I, Kelsey Linstrum, hereby submit this original work as part of the requirements for the degree of Master of Science in Molecular Genetics, Biochemistry, & Microbiology.

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Pbx4 is required to restrict second heart field and ventricular outflow tract size

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Pbx4 is required to restrict second heart field and ventricular outflow tract size

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Abstract

Patterning of the vertebrate heart is a complex process, the failure of which can result in a variety of cardiovascular defects. Congenital cardiac outflow tract (OFT) defects account for 20-30% of all heart abnormalities. Recent work has indicated that mutations in Pbx homeodomain transcription factors are associated with OFT defects in humans, most commonly persistent truncus arteriosus (PTA). However, the mechanisms underlying these defects are not well understood. Here, we demonstrate that Pbx4 is required to limit the size of the OFT during development in zebrafish. We examined heart development in *lazarus* (*lzr*) zebrafish mutants, which are mutant for Pbx4, the functional equivalent of mammalian Pbx1. *Lzr* mutants have elongated hearts with variable ventricular protrusions. Interestingly, through counting cardiomyocytes, we found that *lzr* mutants have a specific increase in ventricular cardiomyocyte (VC) number. *In situ* hybridization for cardiac specification and differentiation markers indicated that the first heart field (FHF) develops correctly. However, the unique heart morphologies of *lzr* mutants begin to develop as the second heart field (SHF) extends around 24 hours post fertilization, suggesting that Pbx4 is specifically affecting SHF-derived cells. Consistent with an increase in SHF-derivatives, we find that SHF-derived smooth muscle is also significantly increased. Moreover, at these stages qPCR analysis suggests that there is an increase in the expression of SHF progenitor markers. To further test if the surplus VCs in *pbx4* deficient embryos are derived from surplus SHF progenitors, we examined VC addition through use of the photoconvertible Kaede protein and found a significant increase in VC addition compared to wild-type siblings. We show that Pbx4 functions downstream or parallel to other known effectors of OFT development retinoic acid (RA) and FGF. We also demonstrate that BMP10 may be an effector of Pbx4 as there is increased expression of BMP10 in *lzr* mutants. Altogether, our work indicates that Pbx4 is one of the first proteins found that is specifically required to limit addition of the SHF-derived VCs, which provides a novel mechanism by which OFT defects can occur in humans.
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Chapter 1: Introduction

*Heart development is highly conserved in vertebrates*

The heart is the first organ to form and function in all vertebrate embryos, with the mechanisms involved in heart development being highly conserved. Human heart formation begins during the third week of development; this is comparable to seven days post fertilization (dpf) in mice, and 16 hours post fertilization (hpf) in zebrafish. Early heart development is divided into three main phases (Figure 1, [1]): the formation of the primary heart tube and its looping; formation of the chambers; and formation of the arterial trunks [2].

In all vertebrate embryos, myocardial progenitor cells move and come together from both sides of the anterior lateral plate mesoderm. As these progenitor cells come together, they form a crescent shape in mammalian embryos and which is termed the cardiac crescent. As the mammalian embryo develops further, the preemptive heart cells are formed into a centrally located tube. After the heart tube has formed, it must loop in order to create curvature and eventually, the divisions between the developing chambers. During looping, the heart tube bends to the right, which is the first visual evidence of asymmetry that occurs within the embryo. Once the loop has formed, there are inlet and outlet areas that will lead to the eventual inflow and outflow tracts (OFT) of the heart. This process occurs in the 25th day of human development, and the 11th day for the mouse [2].
After looping, the heart tube then divides into atrial and ventricular components separated by the atrioventricular canal with an OFT. During looping, the heart tube has an inner and outer curve. The apical parts of the ventricle balloon from the outer curve; its inner area gives rise to the left ventricle, while the outer gives rise to the right.

In mice and humans, a constriction develops between the inlet and outlet parts of the ventricle, which leads to the separation of the developing left and right ventricles. Trabeculations, or muscular finger-like projections, of the balloon-like structures provide definitive morphology to the ventricles. In order to complete the development of the atrial chambers, the pulmonary system must be formed. Following this, the preemptive atrial walls begin to balloon from either side of the OFT to form atrial appendages. These appendages continue to grow with incorporation from a variety of existing tissues. Simultaneously, the ventricles continue to develop from the existing ventricular loop. Upon completion of the ballooning, and the formation of distinct chambers, the valves between the chambers come together through a variety of reformations and growths of existing boundaries between the chambers [2].

Heart development in zebrafish

Although the initial steps of heart development are conserved in zebrafish, there are a few differences in its relatively simple two-chambered heart compared to the above mentioned, four-chambered mammalian heart development (Figure 2, [3]). In zebrafish, the heart precursors join together in an organized fashion,
with the medially located ventricular precursors advancing ahead of the laterally located atrial precursors [4]. However, after the precursors reach the midline, around 18 hpf, they begin to interact and combine, in a process known as cardiac fusion [4]. Initially, the two parallel populations of ventricular precursors make contact mid-posteriorly. Following this initial contact, the cardiomyocytes posterior to the initial point join each other creating a “V” shape [5]. The fusion of the bi-lateral cardiac fields is complete when the most anterior precursors fuse around the endocardial precursors. Viewed dorsally, the nascent heart looks like a ring of ventricular cells surrounded by atrial cells. Viewed laterally, the cardiomyocytes have begun form a cone with the ventricular apex raised above the atrial base [4]. The cardiac cone extends after fusion, and converts to a linear tube around 24 hpf. The cone extension is initiated by the tilting of its apex toward the right side of the embryo [4] and then the cone lengthens and stretches in a posterior direction. As the tube forms, the endocardial cells form the heart’s inner lining [5]. Between 24 and 48 hpf, the heart tube begins to bend gradually at the boundary between the ventricle and the atrium, creating a S-shaped loop. As a result of the looping, the ventricle ends up to the right of the atrium anatomically [6, 7].

As cardiomyocytes can beat by 24 hpf, the heart begins to drive circulation as soon as it forms. By 36 hpf, the ventricle and the atrium exhibit distinct contractions [8]. Although the heart is functioning, cardiac differentiation and morphogenesis continue. Specifically, establishment of the atrioventricular canal
begins with the development of the endocardial cushions at the atrioventricular boundary at 48 hpf; these cushions are later replaced with valve flaps that prevent retrograde blood flow [9, 10]. Moreover, the ventricular wall begins to thicken and the trabeculae begin to form from the outer curvature of the ventricle around 72 hpf [9, 10]. Overall, despite its simplicity, the formation of zebrafish heart is still highly regulated and many developmental processes are conserved from mammalian heart development.

*Distinct origins of cardiomyocytes*

Vertebrate hearts are formed from two origins of heart cells: the first heart field (FHF) and the second heart field (SHF). The FHF are an earlier differentiating population of progenitor cells comprising the cardiac crescent that contributes mainly to the linear heart tube, and later, gives rise to the left ventricle. Whereas the SHF cells are recruited to the tube later, contributing to its further growth, elongation, and rightward looping, ultimately giving rise to right ventricle, the myocardium, smooth muscle and endothelial cells of the OFT, and the right and left atria [11]. SHF cells are characterized by the defining properties of elevated proliferation and differentiation that is delayed relative to cells that give rise to the linear heart tube [12, 13]. In all vertebrates, the SHF population of cardiac progenitor cells is located in the pharyngeal mesoderm adjacent to the FHF progenitors [14]. In the absence of SHF addition, heart tube elongation and looping fail. Additionally, improper specification of the SHF progenitors or the failure of the addition by SHF cells to the heart tube often leads to congenital
heart disease (CHD) in humans and comparable CHD-like phenotypes in mouse models [11, 12].

Although fish do not have a four-chambered heart, the SHF still is conserved in vertebrates. However, in zebrafish the SHF contributes to around 40% of the ventricular cells and the OFT. The existence of the vertebrate SHF likely came about during the transition from aquatic to terrestrial life. Vertebrate hearts acquired an additional set of cardiac components to separate the systemic and the pulmonary circulations, such as the right ventricle, which is mostly derived from the SHF. Thus, the SHF in zebrafish, which does not have separate pulmonary circulation, argues the idea that the SHF and pulmonary circulation coevolved in terrestrial vertebrates [11]. Existence of SHF in fish and amphibians suggests that the progenitor cells of the SHF are not required simply for the development of the four chambered heart, rather, they play a more ancient role in the development of the vertebrate heart [14].

Canonical and non-canonical Wnt, FGF and BMP signaling regulate SHF development

A variety of different factors control heart development to ensure its proper formation. As the vertebrate heart has two main origins of cells, the FHF and SHF, different factors are needed to control either one, or sometimes both heart fields. Cells of the FHF are marked by expression of T-box 5 (Tbx5) and the first wave of Nkx2.5 expression [15, 16]. A number of factors including GATA binding protein 4 (Gata4) and NK2 homeobox 5 (Nkx2.5) are induced in both the
differentiation of the FHF and the SHF to create a cross-regulatory network [17] dependent on various temporal signals (Figure 3, [18]).

Canonical and non-canonical wingless (Wnt), fibroblast growth factor (FGF) signals, and bone morphogenic protein (BMP) signals are integrated to control the differentiation of the SHF during heart tube elongation [14]. Specifically, canonical Wnt signaling promotes proliferation of SHF progenitors, thus inhibiting cardiac differentiation by Islet-1 (Isl1) [1, 19-21] and maintaining it in an undifferentiated state [22]. As have been demonstrated in many other contexts, non-canonical Wnt signaling directly opposes canonical Wnt signaling and promotes cardiac differentiation [20]. In order to progress from cardiac progenitors to myocardium, both the first and second heart fields must down-regulate canonical Wnt signaling, while receiving high levels of BMP signaling, and up regulate non-canonical Wnt signaling [12].

Additionally, precise levels of FGF signaling are required for SHF deployment; specifically, FGF8 has been shown by conditional mutagenesis to be the major FGF ligand driving SHF development [23, 24]. FGF8 promotes proliferation and survival of cells in the SHF through cell type autonomous signaling in the pharyngeal mesoderm. Autocrine FGF8 signaling controls SHF development, which is required later in SHF derived myocardium for robust OFT cushion development and septation [25].

While FGF promotes proliferation, BMP signaling acts to provide a differentiation signal to the cells of the SHF. BMPs might act in a concentration
dependent manner to induce or repress cardiogenesis [26]. In the heart progenitors, BMPs work as a part of transforming growth factor beta (TGF-ß) signaling [12]. BMPs bind to a receptor protein that is able to phosphorylate Similar to Mothers Against Decapentaplegic (SMAD) 1, 5, and 8/9 to induce intracellular signaling [27] and are responsible for inducing cardiac differentiation [12]. Specifically, BMP2/4 induce Nkx2.5, Gata4, and ventricular myosin heavy chain (Vmhc) expression, all of which are SHF precursors [28-30]. After initial heart specification, BMP signaling is still necessary for SHF progenitors to differentiate as myocardium.

Furthermore, the developing heart receives signals from the retinoic acid (RA) signaling pathway. In addition to regulating overall heart development, RA signaling plays a role in establishing the posterior boundaries of the SHF in the embryo [31, 32]. RA signaling is required over E9-10.5 time window for development of the most distal region of the OFT and for subsequent OFT alignment and septation in mice [33]. RA signaling plays sequential roles in SHF development, initially to delimit the progenitor cell territory and later, in the specification of distal OFT progenitor cells [33].

*Nkx2.5 is necessary for overall heart development*

Nkx2.5 plays a critical role in SHF development by inhibiting BMP signaling via Smad1, which holds the second lineage of myocardial cells in a proliferative state as the FHF differentiates [29, 34]. With an Nkx2.5 KO mouse, Prall et al., [34] saw that the SHF differentiated with the FHF to form the initial
cardiac tube, rather than remaining as progenitors for later addition to the heart tube. Moreover, Nkx2.5 has multiple CIS regulatory elements; one drives the expression of the entire heart, while the other drives expression for the right ventricle and the OFT [29]. This suggests that there are other networks that maintain Nkx2.5 expression in the regions of myocardial progenitors that are added after the heart tube has formed [12].

*Mef2cb, Ltbp3, and FGF8 work together to form the SHF in zebrafish*

As mentioned above, there are a number of signaling pathways that work together to control SHF development and formation. SHF precursors are known to express Myocyte enhancer factor 2c (Mef2c) or the zebrafish homolog, Mef2cb, which is an established regulator of SHF development [35] and activates the FGF signaling pathway [35, 36]. Mef2cb and FGF8 are both required for late cardiomyocyte addition of the arterial pole in zebrafish [35, 36]. *Latent TGF-β binding protein 3 (ltbp3)* transcripts mark a field of cardiac progenitor cells that maintain characteristics of the SHF in mammals and in zebrafish [37]. Ltbp3 expressing cells make up the distal half of the ventricular myocardium and the myocardium of the OFT. Zebrafish morphant embryos lacking *ltbp3* fail to form the ventricular OFT [37], confirming its role in SHF development across organisms. Also, SHF progenitors are specified properly in *ltbp3* morphants but they fail to proliferate or self-renew the progenitor pool as they enter the heart. Furthermore, when embryos are treated with TGF-β inhibitor they phenocopy
ltbp3-/− phenotypes, implicating TGF-β signaling in controlling the proliferation or renewal of the SHF progenitors during arterial pole development [37].

Requirements of Pbx proteins in heart development

While the requirements of many factors have been defined in SHF development, we still do not have a complete understanding of all the inputs that control SHF and OFT development. Recent work has indicated that mutations in Pbx homeodomain transcription factors are associated with OFT defects in humans [38, 39]. However, the mechanisms underlying these defects are not well understood. Mouse Pbx1 knockouts (KOs) have demonstrated it is the major developmental Pbx protein, as its loss results in significant branchial arch artery and ventricular OFT defects, in particular persistent truncus arteriosus (PTA) [40]. Despite the requirement for Pbx1, there are likely redundancy or dosage effects. Normally, Pbx2 KOs are homozygous viable and Pbx3 KOs are neonatal lethal. However, these KO embryos are sensitized to loss of a single Pbx1 allele, displaying a spectrum of cardiac malformations in the OFT that range from mild, such as bicuspid aortic valve, to more severe, such as tetralogy of Fallot (TOF) and double outlet right ventricle (DORV) [38]. Based on the murine Pbx KO phenotypes, Arrington et al., [39] hypothesized that defects in the Pbx family may be found in patients with congenital heart defects. In patients screened for mutations in Pbx1-4, they found a missense variant in Pbx3 (p.Ala136Val) in five patients with bicuspid aortic valve/coarctation of the aorta, hypoplastic left heart syndrome (HLHS), DORV, PTA, transposition of the great arteries (TGA), and/or
atrioventricular septal defects. Alanine 136 is the seventh alanine in a nine alanine motif in Pbx3 that is highly conserved between species and in different PBC proteins; a computer based, in silico analysis found this to be mutation likely to cause harm [39]. Together, these studies implicate Pbx proteins to be essential for the formation of the heart.

**Pbx: More than Hox Cofactors**

In a Drosophila screen for mutations affecting early patterning, Extradenticle (Exd) was discovered due to its ability to alter Hox patterning activities [41]. Exd encodes a divergent homeodomain protein related to vertebrate Pre-B cell leukemia homeobox protein (Pbx) [41], which was independently identified due to a t(1:19) mutation in a large number of human pre-B Acute Lymphocytic Leukemia patients [42, 43]. The homologs of Exd and Pbx are joined into the PBC family of three amino acid loop extension (TALE) proteins, referring to the conserved PBC motif N-terminal of their TALE homeodomain [44]. TALE protein family members have the same structure including a conserved homeodomain, which contains a three amino acid loop extension between the first and second helix, giving the family its name. The PBC subclass of proteins also includes Myeloid Ecotropic Integration Site (Meis) proteins [45, 46] and PBX regulatory protein (Prep or Pknox) [47]. All three groups, Pbx, Meis and Prep, contain a DNA binding homeodomain towards the carboxy terminus and two protein interacting domains by the amino terminus (Figure 5). In Pbx proteins, the protein interacting domains are PBC-A, and -B.
Whereas, in Meinox, the protein interacting domains are called the Meis-A and –B domains (Figure 5) [48].

In mammals, there are four *Pbx* genes (*Pbx1*-4). The Pbx proteins are extremely well conserved in the homeodomain, the PBC-A, and the PBC-B domains [49]. Pbx1-3 are about 430 residues long, while the mammalian Pbx4 is shorter due to it missing a part of the PBC-A domain and the carboxy terminal domain. At least Pbx1 and 3 have alternative splicing to code for different variants. *Pbx1* codes for variants Pbx1a and b, while *Pbx3* codes for variants Pbx3a and b. These splice variants differ in the carboxy terminus after the homeodomain [48, 50]. It is important to note that zebrafish Pbx4, which was found in a forward genetic screen, is actually most closely related to the mammalian Pbx3, but it is the functional equivalent of mammalian Pbx1. The divergent mammalian Pbx4 is only found in the testis [51]. Since Pbx proteins are so similar in structure, any functional differences between *pbx* genes are most likely due to differences in their expression rather than in their biochemical properties. For example, the biochemical properties of Pbx1-3 are so similar that they exhibit identical DNA cooperative binding with a subset of Hox proteins in vitro.

*Cellular Functions of Pbx Proteins*

The cellular localization of Pbx proteins has mainly been studied in Drosophila. Pbx homologs have two nuclear localization signals (NLS), NLS1 and NLS2, within the homeodomain [52, 53]. NLS1 is weaker and located on the N-
terminal arm, while NLS2 is stronger and located in helix 3. [48]. The localization of Exd/Pbx is dependent on the balance between nuclear import and export [54], which is mediated by the NLS and the nuclear export signal (NES) in concert with the nuclear import and export pathway [52, 54, 55]. The PBC-A domain of Pbx contains a NES-like sequence that inhibits nuclear localization by binding intramolecularly to its own homeodomain to mask the NLS [53]. Additionally, phosphorylation of serine residues in the PBC-B domain of Pbx1 regulates Pbx nuclear localization [56]. The nuclear localization of Pbx is also dependent on dimerization. Mainly, Exd dimerizes with homothorax (hth), the single Meinox gene that is present in Drosophila and is considered to be the main ancestral ortholog of Meis and Prep1 proteins. In the absence of Hth, Exd remains localized in the cytoplasm [52, 57].

Interactions with other proteins are essential to Pbx functions. The most studied interactions are with Meis/Prep and Hox. Extremely well conserved sequences in Meis and Prep proteins within the Meis-A and -B domains are required for interactions with Pbx proteins. The Meis/Prep domains interact with the PBC-A or -B domains, with the Meis-A domain being crucial for the formation of a complex [58]. The formation of a Pbx-Meis/Prep complex improves the binding with DNA and the selectivity of the protein for its required DNA sequence [58-61]. In order to bind DNA the Meis/Prep-Pbx complex must have both homeodomains available. Mutations in either homeodomain will prevent binding;
thus, it is reasonable to infer that both homeodomains may contact DNA during binding with Pbx.

In addition to Meis/Prep proteins, Pbx proteins interact with Hox transcription factors. Hox genes encode homeodomain-containing DNA binding proteins, with one of their earliest, best studied requirements being patterning of the anterior-posterior body axis of metazoan embryos [41]. Hox genes are able to achieve their necessary DNA binding specificity through co-factors. The first evidence that Pbx acts as a co-factor for Hox was discovered with the identification of paired Hox/Pbx regulatory elements in the promoters of the Hox genes themselves [41]. Pbx cooperates with a broad subset of Hox proteins to bind a paired recognition element on DNA [62, 63]; specifically, Pbx proteins are able to bind with Hox proteins from paralog groups 1-10 [41, 64]. The primary interaction between Pbx/Exd and Hox is through binding of the TALE on Pbx to a hexapeptide motif on the Hox protein [63, 65-69]. Hox recognition elements consist of a paired Hox/Pbx binding site, often with a Meis/Prep site nearby [41]. Together, Hox and Pbx proteins control a number of developmental processes [41]. Like with Meis/Prep, the Pbx-Hox complex increases the DNA binding activity and selectivity of Hox proteins. In the complex, each homeodomain binds one half of an octameric DNA sequence and the third helix of each homeodomain inserts into the main groove of the DNA; this complex then wraps around the DNA molecule [67, 69-71]. The affinity for DNA varies for different Hox proteins; some of them have better affinity when interacting with Pbx, while for some the
interaction has no effect. As a result of the different binding affinities, there are different downstream effects from these types of Hox proteins when the hexapeptide is mutated. For example, posterior Hox proteins, are still able bind to their target, but fail to activate them with a mutated hexapeptide, thereby acting as repressors. Conversely, anterior Hox proteins lacking the hexapeptide will not bind DNA and will recapitulate a loss-of-function phenotype [41, 72].

Importantly, as Hox and Meis bind to different domains on the Pbx protein, the three can form trimeric complexes. To do this, the trimeric complex must bind to a DNA sequence that can accommodate all three homeodomains. However, there are examples where Meis is also able to bind to a complex of Hox-Pbx without binding DNA, though this interaction requires the carboxy terminus of Meis. Of the relatively small number of direct Hox targets that have been defined in vertebrates, all are comprised of paired Hox/Pbx or Hox/Pbx/Meis sites and all require the Pbx binding component and/or the Meis binding component of these sites for their transcriptional activity [59, 73-79].

Although the most studied interactions of Pbx proteins have been with Hox and Meis/Prep factors, they may also act to effect activities of non-homeodomain transcription factors such as Myogenic Differentiation (MyoD) and myogenic factor 5 (Myf5) [80]. MyoD activates expression of Myogenin and others factors required for specification of skeletal muscle [81, 82]. Chromatin remodeling and activation of a subset of these targets involves the binding of MyoD-Pbx-Meis complex to a divergent E-box element in their promoters. Pbx-Meis can bind the
element in undifferentiated cells in the absence of MyoD. Thus, one can postulate that Pbx-Meis acts as a beacon for the subsequent recruitment of MyoD and activation of the myogenic program [41, 44, 83, 84].

Requirements for Pbx proteins in development

In order to study the requirement of Pbx proteins, the first member of the family to be studied through KO methods was Pbx1. Most defects in these mutants affect organ systems where Hox proteins have been shown to control the developmental programs. In 2001, Selleri et al., [49] found that Pbx1 is required for skeletal patterning and programming chondrocyte proliferation and differentiation. Pbx1 may also play a role in patterning the vertebrate limbs, as suggested by its restricted expression within the limb buds, like Exd, and by the malformations in the proximal skeletal elements [49]. Subsequently, Chang et al., [40] found that Pbx1 is required to pattern the great arteries of the heart, branchial arch arteries and cardiac OFT septation in mice. Pbx1-null embryos also lose expression of Pax3 in the cardiac neural crest cells that should ultimately specify the cardiac neural crest cells to function for OFT development [40]. It is possible that Pbx1 acts as a promoter of proliferation of progenitor cells in vertebrates as every organ system that is affected by the Pbx1 mutation is hypoplastic and/or aplastic and is characterized by a reduction in the number of proliferating cells [38].

In contrast to Pbx1, despite being widely expressed during development, Pbx2-null mice are born with expected Mendelian frequencies and show no
detectable abnormalities in development, organogenesis or in reduction of long-term survival [85]. Because Pbx2 KO mice have no phenotype, this suggests that there may be redundancy between members of the Pbx family. The idea that some Pbx genes may be able to compensate for each other was reinforced by Pbx1/Pbx2 double KO mice, which have a much more severe skeletal phenotype than KOs of either gene alone, usually lacking limbs altogether and having a variety of skeletal and bone abnormalities [86]. Furthermore, Pbx1+/−; Pbx2−/− mice die within 24 hours after birth [38]. As referred to above, angiography showed that there is a variety of heart defects in these mice, including an abnormal connection of the right ventricle and ascending aorta, additionally, the aorta straddled both the right and left ventricles implying that Pbx2 has roles in the alignment of the left ventricular OFT [38]. These defects found only with double KOs suggests that there is an important dosage effect of Pbx proteins, as some Pbx proteins can act to replace others when they are absent or their expression is reduced.

While not embryonic lethal, Pbx3 KO mice die within one day of birth as a result of central hypoventilation [87]. The central hypoventilation in Pbx3 deficient mice is due to abnormal activity of inspiratory neurons in the medulla [87]. This is indicative that Pbx3 is essential for respiration and is critical for proper development of the medullary respiratory control mechanisms [87]. Again in support of redundancy between Pbx proteins, embryos with compound Pbx1+/−; Pbx2−/−; Pbx3+/− mutations show heart characteristics that are consistent with
TOF. Moreover, these mutants show a generalized edema that is not present in 
\(Pbx1^{+/+};Pbx2^{-/-}\) embryos. \(Pbx1^{+/+};Pbx2^{-/-};Pbx3^{+/-}\) embryos also have abnormal 
bicuspid aortic and pulmonic valves as compared to their \(Pbx1^{+/+};Pbx2^{-/-}\) 
counterparts [38].

As mentioned above, the zebrafish Pbx4, referred to as \(lzar\), is most closely 
related to mammalian Pbx3, but is the functional equivalent of mammalian Pbx1. 
The \(lzar\) allele is a nonsense mutation that occurs in exon 1 of \(pbx4\), resulting in a 
truncated, nonfunctional protein. The most prominent defects in \(lzar\) mutants are 
hindbrain defects. Specifically, \(lzar\) mutants have a subset of motor neurons that 
are born in rhombomere 4 in the hindbrain which fail to undergo their normal 
posterior migration into rhombomere 6 and 7. The hindbrain defects in \(lzar\) 
mutants phenocopy both Hoxb1 mutants in mice, and \(hoxb1a\) depleted zebrafish 
embryos [88]. In addition to the hindbrain defects, the \(lzar\) mutants have jaw 
defects, lack of pectoral fins, abnormal heart morphology, and poor circulation. 
Despite the variety of defects in \(lzar\) mutants, expression of many of the \(Pbx\) 
genes can rescue the zebrafish \(pbx4^{-/-}\) mutants [89].

Consistent with the physical interactions of Pbx proteins with Meis/Prep 
proteins, Pbx KO and/or knockdown models share phenotypic similarity with Meis 
and Prep mutants. Stankunas et al., [38] found that Meis1 is required for proper 
cardiac development in mice. Meis-/- mutants died between E14.5 and E15.5. 
Cardiac analysis found that the aorta was septated from the main pulmonary 
artery, but overrode both right and left ventricles and the embryos had a ventricle
septal defect. These defects fall within the realm seen with Pbx deficiencies, suggesting that the two work together during heart development [38]. Zebrafish embryos injected with mRNA for Meis or with morpholinos (MOs) against Prep1.1 are able to phenocopy the majority of the hindbrain defects found in lzc mutants [90-93]; however, Prep1.1 morphants have additional mutations not found in lzc mutants, such as impaired migration of facial nerve motor neurons, and the inability for cells to differentiate into chondroblasts. [91].

**Zebrafish use as a developmental model**

Zebrafish have become an increasingly popular genetic and developmental model over the past 20 years due to many experimental and husbandry advantages. Compared to other vertebrate models, zebrafish have short generation times and rapid development. Embryos are readily produced in large numbers and are transparent, allowing for high-resolution live visualization of the heart and other organs during development [94]. Exploring the effects of drugs on development can easily be done through immersion of the embryos in treated water. Zebrafish transgenic lines can be created easily to visualize certain areas of interest or even modify an area of interest. Additionally, zebrafish can also be easily manipulated genetically with CRISPR/Cas9 techniques, and molecular signaling pathways with RNA or MO injection. Moreover, the zebrafish remains largely optically clear for about 40 hpf, after which they can remain optically clear with chemical treatments. With respect to the heart, the embryos can survive without circulating blood until the larval stages, as it is able to get
oxygen via absorbance though the skin. This feature in particular allows for study of a dysfunctional heart and cardiovascular system [95]. Significant cardiovascular defects can easily be observed in live embryos, while more subtle defects can be seen with molecular markers or transgenic embryos [5, 94, 96, 97]. Therefore, the use of zebrafish to study cardiac formation provides significant advantages over other model organisms.

**Significance of Outflow tract defects**

Heart development in vertebrates is tightly controlled, with the early steps being particularly highly conserved. The vertebrate heart is very sensitive to perturbations from many different signals. The intricacies of the complex inputs for normal heart development are evident by the high frequency of CHDs, which occur in 1% of all births per year in the United States [98]. More specifically, anomalies in the OFT account for 20-30% of all congenital heart defects [99-102]. Common OFT defects are PTA, TGA, DORV, and TOF. PTA occurs when there is a single outflow tract instead of a separate aorta and pulmonary artery. TGA consists of the great arteries being switched so that the aorta arises from the right ventricle and the pulmonary artery from the left ventricle. DORV has both great arteries stemming from the right ventricle. Lastly, TOF consists of multiple defects including, septal defects, right ventricular outflow tract obstruction, aorta overriding the ventricular septum and right ventricular hypertrophy. Some OFT defects are associated with chromosomal abnormalities such as trisomies 13, 18 and 21 and about 6% of OFT occur with 22q11 microdeletion syndrome [103-
110]. Most other causes and mechanisms of OFT defects are not well understood. As Pbx proteins have been shown to function in the formation of the OFT, it is beneficial to study the mechanisms underlying abnormal OFT development to allow for a greater understanding of OFT defects and perhaps provide potential for novel therapies or prevention of these defects.
Chapter 1 Figures

Figure 1: Heart Development in Mammals (Adapted from Laugwitz et al., 2008).
(A) Progenitors of the cardiogenic mesoderm are first recognizable under the head folds of the mouse embryo at E7.5, then move ventrally to the midline and form the initial linear heart tube and then, ultimately, the four chambered heart. After the looping of the heart tube (E8.5), the cardiac neural crest progenitors migrate to the tube to contribute to the vascular smooth muscle cells of the outflow tract. Around E14, the chambers separate due to septation and are connected by the pulmonary trunk and aorta. AA, aortic arch; IVS, interventricular septum; LA, left atrium; LV, left ventricle; PhA, pharyngeal arches; PLA, primitive left atrium; PRA, primitive right atrium; RA, right atrium; RV, right ventricle.
Figure 2: Heart Development in Zebrafish (Adapted from Bakkers, 2011). (A) At 5 h post fertilization (hpf) the cardiac progenitor cells are located bilaterally in the lateral marginal zone. Atrial progenitor cells (pink) are located more ventrally than the ventricle progenitor cells (light blue). Cardiogenic differentiation is initiated in the future ventricle myocardial cells by the expression of cardiac myosins (purple) at the 12-somite stage (B). During mid- and late-somite stages, the myocardial tissue expands by continuous cardiogenic differentiation into more lateral regions of the ALPM by the cardiogenic differentiation of future atrial myocytes (orange; venous differentiation). Whilst the endocardial cells (light green) have already migrated from the ALPM towards the mid-line, myocardial cells follow this behaviour slightly later (C). When the bilateral heart fields fuse at the mid-line, they form a cone, with a ring of ventricular cells surrounding by atrial cells (D). Cardiac morphogenesis transforms the cardiac cone into a cardiac tube (E). (F) Cardiogenic differentiation continues at the arterial pole, and as a result new cardiomyocytes are added to this region (purple gradient). At 36 hpf, cardiac looping has started, with a displacement of the ventricle towards the mid-line, and the constriction at the position of the AV canal is first visible (F). The heart tube continues to loop and forms an S-shaped loop (G).
Figure 3: Phylogeny and structure of Hox cofactors (Adapted from Moens and Selleri, 2006). Homeodomain proteins are divided into two groups: the PBC family, including the vertebrate Pbx proteins, fly Extradenticle and worm Ceh-20, and the MEIS family, including vertebrate Meis and Prep, fly Homothorax (Hth) and worm Unc-62. Orange letters indicate mouse proteins, purple lettering indicates their zebrafish orthologs.
Figure 4: Cardiac gene regulatory network (Adapted from Paige et al., 2015). The diagram shown is a brief overview of a subset of all known transcription factor interactions and signaling pathways that drive the differentiation of first heart field (FHF; orange) and second heart field (SHF; blue) cardiac progenitor cells during development. Factors colored by both orange and blue represent regulators of both FHF and SHF. Arrows indicate increased expression of 1 transcription factor or signaling molecule because of activity of another transcription factor. Signaling pathways that activate expression of certain transcription factors are shown in red. BMP indicates bone morphogenetic protein; and SHH, sonic hedgehog.
Figure 5: Structure of TALE homeodomain proteins. All TALE proteins contain a homeodomain containing the Three Amino Acid Loop Extension (TALE) between the first and second alpha-helices. Pbx proteins contain an extra domain, HCM, which contacts DNA.
Materials and Methods

Zebrafish Husbandry and Lines:
Zebrafish were raised and housed under standard conditions [111]. The following transgenic and mutant lines were used alone and in combination: \textit{Tg(myl7:EGFP)} [112], \textit{Tg(-5.1myl7:DsRed-NLS)} [113], \textit{TgBAC(-36nkx2.5:zsyellow)} [37], \textit{Tg(myl7:Kaede)} [36], and \textit{Izr} [88, 114].

Drug Treatments:
For treatment with RA, and SU5402 (FGF inhibitor) at 24 hpf, 30 embryos were placed into glass vials with RA (0.1x10^{-6} M) or SU5402 (10µM). Control embryos were placed into vials containing only blue water. Embryos were placed on a nutator at 28°C overnight and were fixed for analyses at 48 hpf.

\textit{In Situ Hybridization (ISH)}:
Standard protocol was followed as described previously [115]. The following probes were used: \textit{amhc} (ZDB-GENE-031112-1), \textit{cmic2/myl7} (ZDB-GENE-991019-3), \textit{eln2} (ZDB-GENE-061212-2), \textit{mef2cb} (ZDB-GENE-040901-7), \textit{nkx2.5} (ZDB-GENE-980526-321), \textit{nppa} (ZDB-GENE-030131-95), \textit{vmhc} (ZDB-GENE-991123-5)

\textit{Immunohistochemistry and Cell Counting}:
Wholemount immunohistochemistry (WIHC), cell counting and statistical analysis were done as described previously [116]. For the Phospho-Histone H3 (PHH3) staining, the standard IHC was followed on \textit{Tg(nkx2.5:ZsYellow)} embryos. An
anti-PHH3 antibody (Abcam) was used along with a Living Coral Reef (LCR) antibody (Clontech). To examine the amount of BMP signaling through the pSMAD assay, \textit{lzr}; \textit{Tg(cmlc2:EGFP)} embryos were used, and underwent the ICH protocol with a Chicken anti-GFP antibody (Abcam) and a pSMAD1/5/9 antibody (Cell Signaling).

\textit{Kaede analysis of SHF addition:}

Embryos from a \textit{lzr}^{+/c}; \textit{Tg(myl7:Kaede)} incross were photoconverted using a DAPI filter at 36 hpf, sorted into wild-type (WT) siblings and \textit{lzr} mutants, and then imaged at 48 hpf using a Nikon A1-Confocal microscope. \textit{Tg(myl7:Kaede)} embryos injected with a Pbx4 MO cocktail at the 1 cell stage were handled in the same manner. The area of the OFT was measured and compared between mutants/Pbx4 depleted embryos and their WT siblings using ImageJ and a Student's t-test.

\textit{mRNA and MO Injections:}

Injections were performed at the one cell stage. MOs for Pbx4 were injected as described in Maves et. al, 2007 \cite{80}; \textit{pbx4}-MO1, AATACTTTTGAGCCGAATCTCTCCG and \textit{pbx4}-MO2, CGCCGCAAAACCAATGAAAGCGTGTT, were injected together at a dose of 0.5 mg/mL each.
Reverse transcriptase quantitative PCR (RT-qPCR):

RT-qPCR was performed as previously described [117, 118]. Whole embryo RNA was obtained from groups of 30 embryos, which were homogenized in Trizol (Ambion) and isolated using the Purelink RNA Microkit (Invitrogen). cDNA synthesis was made using 1 µg of RNA using the ThermoScript Reverse Transcriptase kit (Invitrogen). Quantitative real time PCR was done with Power Sybr Green PCR Master Mix (Applied Biosystems) using standard PCR conditions in a BioRad CFX-96 PCR Machine. Expression levels were standardized to β-actin expression levels and analyzed using the $2^{-\Delta\Delta CT}$ Livak Method. Primer sequences are shown in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>ltbp3</td>
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<td>GTTTTGGTACTGTGAGGCTTG</td>
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Table 1: Primer Sequences for RT-qPCR
Chapter 2: Results

*Pbx4 is required to limit the size of the ventricle*

Previous studies indicated that Pbx proteins are required for proper heart development in vertebrates [38-40]. However, the mechanisms underlying the heart defects in Pbx deficient animals, including *lzr* mutants, are not completely understood. The first characterization of *lzr* mutants mentioned they have thin, weakly beating hearts that are accompanied by severe pericardial edema [88]. Examining the hearts of *lzr* mutants in live embryos we were able to confirm these phenotypes (Figure 1). In order to understand the cardiac defects in *lzr* mutants, we began by examining their hearts at 48 hpf with WIHC using the sarcomeric actin (MF20) and atrial myosin heavy chain (S46) antibodies (Figure 2A,B) and with ISH using probes for the pan-cardiac marker, *myosin light chain 7* (*myl7*, also known as *cm1c2*) and the cardiac stress marker *natriuretic peptide A* (*nppa*, also known as *anf*). Both these assays indicated that *lzr* mutant hearts were not looped properly and had variable ventricular outpocketing and atrial ballooning (Figure 2E,F). We are not aware of any other published zebrafish mutants having a similar cardiac defect. Therefore, *lzr* mutant hearts have a unique heart morphology.

In order to determine how early during development we can discern cardiac defects in *lzr* mutant embryos, we used ISH embryos and probed for *myl7* in embryos at the 20 somite stage, 24 hpf, 30 hpf, and 48 hpf. We were not able to distinguish *lzr* mutants from their WT siblings at the 20 somite stage (not shown),
when the cardiomyocytes have begun to form a cone. Additionally, using the pbx4 MOs we found that there is no difference in cardiac markers in pbx4 deficient embryos prior to 24 hpf (Figure 3A,B). However, we found that as early as 24 hpf when the heart is beginning to extend, the lzr mutants, i.e. around 25% of embryos had developed ventricular outpocketings, which were also evident at 30 hpf (Figure 3C-H). Therefore, these results indicate that the initial wave of cardiac migration and differentiation occurs appropriately in lzr mutants, suggesting that Pbx4’s role in heart development occurs later.

Next, we wanted to determine if the differences in cardiac morphology correlated with defects in cardiomyocyte number. To quantify cell number in the heart in the lzr mutants we crossed the allele into the Tg(myl7:nuc-DsRed2) line, which expresses nuclear DsRed in all chambers of the heart and counted the cardiomyocytes in each chamber following WIHC at 48 hpf, as previously described [116]. Surprisingly, we found that there was a significant increase in the number of ventricular cells in the lzr mutants, whereas there was no change in the number of atrial cells (Figure 4D). Depletion of Pbx4 with MOs gave a virtually identical significant increase in ventricular cell number with no change in the number of atrial cells (Figure 4E). Additionally, we found that the increase in ventricular cell number correlated with increases in cardiac differentiation markers through RT-qPCR at 48 hpf (Figure 5). Therefore, lzr mutants have a specific increase in ventricular cardiomyocyte number,
suggesting Pbx4 is one of the only identified protein that is required to specifically limit the number of ventricular cells.

**Pbx4 is required to limit the amount of SHF addition**

Since we were not able observe a difference in the early wave of cardiomyocyte differentiation at the 20s stage with markers including vmhc (Figure 6A,B), this suggested that there might be defects in the SHF development. Therefore, we first examined the SHF marker mef2cb using ISH at the 20s stage. However, we found that there were no differences in expression of mef2cb (Figure 6C,D), suggesting the SHF is initially specified correctly. We next examined markers of the SHF through RT-qPCR at 36 and 48 hpf and found that at these later stages lzr mutants do have an increase in SHF progenitor marker expression (Figure 6E). Since our marker analysis suggested that lzr have an increase in SHF progenitors at stages after the SHF is specified, we examined SHF-derived smooth muscle using ISH for elastin2 at 72 hpf, its earliest onset of expression [119]. We found that it, too, was increased in lzr mutants (Figure 6F-H). Therefore, these results suggest the FHF and SHF are initially specified correctly in lzr mutants. However, the lzr mutants have an increase in the expression SHF progenitor markers and SHF-derived OFT smooth muscle, suggesting Pbx4 plays a role later restricting the size of the SHF progenitor pool.

Because our analysis indicated we were getting a specific increase in SHF-derived lineages after the SHF is initially specified, we wanted to explicitly test if there was an increase in SHF-derived ventricular cardiomyocytes. To
determine the amount of SHF addition in _lzr_ mutant embryos, we crossed adult _lzr_ carriers to the _Tg(myl7:Kaede)_ line. In this transgenic line, the photoconvertible protein Kaede is expressed specifically in cardiomyocytes and has previously been used as a tool to assess the amount of cardiomyocyte addition from the SHF [36]. Embryos from a _lzr^{+/-};Tg(myl7:Kaede)_ cross were collected and screened at 24 hpf for green Kaede-expressing hearts. The embryos with green Kaede+ hearts were photoconverted to red at 36 hpf using the DAPI filter of a Zeiss M2Bio dissection microscope and then imaged at 48 hpf with confocal microscopy (Figure 7A). We found that there was an increase in the amount of later differentiating, newly expressing green only, SHF-derived ventricular cardiomyocytes added to the OFT in _lzr_ embryos compared to their WT siblings (Figure 7C,E). Similar results were found performing the experiment with the _pbx4_ MO depleted embryos. (Figure 7D,F). Therefore, the _lzr_ mutants’ increase in ventricular cell number and outflow tract size is likely due to an increase in addition from the SHF.

*Pbx4 functions downstream of known OFT effectors*

Although many factors have been shown to promote SHF addition, Pbx4 is unique in its ability to specifically restrict SHF addition and one of only two identified factors to have this requirement. Therefore, we next wanted to understand the relationship between Pbx4 and some of the known effectors of OFT and SHF development in zebrafish. Recent unpublished work from the Waxman lab has indicated that increasing retinoic acid (RA) signaling limits SHF
addition. RA signaling represses and is functionally upstream of FGF signaling which promotes SHF progenitor proliferation in vertebrates [23, 24]. To determine the relationship of Pbx4 to RA and FGF signaling in SHF development, we tested the effects of increased RA and decreased FGF signaling on pbx4 depleted embryos treating with RA, or the FGF receptor 1 inhibitor, SU5402 beginning at 24 hpf. Previous studies have shown that treatment with RA and SU5402 will reduce ventricular number [116, 120, 121]. Consistent with these studies, we found that embryos treated with RA or SU5402 had a reduction in the number of ventricular cells when compared to their WT siblings (Figure 8A, B-D). Interestingly, we found that the same drug treatments administered to Pbx4 depleted embryos did not produce an equivalent reduction in ventricular cell number (Figure 8A, E-G). Therefore, these observations suggest that Pbx4 is functioning downstream or parallel to RA and FGF signaling in determination of SHF size.

*BMP10 and FoxH1 are candidate downstream effectors to Pbx4*

To garner a further understanding of the mechanisms by which Pbx4 functions in SHF development, we performed transcriptome analysis with RNA-seq of lzf mutants and their WT siblings at 36 and 48 hpf. In addition, we compared these results to RNA-seq from embryos and isolated hearts with increased RA signaling. Two of the notable candidates we found with increased expression in lzf mutants were BMP10 and forkhead box H1 (FoxH1). Additionally, cyclin-dependent kinase inhibitor 1a (Cdkn1a) was downregulated in
the lzd mutants. This was particularly interesting because BMP10 and FoxH1 are expressed in the heart and are required for its development. BMP10 mouse KOs have a variety of heart defects, including a decrease in ventricular wall thickness, and defects in ventricular growth regulation [122]. KOs of BMP10 also have decreased proliferation and differentiation [123]. FoxH1 is required for SHF development in mice. FoxH1 KOs in mice lack both the OFT and right ventricle; it has also been shown that FoxH1 has a significant role in heart development through interactions with Nkx2.5 in order to mediate activation of TGF-β signaling and Mef2c [124]. Interestingly, promoter analysis of bmp10 using the UCSC genome browser has indicated it has a highly conserved FoxH1 binding site (not shown).

To test the hypothesis that BMP signaling is increased in lzd mutants, we performed WIHC to measure the amount of phospho-SMAD (pSMAD) signaling occurring in or near the OFT of the heart. The amount of pSMAD1/5/9 that is present is proportional to the amount of BMP signaling in a tissue [125], as BMP is able to activate SMADs1/5/9 indirectly through type 1 receptors. We found that lzd mutants have a significant increase in the number of cells with pSMAD1/5/9 in the newly differentiating ventricular cells of the OFT and in the cells adjacent to join the OFT of heart (Figure 9). In Lzr mutants, 91% (n=63/69 cells) of differentiated or newly differentiating cells expressing GFP in the OFT also express pSMAD1/5/9, whereas in their wildtype siblings, only 74% (n=40/54 cells) of differentiated or newly differentiating cells expressing GFP are also
expressing pSMAD. Therefore, *lzr* mutants have an increase in BMP signaling in the OFT, which may be contributing to the ventricular and OFT growth errors seen in *lzr* mutants.

We were also intrigued that Cdkn1a expression was downregulated from our RNA-seq data. Cdkn1a is a known cell cycle regulator, monitoring the transition between G₁ and S phase. Previous work has implicated Cdkn1a to have roles in both regulation of proliferation and apoptosis [126]. Blundell et al., [126] found that Cdkn1a-null mice had increased proliferation and protection from apoptosis in airway epithelial cells. Examining Pbx4 depleted *Tg(nkx2.5:zsyellow)* embryos, which allowed the visualization of SHF cardiac progenitors, we found that the number of SHF progenitors was increased compared to control sibling embryos, consistent with our qPCR data (Figure 10A,B). To test if there is increased proliferation in the SHF progenitors of *Pbx4* deficient embryos, we performed WIHC on the Pbx4 depleted *Tg(nkx2.5:ZsYellow)* embryos and analyzed the amount of phospho-histone H3 (pHH3), a marker of mitosis, present in the heart at 30 hpf. From this, we found that Pbx4 deficient embryos have an increase in the percentage of pHH3 expressing cells present in the OFT (Figure 10D,E). Therefore, in the absence of Pbx4, there is an increase in the amount of SHF progenitor proliferation, which may be contributing to the increase in SHF-derived OFT cells.
Figure 1: *lzr* mutants have a variety of defects throughout the embryo. (A, B) Wholemount images of a wildtype sibling and *lzr* embryo at 48 hpf. *lzr* embryos display pericardial edema, lack of pectoral fins, jaw defects and abnormal heart morphology. Embryos are shown in lateral positions.
**Figure 2: IZr mutants have dysmorphic hearts.** (A,B) WIHC of wildtype siblings and IZr mutants showing ventricular outpocketing and a bulbar atrium at 48 hpf. The atrium is stained with S46 and is shown in green, while the ventricle is stained with MF20 and is shown in red. (C-F) ISH images of wildtype siblings and IZr mutants at 48 hpf. IZr mutants have variable ventricular outpocketing (arrowheads, C and D), and bulbar atriums. All embryos are shown in frontal positions with anterior up.
Figure 3: *lzr* mutants develop dysmorphic hearts as early as 24 hpf. (A, B) Morphants injected with a MO that phenocopies all aspects of the *lzr* phenotype show no change prior to 24 hpf, at 20s. (C-H) ISH of wildtype siblings and *lzr* mutants, beginning at 24 hpf, the *lzr* embryos begin to display abnormal heart morphology including ventricular outpocketing (arrowheads). In A-D, F and G embryos are shown with dorsal views with anterior down. In E, H embryos are shown with dorsal views and anterior up.
Figure 4: pbx4 deficient embryos have an increase in ventricular cardiomyocytes number. (A,B) Wholemount immunohistochemistry (WIHC) of lzr embryos and wildtype siblings at 48 hpf. The atrium is stained with S46 and is shown in green, while the ventricle is stained with MF20 and is shown in red. (C) WIHC of a pbx4 depleted embryo at 48 hpf. (D-E) Graphs showing the amount of cardiomyocytes in (A-C). Embryos are shown in frontal positions with anterior up. Asterisks indicate p<0.05 and bars represent standard error for all graphs.
Figure 5: *lzr* mutants have an increase in expression of heart field markers. *lzr* mutants have a statistically significant increase in markers of differentiated cardiomyocytes, *amhc*, *vmhc* and *cmlc2*. 
**Figure 6: pbx4 deficient embryos have properly specified FHF and SHF.** (A,B) Wildtype embryo and pbx4 deficient embryo ISH stained for vmhc. (C,D) Wildtype sibling and pbx4 deficient embryo stained for mef2cb. (E) pbx4 deficient embryos have an increase in expression of Mef2cb and and Ltbp3, second heart field progenitor markers, at 48 hpf. (F,G) lzr embryos have an increase in the length of elastin2b staining indicated by brackets. (H) Quantification of (F, G). In A-D embryos are shown dorsally with anterior down. In F and G embryos are shown laterally with anterior to the left.
Figure 7: pbx4 deficient embryos have an increase in SHF addition. (A) Schematic of the Kaede experimental timeline, fish were collected (MO was injected at 1 cell stage), screened for the transgene at 30 hpf, positive fish were then photoconverted at 36 hpf and then imaged 48 hpf. (B-D) Confocal images of zebrafish hearts from Tg(myl7:Kaede) at 48 hpf. Brackets indicate the amount of addition. (B,C) Images of wildtype siblings and lzr embryos. (D) Image of pbx4 morphant embryo. (E, F) Graphs showing the amount of SHF addition in (B-D) shown with brackets. Embryos are shown in frontal views with anterior up from Nikon A1 Confocal slices.
Figure 8: *pbx4* functions downstream or parallel to FGF/RA signaling. (A) Treatment to embryos either wildtype or *pbx4* morphants; treated morphant embryos show a rescue in the number of ventricular cells. (B, E) WT embryos uninjected or injected with Pbx4 MO under control treatment show the same increase in ventricular cells as shown previously. (C) Uninjected WT embryo treated with RA show a decrease in the size of the ventricle (F) Embryo injected with Pbx4 MO under RA treatment show an increase in the number of ventricular cells (F) compared to (C). (D) Uninjected WT embryo treated with SU5402 show a decrease in the size of the ventricle. (G) Embryo injected with Pbx4 MO and treated with SU5402 show an increase in the size of the ventricle compared to (D). Embryos are shown in frontal positions with anterior up.
Figure 9: *lzr* mutants have an increase in the amount of BMP signaling in the heart. (A,B) Confocal images of wildtype (A) and *lzr* (B) fish WIHC stained for pSMAD, representative of the amount of BMP signaling that is occurring in the heart. (C) Quantification of total cardiomyocytes and cardiomyocytes expressing pSMAD in the OFT from images (A,B). Embryos are shown in frontal positions with anterior up from Nikon A1 Confocal slices.
Figure 10: lzr mutants have an increase in the amount of proliferation in the OFT. (A,B) Confocal images suggesting the amount of proliferation was increased in lzr mutants, based on the location of the border between differentiated and undifferentiated cardiomyocytes (arrowheads), so (C,D) analysis of proliferation was done with pHH3 staining. (E) Quantification of the amount of proliferation in the OFT as marked by cells co-expressing Nkx2.5;ZsYellow and pH3 (arrowheads). Embryos are shown at 30 hpf and in frontal positions with anterior up from Nikon A1 Confocal slices.
Chapter 3: Discussion

We have examined the role of Pbx4 in heart development of zebrafish. We have found that in addition to previous known roles of Pbx4 in the hindbrain and forelimbs [88], Pbx4 plays a unique role specifically restricting addition of the SHF-derived OFT. Notably, Pbx4 deficiency results in an increase in SHF-derived ventricular cardiomyocyte number, smooth muscle, and the overall length of the OFT. The requirements restricting SHF development are downstream or parallel to RA and FGF signaling. Furthermore, our results suggest that Pbx4 may perform these functions through restricting SHF progenitor proliferation by promoting Cdkn1a expression, as well as limiting cardiomyocyte differentiation through repressing BMP10 expression. Therefore, these results indicate that *lzr* is the first mutant identified that is specifically required to limit the size of the SHF in vertebrates.

A new player in the formation of OFT defects

Previous studies have associated Pbx3 with heart development in humans [39], and other studies have found a requirement for Pbx1 in the development of the OFT in mice [38, 40]. Here, we show that zebrafish Pbx4, the functional equivalent of mammalian Pbx1, and a homolog of Pbx3, also plays a crucial role in the development of the OFT and the heart. KO studies of different combinations of Pbx1-3 proteins in mice have shown that there may be dosage requirements and redundancy for Pbx proteins in the development of the heart [38]. Although a previous study demonstrated that the PTA in Pbx1 KO mice is, in
part, due to cardiac neural crest defects, the mechanisms underlying OFT defects in Pbx deficient animals are not completely understood [40]. Despite the previous studies exploring the overt phenotypes caused by mutations in Pbx proteins, the OFT is composed of ventricular cardiomyocytes and defects in these cells were not explicitly examined in Pbx1 KO mice [38, 40]. Because our study suggests that Pbx4 is required to restrict OFT ventricular cardiomyocytes, it suggests the idea that PTA, and potentially other OFT defects, may be caused by multiple different underlying cellular defects, in particular the OFT having too many SHF-derived cells.

Pbx is known to function in a trimeric complex with both Hox and Meis proteins [59, 64], as stated earlier. Stankunas et al., [38] also found that disruption of Meis1 results in heart abnormalities that are similar to those caused by mutations in the Pbx proteins. Furthermore, Chang et al., [40] found that misregulation of Hox activity that is dependent on Pbx1 also contributes to the arch artery phenotype seen in Pbx1 KOs. Currently, there is no evidence in the *lzr* mutant that the cardiac phenotype is developing as a result of cooperation between Pbx, Hox and/or Meis. Future analysis of these relationships will be essential to determining the mechanisms by which the *lzr* phenotype develops.

**Possible mechanisms restricting SHF addition**

Our evidence suggests that Pbx4 acts downstream or parallel to previously known effectors of the SHF in zebrafish, RA [31, 33] and FGF signaling [24, 25, 32]. As heart development can be seen as a network of cross-
regulatory signals, this connection is significant in determining further mechanisms of Pbx4 with RA and FGF signaling in the SHF. While there are a number of genes that are known to effect the development of the second heart field in zebrafish, including Mef2cb [35], FGF [24, 25], Ltbp3 [37], Islet-1 (Isl1) [19, 21], lizr is the first mutant to have been found to specifically limit the size of the ventricle and the OFT. The other known effector of SHF size in zebrafish is cell adhesion molecule4 (Cadm4). Cadm4 plays a role for cell adhesion to restrain the SHF deployment to the OFT downstream of FGF signaling, which when disrupted causes OFT defects [127]. Despite the promising knockdown data, there are not currently published mutants demonstrating that Cadm4 is required to restrict OFT addition. Furthermore, we did not detect an effect on Cadm4 expression from our RNA-seq analysis, suggesting Pbx4 functions independent of or downstream of Cadm4 too.

During the initial description of the lizr mutant, Popperl et al., [88] mentioned briefly that there was a significant heart defect present in the mutant. Later, Maves et al., [128] suggested that there are redundant requirements for Pbx2 and Pbx4 proteins in the development of the heart, but this was done only through analysis of MOs. As other studies have found redundancy between Pbx2 and -4 in the hindbrain [128], it would not be surprising if there were redundancy in other tissues. Maves et al., [128] found that Pbx2 and Pbx4 were redundantly required to promote the differentiation of cardiomyocytes. Our results suggest that Pbx4 alone is required to limit OFT addition (Figure 4), obviously contrasting
with these previous results. Although these differences could be due to analysis using MOs vs. mutants, depletion of Pbx4 from MOs gave the same results. Moreover, we did deplete Pbx2 in lzr mutants and did not find a further increase in OFT cells (not shown). Therefore, while we cannot rule out that Pbx2 and Pbx4 may have redundant roles promoting cardiomyocyte differentiation, they are not redundantly required to limit OFT addition.

Our data supports, that in the absence of Pbx4, the amount of SHF progenitor proliferation is increased. This increase in SHF proliferation correlates with a decrease in the amount of Cdkn1a (also known as p21) in lzr mutants from our RNA-seq analysis. Cdkn1a has been found to control the cell cycle as a negative regulator of proliferation [126]. It would be advantageous in the future to determine if cdkn1a is directly targeted in SHF progenitors by Pbx4 to limit the amount of proliferation in the OFT potentially through WIHC and mutant models of Cdkn1a and lzr mutants.

In addition to an increase in proliferation from Cdkn1a down-regulation as a cause of the excess SHF-derived cells, our results suggest that an increase in the amount of BMP signaling in lzr embryos may contribute to increased ventricular and OFT size (Figure 9). For proper OFT development, SHF progenitor proliferation and differentiation are tightly coupled through interactions between FGF and BMP signaling. BMP10 has a number of known roles in the heart, including differentiation and proliferation as well as ventricular formation [122, 123]. BMP10 may also provide a positive growth signal for cardiomyocytes
that antagonizes negative regulators, p57 and Cdkn1a [122]. BMP10 is known to maintain expression levels of heart field and SHF progenitor markers, Nkx2.5 and Mef2cb, both of which are upregulated in IZr embryos (Data not shown and Figure 6) and are known to be critical during heart development and SHF differentiation [122]. This suggests that the increase in BMP10 signaling could be a major contributor to the addition of SHF and OFT size in pbx4 deficient embryos. As FoxH1 expression was also increased in the RNA-seq of IZr mutants, and is known to have a significant role in heart development, the fact that BMP10 maintains a highly conserved FoxH1 binding site suggests that FoxH1 may also play a role in the production of the IZr phenotype, upstream of BMP10. Clearly, future analysis will specifically examine the requirements of these factors downstream of Pbx4 in restricting OFT addition.

Overall, incorporating our observations here with recent studies involving RA signaling, we propose a working model where Pbx4 acts on FoxH1 which in turn is able to act on BMP10 to affect the amount of SHF addition and ventricular OFT size. This is in parallel to RA and FGF signaling acting in converse with Pbx4 on FoxH1 (Figure 1).

Summary

The present study is significant relative to understanding the underlying molecular etiology CHDs and OFT anomalies in humans. Our studies have shown through the use of IZr mutants that Pbx4 has a unique requirement limiting the size of the ventricle and the SHF, potentially by limiting the amount of
proliferation and differentiation in the OFT of the heart. Our studies provide a novel mechanism by which OFT defects may be caused in humans and suggest that similar overt congenital cardiac malformations in humans can be caused by differing underlying molecular and cellular mechanisms.
Figure 1: Proposed Model for the role of Pbx4 in heart development.
References:


