only useful for targeting reagents to the endoderm not mesoderm but overall provides a useful method to target the endoderm.

Applying what we know about pancreas patterning to develop potential therapies

Understanding how the gut tube is patterned to make different organs and identifying genes and signaling factors that direct their differentiation into specific tissues and organs allows future opportunities to design therapies to target pancreatic diseases (Grapin-Botton and Melton 2000). For example, Diabetes is the most well known disease associated with the pancreas. Diabetes mellitus, often just called diabetes, is a condition in which the body either does not produce enough, or does not properly respond to insulin, a hormone produced by β-cells in the pancreas. Insulin allows cells to absorb glucose in order to produce energy. This causes glucose to accumulate in the blood, often leading to various complications for example heart disease and kidney failure (Tierney LM 2002; Rother 2007).

The three primary types of Diabetes are Type 1, Type2 and Gestational (Tierney LM 2002). Patients with Type 1 fail to produce insulin, Patients with Type 2 exhibit a resistance to insulin and cells are incapable of utilizing insulin, whereas gestational diabetes occurs in pregnant women, whom have never had diabetes before, but have high blood sugar levels during pregnancy. Other forms of diabetes exist and are categorized separately. In particular, several forms of monogenic diabetes called Maturity onset diabetes of the young (MODY) (Tierney LM 2002). MODY is a form of dominantly inherited type II diabetes and is characterized by an onset of pancreatic β-cell dysfunction by the age of 25 or younger. MODY5 is associated with nondiabetic, early-onset renal diseases (Horikawa, Iwasaki et al. 1997; Nishigori, Yamada et al. 1998; Lindner, Njolstad et al. 1999; Bingham, Bulman et al. 2001). In addition, some patients with GCKD (glomerulocystic kidney disease), a kidney disease characterized by cystic dilation of the Bowman space and the initial proximal convoluted tubule, display early-onset diabetes (MODY) or impaired glucose tolerance (Rizzoni, Loirat et al. 1982; Bingham, Bulman et al. 2001) linking kidney disease with MODY5.

Various genes have been linked to MODY. Conditional inactivation of *Pdx1* in adult mouse β-cells leads to diabetes (Ahlgren, Jonsson et al. 1998) therefore linking it to MODY4 (Stoffers, Zinkin et al. 1997). Mutations in *vhnf1* results in kidney cysts, underdevelopment of the pancreas and liver, reduction in otic vesicle size (Sun and Hopkins 2001), and is associated with MODY5, GCKD and MODY3 (Yamagata, Oda et al. 1996; Horikawa, Iwasaki et al. 1997;

Nishigori, Yamada et al. 1998; Lindner, Njolstad et al. 1999; Bingham, Bulman et al. 2001). The discovery of these genes and their linkage to diabetes not only helps pinpoint the underlying cause of MODY but can lead to potential treatments. Therefore studying these genes and gathering insight into their regulation may aid in therapy for MODY Patients.

Thus far, the only cure for Diabetes is a pancreas transplant. Although, insulin became medically available in 1921 and has been the treatment of choice for diabetes, it is not a cure. Gestational diabetes usually resolves after delivery, not requiring a transplant, but other forms of Diabetes persist. Diabetes and its treatments can cause many complications, including hypoglycemia (low blood sugar), diabetic ketoacidosis (the body switches to burning fatty acids, producing acidic ketone bodies that build up), or nonketotic hyperosmolar coma (diabetic coma associated with a high mortality. Serious long-term complications include cardiovascular disease, chronic renal failure, retinal damage that can lead to blindness, several types of nerve damage, microvascular damage, and poor wound healing. Poor healing of wounds, particularly of the feet, can lead to gangrene, possibly requiring amputation (Tierney LM 2002). Therefore, a cure is ideal to eliminate these debilitating side effects. Understanding how the pancreas forms and the upstream regulators may lead to methods to cure Diabetes.

Managing diabetes with insulin therapy is far from a cure. Replacing diseased pancreatic islets in diabetic patients with healthy functional cells is the

ideal cure. Success of a new technique called the Edmonton protocol (Shapiro, Lakey et al. 2000; Shapiro, Ricordi et al. 2006) where cadaver-derived islets are transplanted into diabetic patients, indicates that this is a feasible therapeutic avenue (Kinkel and Prince 2009). However, there is a limit to availability of harvested islets and patients require long-term immunosuppressive drugs (Shapiro, Ricordi et al. 2006). These limitations could be better met by developing more functional islets derived either from stem cells or to induce regeneration of pancreatic cells. Stem cell research demonstrates a new method where islet cells from a treated individual can regrow thus eliminating the need for immuno-suppressant drugs (Vinik, Fishwick et al. 2004). This new method is called autologous nonmyeloablative (HSTC) and has led to a sufficient enough repopulation of functioning insulin-producing beta cells in the pancreas of affected patients that they no longer require insulin administration (Couri, Oliveira et al. 2009). Therefore, understanding the signaling factors capable of inducing stem cells to develop into β-cells is an essential step in developing effective therapeutics.

Much of what we know about pancreas development today has been contributed from mice. However, the zebrafish pancreas shares the basic structure and cellular makeup of mammalian pancreas, indicating that what we learn from zebrafish can be broadly applied. From zebrafish, we can learn the fine-tuned process of building a pancreas over millions of years of evolution, how

to coax undifferentiated embryonic stem cells into differentiated cell types that make a functional pancreas.

Future Directions

Ultimately, we would like to determine the gene affected in *kiribati* mutants. *Sox17* MOs have been utilized in *Xenopus* embryos and are successful in knocking down *XSox17* genes and results in defective endoderm formation (Clements and Woodland 2003). *sox17* MOs have not been utilized in zebrafish so we designed a *sox17* splice MO against the intron 1/exon 2 splice junction and plan to inject it. We will allow the embryos to develop to determine their phenotype and compare it to *kiribati* mutant embryos. *sox17* morphants will be screened with various endoderm markers to detect if their expression is present. Also, the rescue experiments will be repeated to see if endoderm markers are rescued. These experiments will allow us to conclude whether or not we have a *sox17* mutant. However, in the event that these experiments indicate that we do not have a *sox17* mutant, we will try to map the mutation using methods other than CA repeats.

In Chapter III we described another mutant, *curvy*, from our pilot ENU screen, which exhibits a broad phenotype that affects early development, not limited to the endoderm. While endoderm marker analysis has been done on mutant embryos, the mutation itself remains elusive. We tried to map the mutation using a CA panel but nothing conclusive resulted. This could be

another interesting gene to map since it affects other regions besides the endoderm to determine where it fits in endoderm development or in the overall development of the embryo; its role, its function, and what other factors it interacts with.

The information gathered from these experiments and mutants will lead to further insight into the early endoderm development pathway in zebrafish. Information gathered can be utilized to understand pancreas diseases that affect humans and potential treatments for patients.

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