UNIVERSITY OF CALGARY

Transcriptional Regulation of the *Hand1* Gene in the Developing Mouse Conceptus

by

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Abstract

The *Hand1* gene encodes a basic helix-loop-helix transcription factor that is essential for heart, yolk sac and placental development in mouse. The *Hand1* expression domain is highly restricted in all of these tissues. Gene expression in multiple tissues is frequently regulated by modular enhancers, as is the case for *Drosophila Hand* and mouse *Hand2*. Previous results in the Cross laboratory suggested that the mouse *Hand1* intron contained cardiac-specific enhancer activity. A combination of comparative genomics, comparative expression analysis, and a knockout mouse model lacking the endogenous *Hand1* intron were used to investigate this possibility. The evidence presented in this thesis suggests that the *Hand1* intron does not contain cardiac-specific enhancer activity but may regulate *Hand1* expression levels.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AHF	Anterior Heart Field
ASD	Atrial Septal Defect
At	Atria
AVC	Atrioventricular Canal
CCS	Central Conduction System
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
E	Embryonic day
EMT	Epithelial-Mesenchymal Transition
Kb	kilobases
LVt	Left Ventricle
N/A	Not Associated with phenotype
N/R	Not Reported
OFT	Outflow Tract
PA	Pharyngeal Arches
PFA	Paraformaldehyde
RVt	Right Ventricle
SHF	Secondary Heart Field
ToF	Tetralogy of Fallot
VSD	Ventricular Septal Defect

Chapter One: Introduction

1.1 General Introduction

The *Hand1* gene encodes a basic helix-loop-helix transcription factor that is essential for cardiac, yolk sac and placental development in mouse (Firulli et al., 1998; Morikawa and Cserjesi, 2004; Riley et al., 1998; Riley et al., 2000). The *Hand1* expression domain is highly restricted in all of these tissues. During heart development, for example, expression is restricted in all three body axes to a region that will form the outer curvature of the left ventricle, at a stage when all other cardiac transcription factors are expressed more broadly. The overall goal of this project was to understand how the *Hand1* gene expression pattern is established, with the aim of identifying tissue-specific enhancers and the transcription factors underlying their activity.

In this thesis, I present my work studying the transcriptional regulation of *Hand1* in the developing mouse heart. I have utilized bioinformatic techniques to search for conserved elements that may act as enhancers, and identify the *Hand1* intron as a putative enhancer. I have employed a comparative expression analysis to identify putative regulators of *Hand1*. Finally, I have examined the *Hand1* expression pattern in mouse embryos lacking the *Hand1* intron. The Introduction reviews heart development and evolution, bioinformatic techniques used to identify regulatory elements, the *Hand1* gene family, and the overall rationale for focusing on *Hand1* gene regulation.

1.2 Heart Development

Heart development involves cell fate specification, migration, differentiation, proliferation, and morphogenesis. Briefly, cells from the mesoderm migrate to the midline where they form the linear heart tube at embryonic day (E) 8.0, which undergoes

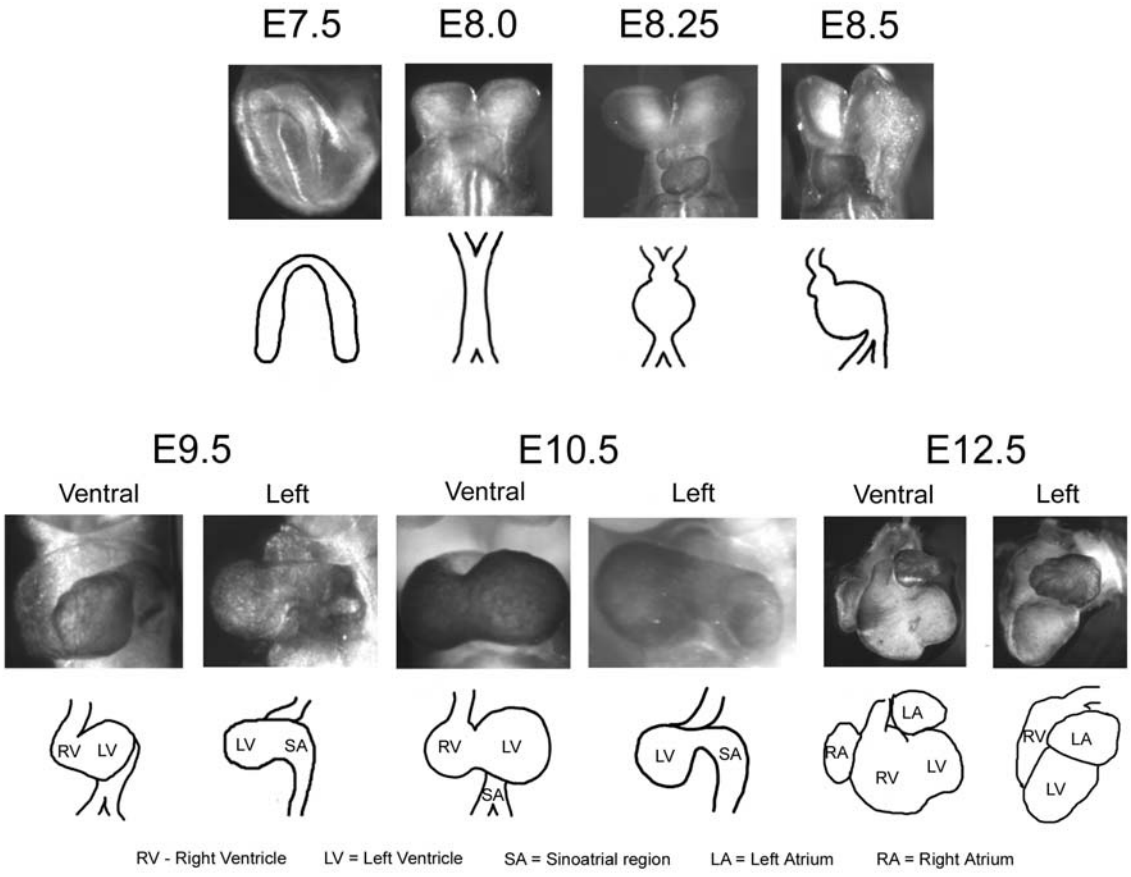


Figure 1.1. Overview of mouse heart development. Embryos were processed by *in situ* hybridization to detect the α-cardiac actin mRNA, a cardiac marker. Below each embryo, a cartoon of the heart is provided. At E7.5, the primary heart field is detected in a horseshoe-shaped crescent in the anterior lateral plate mesoderm. A second field of precardiac cells is present, positioned medially and extending anterior and posterior of the primary heart field. The cells of the primary heart field migrate to the midline and form the primary heart tube at E8.0, which undergoes looping (E8.0-8.5). At E9.5, the future left and right ventricles and the sinoatrial region have formed. At E10.5, the ventricular chambers are well-defined but the atrial regions have not developed yet. At E12.5, the atria have developed from either side of the sinoatrial region, and by E14.5, septation and valvulogenesis is underway.

looping morphogenesis as more cells are recruited (Fig 1.1). Within the looped heart tube, specific regions differentiate to form the future left and right ventricles and the future atria (Fig 1.1). These regions expand to form their respective chambers and are eventually separated by septae, and valves form to regulate blood flow.

1.2.1 Evolution and Phylogeny

The heart has an interesting evolutionary history. Present in insects (*Drosophila*) and chordates cardiac complexity has increased as evolution progressed, ultimately forming the four-chambered organ with valves and conduction system with which we are most familiar. As several forms of hearts are found in experimental organisms, and a common genetic network is involved in the development of hearts amongst diverse organisms, comparative genomics can provide considerable insight into the molecular control of development. With that in mind, a brief discussion on evolution, and cardiac evolution is appropriate.

Evolutionary theory proposes that all organisms on earth are related and evolved from a common ancestral organism. As the organism acquired and refined features, the population diversified and continued to acquire and refine features, leading to prokaryotes and eukaryotes, plants and animals, fish and humans, with each successive organism acquiring features suited to survival in their environment. To understand evolution and estimate evolutionary distance between organisms, researchers study physical attributes of organisms during their embryonic and adult stages, and study the degree of conservation and divergence of genomic sequences (Carroll et al., 2001).

One driver of evolution is duplication and divergence of genes and changes in gene function, which change organism traits. Additionally, regions that regulate the

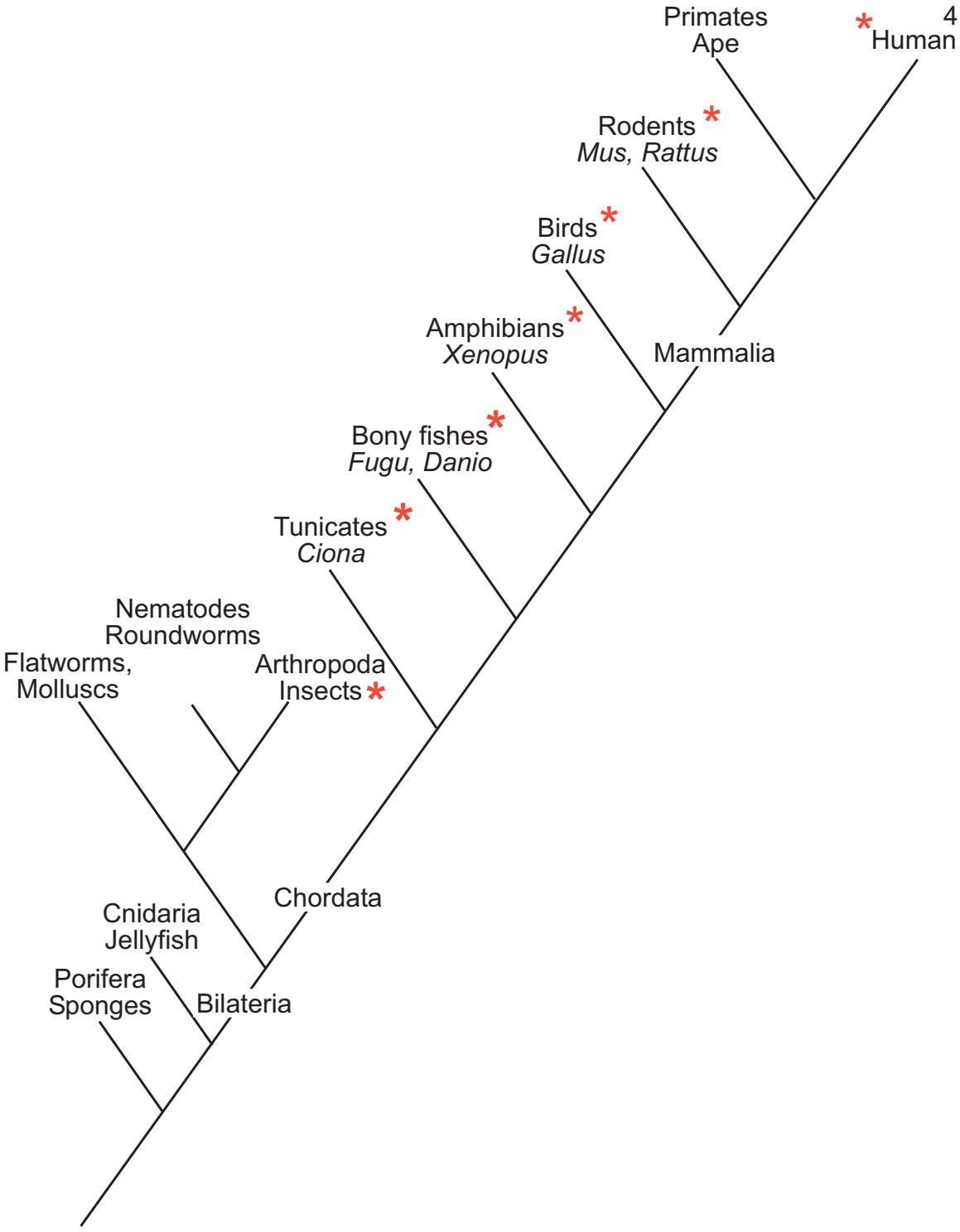


Figure 1.2. Overview of eukaryotic evolution. Eukaryotic phylogenetic tree showing evolution of mammals and higher chordates. Organisms marked with a red star are useful for identifying regulatory elements in heart development, as they cover the broad range of cardiac morphologies. Tree modeled after Bishopric, 2005; Carroll et al., 2001.

expression of developmental patterning genes, like the *Hox* gene cluster, are acquired or changed over time, altering the body plan of the organism (Carroll et al., 2001).

The ancestral eukaryote was a single cell organism that had mitochondria for energy metabolism, and a nucleus to house chromosomes. From that ancestral organism developed multicellular organisms that had two germ layers, ectoderm and endoderm (diploblasts, sponges and jellyfish) (Fig 1.2). The formation of a third germ layer, mesoderm, along with a bilateral axis of symmetry, is a defining feature of Bilateria. Within bilateria, considerable diversification has occurred, as shown in Figure 1.2, including organisms from flatworms to insects to humans. The major branch points leading to humans, with reference to species that are discussed within this thesis, include the divergence of tunicates (*Ciona intestinalis*), bony fishes (*Fugu rubripes* and *Danio rerio*), amphibians (*Xenopus laevis*), birds (*Gallus gallus*) and mammals (*Mus musculus, Rattus norvegicus* and *Homo sapiens*) (Kardong, 2005).

1.2.2 Evolution of the Heart

Hearts are found in various forms from the complex human four-chambered heart down to a simple linear pump in primitive bilateria (Bishopric, 2005). In all organisms considered here, the heart is derived from mesodermal cells that migrate to the midline to form a linear heart tube. In *Drosophila*, the heart tube forms at the dorsal midline, whereas in fish, amphibians, mammals and birds, the heart tube forms at the ventral midline. Blood flows posterior to anterior in the heart tube, and in organisms with chamber differentiation, the inflow chambers form in the posterior portion of the heart tube, the ventricles form centrally, and the anterior region forms the outflow tract. The cardiac conduction system is an adaptation in vertebrates which allows synchronous

contraction of the multi-chambered heart. Due to the linear tube nature of invertebrate hearts, intercellular communication between myocytes is sufficient for synchronous contraction of the linear heart tube. Sketches of the hearts in organisms discussed in this section are provided in Figure 1.3.

In *Drosophila*, the heart is a linear tube consisting of a layer of contractile cardial cells, surrounded by a layer of non-contractile pericardial cells (Bodmer and Venkatesh, 1998). Haemolymph enters the posterior end of the heart through specialized cardial cells (ostia) which act as valves, is pumped anteriorly, and leaves the anterior portion of the heart, the anterior aorta, to bathe the tissue. Some functional differences arise within the cardial and pericardial cell populations but there is no further morphogenesis after tube formation (Olson, 2006). The presence of a heart in *Drosophila*, which develops similarly to that in vertebrates and uses many of the same molecules for its development and patterning, argues for the origin of a heart-like organ prior to the divergence of Deuterostomes and Protostomes, probably shortly after the development of triploblasty. However, a distinct heart organ is not present in all Protostomes, like *Caenorhabditis elegans*, although the pharynx has some heart-like properties (Haun et al., 1998).

In *Ciona intestinalis*, a primitive chordate, the heart consists of a linear myocardial tube surrounded by a pericardium, as reviewed by Davidson (2007). This heart lacks valves and an endocardial lining, instead being lined by the basal lamina of the myocardial cells. Interestingly, the *Ciona* heart regenerates throughout its life, replacing degenerating myocardial cells. This makes *Ciona* an interesting organism for studying cardiac regeneration.

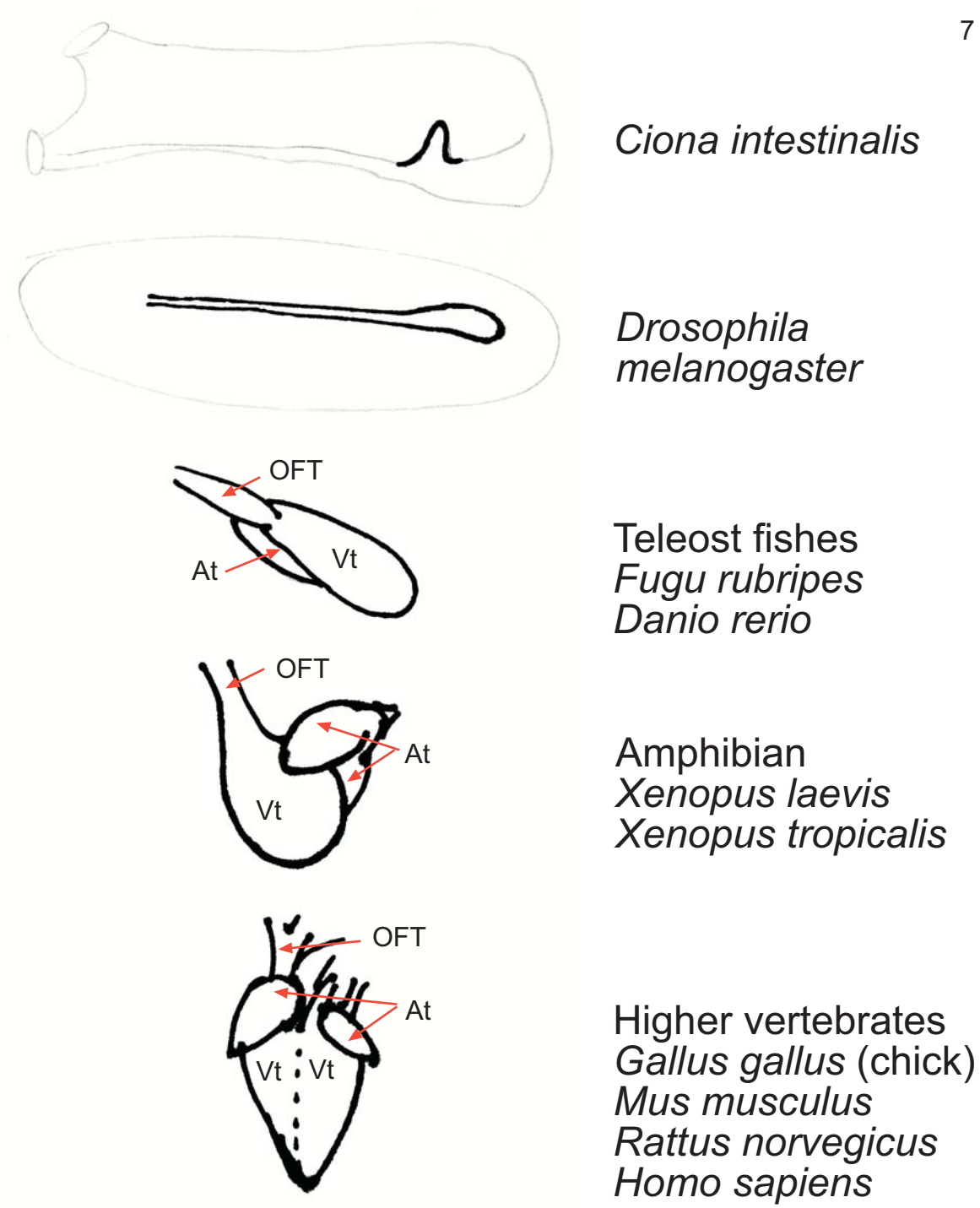


Figure 1.3. Structure of the heart in eukaryotes. A heart-like organ, which forms inline within the ventral vessel, and is formed by a layer of contractile cardiomyocytes, surrounded by a pericardium, is found in *Ciona intestinalis,* a primitive chordate. The heart of *Drosophila melanogaster* forms as a simple linear tube, closed at one end, with a layer of myocardial cells surrounded by pericardial cells, and pumps hemolymph throughout an open circulatory system. The hearts of teleost fishes have a single atrium and ventricle, with an outflow tract. The amphibian heart has two atria, a single ventricle, and an outflow tract. The hearts of higher vertebrates have two atria and two ventricles, and outflow vessels from each ventricle. Atria (At), Ventricle (Vt), Outflow Tract (OFT).

In *Danio rerio* (zebrafish), the mature heart consists of an atrium and a ventricle. Unlike *Drosophila,* the linear heart tube consists of contractile cardiomyocytes, lined by an endocardial layer, with a pericardial layer surrounding the heart at later stages. The atrium and ventricle form by differentiation of cardiomyocytes at posterior and central positions, respectively (Yelon, 2001). The chambers are separated by a region of undifferentiated tissue, which is later induced by the endocardium to form the atrioventricular valve, which regulates blood flow from the atrium (Yelon, 2001). The heart loops during chamber formation to position the ventricle to the right of the atrium, completing morphogenesis.

In *Xenopus laevis* (frog), the mature heart consists of two atria and a ventricle (Mohun et al., 2000). It has been argued from morphological and angiocardiographic studies that the single ventricle has two functions: a right-sided lumen, which handles deoxygenated blood from the right atrium, and a left-sided spongy wall, which handles oxygenated blood from the left atrium, and that blood flow from these ventricular compartments is kept separate by the spiral valve in the outflow tract, directing oxygenated blood to the systemic circulation and deoxygenated blood to the pulmonary circulation (Angell et al., 1966; Victor et al., 1999). This suggests that separate systemic and pulmonary circulation was established prior to the formation of two separate ventricles. As in zebrafish, the linear heart tube in *Xenopus* has cardiomyocyte and endocardial layers. The linear heart tube undergoes looping, chambers form by differentiation of the cardiomyocytes, and the endocardial layers participate in atrioventricular and outflow valve formation, and septation of the atria (Mohun et al.,

2000).

The hearts of birds and mammals have the highest structural complexity, with four chambers: two atria and two ventricles. Two populations of mesodermal cells are known to contribute to the heart in these organisms. The primary heart field forms a linear heart tube that gives rise to the left ventricle and part of the atria (Cai et al., 2003). As this tube initiates looping, cells from the secondary heart field migrate into the heart to contribute to the rest of the heart (Cai et al., 2003). During looping, regions of the heart tube differentiate to chamber phenotypes, with undifferentiated regions between the chambers later forming the septae and valves, as in fish and frog. The atria differentiate from a posterior region of the heart tube, and the ventricles form in a central position. Septation divides the common atria into left and right halves, which align with the right and left ventricles. The atrioventricular septum divides the atria and ventricles, and the atrioventricular valves form within this septum.

In conclusion, heart form has evolved considerably between *Drosophila* and mammals. Features like chambers, valves, a conduction system and separation of oxygenated and deoxygenated blood, have been added progressively, in a manner suggesting conservation of developmental mechanisms throughout cardiac evolution. Understanding how the heart has evolved, and the common and divergent features of evolutionarily diverse hearts, allows us to use comparative approaches to study heart development and transcriptional regulation in the heart.

1.2.3 Cardiac Specification and Determination

During development, cells within an initially identical population acquire different phenotypes and gene expression patterns, undergo morphogenesis and form

structures. Cells first become specified, then determined to a fate, and finally differentiate according to their fate. Cells are said to be specified when they will differentiate according to their normal fate if cultured in a neutral environment, but when transplanted to a different position in the embryo or explanted to culture and exposed to different signals, the cells will differentiate to a different fate in response to the new signals (Slack, 1991). Cells are said to be determined when they are no longer able to adjust differentiation according to a new position or signal, and differentiate according to their original fate (Slack, 1991).

Precardiac cells are specified in chick embryos by Hamburger-Hamilton(HH) stage 6 and in mouse embryos by embryonic day (E) 6.5 (Auda-Boucher et al., 2000; Montgomery et al., 1994). Ingression through the primitive streak is critical for specification to the cardiac fate (Montgomery et al., 1994). Additionally, anterior-posterior identity of the cells is specified at stage 6. Prior to stage 6, cells in the heart field can acquire new anterior-posterior identities when the heart field is excised and rotated, but this plasticity is lost after stage 6 (Patwardhan et al., 2000). In the mouse embryo, cardiac cells are determined by the late streak stage at E7.5 (Auda-Boucher et al., 2000). In the chick embryo, cardiac cells are determined by the time the bilateral cardiac fields fuse at stage 8 (Yutzey et al., 1995).

1.2.4 Primary and Secondary Heart Fields

Until recently, it was believed that all cells contributing to the myocardium were present in the linear heart tube (Zaffran and Frasch, 2002). Several findings in chick and mouse have since demonstrated that the myocardium is constructed from cells derived from two heart fields, which are recruited at separate times and likely originate from the

same cell population. Figure 1.4 provides an overview of the primary and secondary heart fields, and their relative contributions to the heart.

Fate mapping in the chick heart by de la Cruz and colleagues suggested that the linear heart tube contributes only a portion of the mature heart, with the rest of the heart added after initiation of looping (1989; 1977). Subsequent fate mapping experiments, utilizing dye and retroviral reporter gene construct injection in chick embryos, confirmed that primary heart tube produces only a portion of the mature heart, and demonstrated that the conotruncal myocardium is derived from a second field of cardiac cells, referred to as the secondary heart field (Mjaatvedt et al., 2001; Waldo et al., 2001).

Work in the mouse embryo using genetic lineage tracing techniques supports the general conclusions about the existence of a secondary heart field. Genetic lineage tracing utilizes expression of a reporter under control of a regulatory element, or activated in a specific tissue or cell population through a technique like Cre/loxP to mark cell populations and follow their behaviour through development. Unfortunately, different approaches to lineage tracing have led to different conclusions about the regions of the heart derived from the secondary heart field. Lineage tracing with the *Mef2c*-AHF-Cre transgene and a transgenic reporter under control of *Fgf10* regulatory sequences suggests that only the interventricular septum, right ventricle and outflow tract, and not the left ventricle or atria, are derived from the secondary heart field (Kelly et al., 2001; Verzi et al., 2005). The *Isl1*-Cre mice suggest that the secondary heart field contributions are found throughout the heart, except for in the outer curvature of the left ventricle (Cai et al., 2003). *Isl1* is expressed in a broader population of cells than *Mef2c* and *Fgf10*, and therefore *Isl1* is likely a marker for the complete secondary heart field, while *Mef2c* and

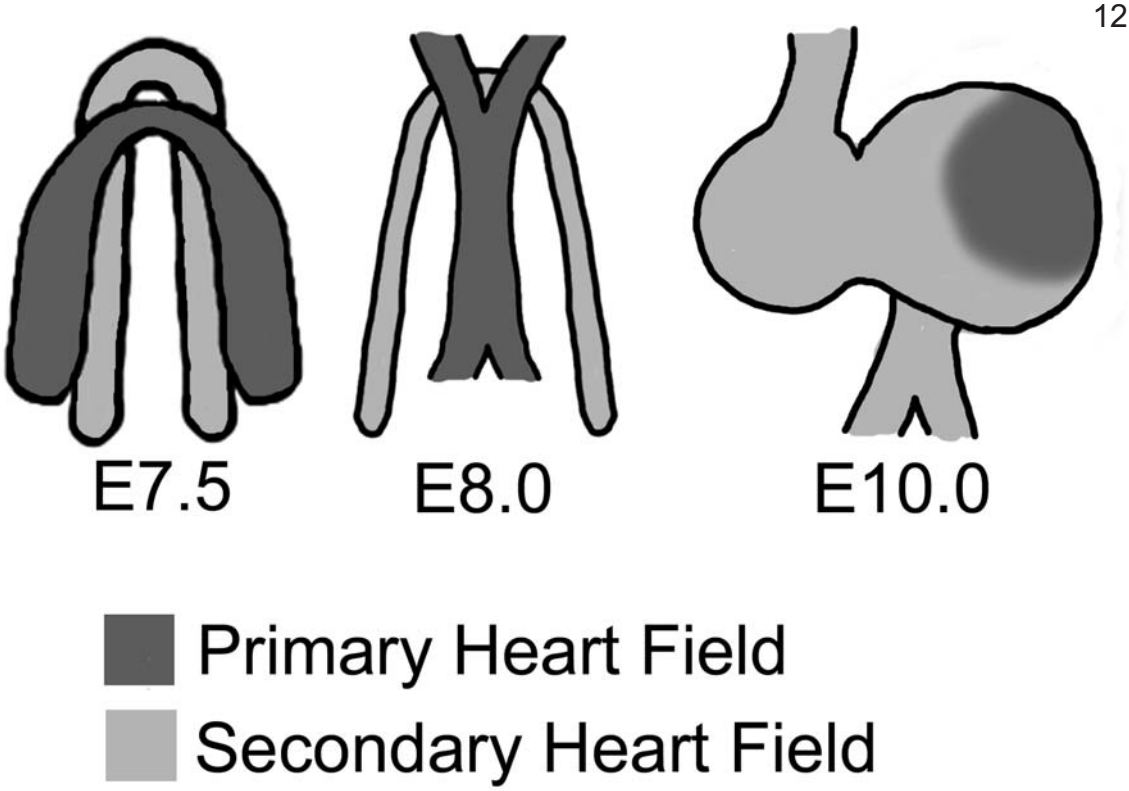


Figure 1.4. Heart development and the primary and secondary heart fields. The cardiomyocytes of the heart are derived from two heart fields. The primary heart field (dark grey) is recruited to form the linear heart tube, while the secondary heart field cells (light grey) remain in the mesoderm. When the primary heart tube initiates looping, the secondary heart field cells are recruited, and migrate into the heart. The primary heart field contributes part of the atria, and part of the left ventricle, including the left ventricle. The secondary heart field contributes the outflow tract, the right ventricle, part of the left ventricle, but not the outer curvature of the left ventricle, and part of the atria.

Fgf10 are likely markers of a subregion of the secondary heart field, often referred to as the anterior heart field as it does not include precursors of the posterior portions of the heart.

The primary and secondary heart field cells are derived from a common set of precursor cells. Meilhac et al. (2004a) generated myocardial clones expressing lacZ by using a transgenic reporter, nlaacZ, which is only expressed following the relatively rare event of mitotic recombination, under the control of the α-cardiac actin promoter. Three clonal behaviours were observed: clones marked cells in only derivatives of the primary heart field, only derivatives of the secondary heart field, or clones were large and marked derivatives of both primary and secondary heart fields. Meilhac et al. (2004a) concluded that the large clones were likely derived from mitotic recombination events that occurred prior to the separation of primary and secondary lineages from a common precursor.

The primary and secondary cardiac lineages can be distinguished at E7.5, with the primary heart field expressing *Nkx2-5*, and the secondary heart field expressing *Isl1* (Cai et al., 2003). At this stage, both are bilateral, horseshoe shaped fields of cells in the lateral plate mesoderm. By E8.25, the primary heart field cells have migrated to the midline, fused, and formed the linear heart tube. When looping of this tube is initiated, cells from the secondary heart field are recruited. These cells migrate into the heart at the anterior and posterior poles, and integrate into the heart (Cai et al., 2003). Currently, the cues involved in secondary heart field recruitment, migration, and differentiation of those cells after integration into the heart are unknown.

Several mutant phenotypes support the overall primary and secondary heart field model that was initially based exclusively on the lineage tracing experiments discussed

above. *Hand2* mutant embryos lack a right ventricle (Srivastava et al., 1997), and *Nkx2-5/Hand2* double mutants lack a ventricle altogether (Yamagishi et al., 2001). *Isl1* mutation and cardiac-specific *Fgf8* deletion both result in failure to form the right ventricle and outflow tract (Cai et al., 2003; Ilagan et al., 2006). *Tbx1* mutant embryos have shortened outflow tracts, and distribution of secondary heart field cell contribution to the arterial and venous poles of the heart is disrupted (Kelly and Papaioannou, 2007).

As the secondary heart field contributes to most of the right ventricle, it could be suggested that the secondary heart field evolved with the origin of a two ventricle heart, when the need to separate oxygenated and deoxygenated blood arose in an evolutionary ancestor of birds and mammals. However, there are no publications to date investigating the possibility of a secondary heart field in frog, which has a single ventricle but is thought to separate oxygenated and deoxygenated blood (Angell et al., 1966; Victor et al., 1999). In zebrafish, which does not separate oxygenated and deoxygenated blood, fate mapping suggests that the atrial and ventricular lineages are segregated in the midblastula embryo (Stainier et al., 1993). This led Meilhac et al. (2004a) to propose that two myocardial lineages were established in an ancestor common to fish and mammals, and that the fate of these lineages has diversified in mammals to create the four chambered heart.

1.2.5 Cardiac Morphogenesis and Patterning

The mammalian heart undergoes an extensive morphogenetic process to transform from a bilateral field of cells to a four chambered heart. The current model describing heart development is the ballooning morphogenesis model, which incorporates several key observations, particularly that chambers form along the outer curvature of the

heart, and the inner curvature is used for septation and valvulogenesis (Christoffels et al., 2000). The ballooning morphogenesis model replaced the previously dominant model of heart development, the "segmental model", which proposed that the cardiac chambers form from discrete segments of the linear heart tube (Srivastava and Olson, 2000). The major transcription factors involved in cardiac development and patterning, their expression patterns and roles in heart development are listed in Table 1.1.

1.2.5.1 Heart Tube and Chamber Formation

At E7.5, the cardiac precursor cells are located in the primary and secondary heart fields, forming bilateral crescents (Fig 1.1). Cells of the primary heart field migrate to the midline and form the primary heart tube. This tube expresses a uniform primitive cardiac phenotype, which is characterized by an absence of 'chamber myocardium' gene expression (*ANF, Chisel, Cx43, SERCA2a, Irx5*), slow conductivity and pacemaker-like action potentials (Christoffels et al., 2000; Moorman et al., 1998; Moorman et al., 2000). The primary cardiac crescent expresses the transcription factors *Nkx2-5* and *Gata4*, which act together to regulate cardiac expression of many cardiac genes (Heikinheimo et al., 1994; Lints et al., 1993). *Nkx2-5* and *Gata4* have several roles in development and morphogenesis of the heart tube, as demonstrated by the diverse phenotypes exhibited by full and conditional mutants of these genes (Biben and Harvey, 1997; Jay et al., 2004b; Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1997; Watt et al., 2004).

Table 1.1. Expression, Roles, and Associated Cardiac Phenotypes of Cardiac Transcription Factors.

Transcription Factor	Cardiac Expression	Role	Human Cardiac Phenotype	References
Nkx2-5	*pan*-cardiac	Cardiac specification and patterning, conduction system development	ASD, VSD, conduction	(Biben and Harvey, 1997; Habets et al., 2002; Jay et al., 2004a; Jay et al., 2004b; Lyons et al., 1995; Pashmforoush et al., 2004; Tanaka et al., 1999)
Gata4	*pan*-cardiac	Cardiac differentiation, ventral morphogenesis	ASD, VSD,	(Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1997; Peterkin et al., 2005; Watt et al., 2004)
FOG2	*pan*-cardiac	Gata4 cofactor, modulator	ASD, ToF	(Crispino et al., 2001; Finelli et al., 2007; Tevosian et al., 1999; Tevosian et al., 2000)
Mef2c	*pan*-cardiac	Specifies cardiomyocytes	N/A	(Dodou et al., 2004; Edmondson et al., 1994; Vong et al., 2006)
FGF10	SHF	Ventricular patterning	N/A	(Kelly et al., 2001; Marguerie et al., 2006)
Isl-1	SHF	SHF development	N/A	(Cai et al., 2003)
Hand1	LVt, AVC, OFT	Cardiac patterning, proliferation	N/A	(Cross et al., 1995; Cserjesi et al., 1995; Firulli et al., 1998; Hollenberg et al., 1995)
Hand2	RVt, Endocardium	Cardiac proliferation, RVt identity	N/A	(Cross et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995; Srivastava et al., 1997)
Tbx1	PA	SHF proliferation, OFT development	Conotruncal abnormalities	(Chapman et al., 1996; Xu et al., 2004; Yamagishi and Srivastava, 2003)
Tbx2	Primitive myocardium	Inhibits chamber differentiation	N/A	(Christoffels et al., 2004; Harrelson et al., 2004)
Tbx3	Primitve myocardium, CCS	Inhibits chamber differentiation; Sinoatrial node patterning	N/A	(Hoogaars et al., 2004; Mommersteeg et al., 2007)
Tbx5	LVt, At	Atrial and left ventricle identity	ASD, VSD, conduction defect	(Bruneau et al., 1999; Bruneau et al., 2001b; Mori et al., 2006)
Tbx20	*pan*-cardiac	Repress *Tbx2*, endocardial cushion development	N/A	(Kraus et al., 2001; Shelton and Yutzey, 2007; Singh et al., 2005; Takeuchi et al., 2005)
Irx4	LVt, RVt	Promotes ventricular identity	N/A	(Bao et al., 1999; Bruneau et al., 2001a; Wang et al., 2001)

Legend: ASD: Atrial Septal Defect; VSD: Ventricular Septal Defect; ToF: Tetralogy of Fallot; N/A: Not Associated with Phenotype; SHF: Secondary Heart Field; N/R: Not Reported; LVt: Left Ventricle; AVC: Atrioventricular Canal; OFT: Outflow Tract; RVt: Right Ventricle; PA: Pharyngeal Arches; CCS: Central Conduction System; At: Atria

By E8.25, the linear heart tube has initiated looping (Fig 1.1), which will ultimately position the ventricles at the apex of the heart, the atria at the base, and align the chambers. As the heart loops, it rotates to the left, and the original dorsal side becomes the inner curvature of the looped heart. Notably, *Hand1* is expressed exclusively on the ventral side of the linear heart tube, suggesting that it may have a role in dorsal-ventral patterning (Biben and Harvey, 1997; Thomas et al., 1998). Looping is driven in part by unequal forces exerted on the left and right sides of the inflow region of the heart by mesoderm at the posterior end of the heart and the incoming veins (Voronov et al., 2004), and by proliferation of the outer curvature of the heart (Christoffels et al., 2000). Three regions of the heart tube differentiate to the chamber phenotype, with the dorsal aspect (future inner curvature) of the heart tube and regions separating the chamber myocardium retaining the primitive phenotype (Fig 1.5) (Christoffels et al., 2000). The chamber phenotype is characterized by expression of the chamber myocardium genes, increased proliferation causing an outward ballooning of the region, and acquisition of atrial- and ventricular-type action potentials (Christoffels et al., 2000; Meilhac et al., 2004b; Moorman et al., 2000).

The common atria require retinoic acid signalling and the *Tbx5* gene for patterning. Retinoic acid signalling promotes *Tbx5* expression, which in turn promotes the atrial identity in the early linear heart tube during chamber differentiation, thereby determining the size of the common atrium (Hochgreb et al., 2003; Liberatore et al., 2000; Niederreither et al., 2001). Atrial development is inhibited by the *Irx4* gene (Bao et al., 1999).

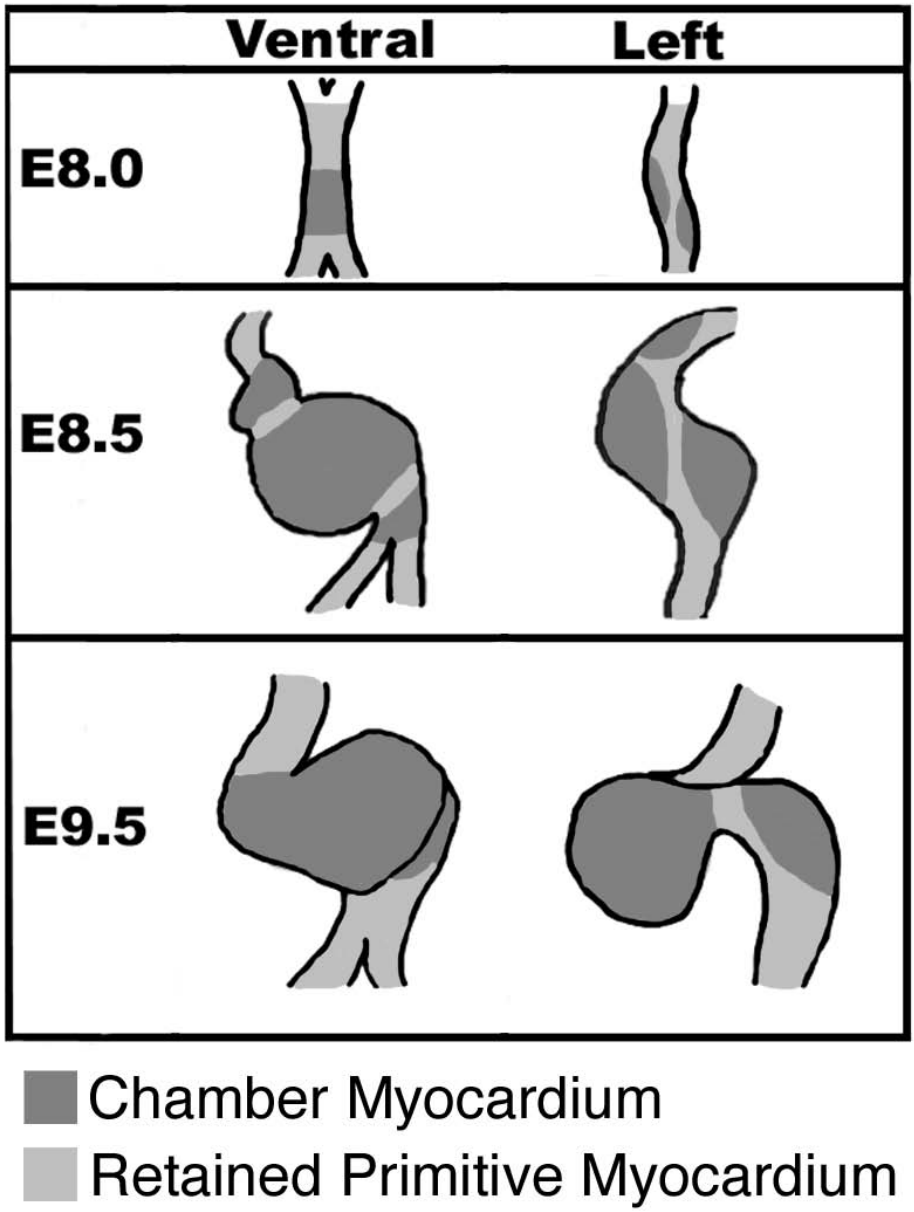


Figure 1.5. Differentiation of the myocardium. The heart tube initially exhibits a uniform, primitive phenotype. Regions of the heart tube, as marked in dark grey, differentiate to a chamber phenotype, characterized by elevated proliferation, expression of chamber myocardium markers, and ballooning morphogenesis. Tissue between the chambers and in the inner curvature retain the primitive phenotype and exhibit a low proliferation rate until they are recruited to form septae, valves, and portions of the cardiac conduction system at later stages.

Ventricular development requires expression of *Irx4*, which promotes the ventricular identity and represses the atrial identity (Bao et al., 1999; Bruneau et al., 2001a). In the left ventricle, which is largely derived from the primary heart field, *Tbx5* and *Hand1* are expressed (Bruneau et al., 1999; Cserjesi et al., 1995). *Tbx5* is required for left ventricle fate specification, in its absence the heart arrests prior to looping (Bruneau et al., 2001b). *Hand1* is expressed in the future left ventricular region of the linear heart tube, and later in the outer curvature of the left ventricle, and in its absence the heart arrests at the linear tube stage without undergoing chamber morphogenesis (Biben and Harvey, 1997; Firulli et al., 1998; Riley et al., 1998).

The right ventricle, which is derived from the secondary heart field, expresses *Tbx20* and *Hand2*. *Isl1* is necessary for secondary heart field development and formation of the right ventricle, supporting proliferation and survival of these cells prior to their recruitment to the heart (Cai et al., 2003). In *Tbx20* mutants, the right ventricle and outflow tract fail to form but secondary heart field markers are unaffected, suggesting that the cells may be recruited to the heart, but they fail to proliferate (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005). *Hand2* mutant embryos lack the right ventricle (Srivastava et al., 1997), although it is not clear if the defect is in the secondary heart field recruitment, survival or proliferation, or differentiation of those cells, as the mutants have not been revisited since the discovery of the secondary heart field. *Hand2;Nkx2-5* double mutants lack ventricular tissue altogether, supporting the idea that *Hand2* mutants lack the right ventricle and *Nkx2-5* mutants lack the left ventricle (Yamagishi et al., 2001).

Within the regions retaining the primitive cardiomyocyte phenotype, expression

of the transcriptional repressor Tbx2 is initiated (Christoffels et al., 2004; Yamada et al., 2000). *Tbx2* represses the chamber phenotype, and it is in turn repressed by *Tbx20*, which is expressed throughout the heart tube at this stage (Cai et al., 2005; Christoffels et al., 2004; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005). It has been hypothesized that Tbx20 represses *Tbx2* expression, and relief of that repression permits *Tbx2* expression in the primitive myocardium, but how this repression is relieved has not been demonstrated (Takeuchi et al., 2005). The inner curvature and other *Tbx2* expressing regions exhibit low cell proliferation and remain undifferentiated while the chamber regions form and the heart loops (Sedmera et al., 2003). These regions will later form septae, valves and portions of the cardiac conduction system (Lamers and Moorman, 2002; Moorman et al., 1998).

1.2.5.2 Chamber Morphogenesis, Septation and Valvulogenesis

By E9.0, the heart is looped and three chamber regions can be distinguished: the common atrium, the future left ventricle and the future right ventricle, separated by the atrioventricular canal and the interventricular groove. At this stage, the ventricles are essentially outpockets of the heart tube. Their growth continues, with cells proliferating in a clonal and oriented manner to generate the characteristic shape of the ventricles (Meilhac et al., 2004b). The ventricle walls thicken as the myocardium forms two layers; the inner, loosely packed and highly branched trabecular layer and the compact outer layer. Trabeculae are observed along the outer curvature of the ventricles at E10.5. Myocardial cells in the ventricular wall proliferate rapidly, and trabeculation is induced by contact with endocardial cells, which secrete neuregulins and FGF9 (Corfas et al., 1995; Gassmann et al., 1995; Kramer et al., 1996; Lavine et al., 2005; Lee et al., 1995;

Meyer and Birchmeier, 1995). Trabeculation allows the ventricle to expand outward, thicken its wall and increase the number of cells available to contract without requiring vascularisation of the heart until later stages, as the blood passing through the heart can easily infiltrate the trabecular layer (Sedmera et al., 2000). Trabeculation proceeds until E14.5, when vascularisation is initiated, permitting the trabeculae to compress without compromising blood supply, and contributing to the compact myocardium of the ventricular wall (Sedmera et al., 2000).

The interventricular groove, a band of tissue which does not balloon, forms a restriction between the left and right ventricles. It undergoes proliferation during E9.0-14.5, directed towards the inner curvature of the heart, to form the muscular portion of the interventricular septum (Franco et al., 2006). The interventricular septum is completed by a membranous portion, derived from the endocardial cushions of the atrioventricular canal later in development (Lamers and Moorman, 2002). The position of the interventricular septum is determined in part by expression of *Tbx5, Hand1* and *Hand2*. *Tbx5* is expressed in the future atria and left ventricle, with a defined anterior border, which corresponds to the position of the interventricular septum. Manipulation of this anterior border by ubiquitous expression of *Tbx5* precludes interventricular septum formation. Conversely, ectopic expression of *Tbx5* within the right ventricle causes formation of a second interventricular septum (Takeuchi et al., 2003). *Hand1* is a likely candidate for mediating the effect of *Tbx5* on the interventricular septum, as it is induced by ectopic *Tbx5* expression (Takeuchi et al., 2003). *Hand1* is normally expressed in the outer curvature of the left ventricle (Biben and Harvey, 1997), but when it is ectopically expressed throughout the heart, the interventricular septum fails to form (Togi et al.,

2004). Similarly, *Hand2* is normally expressed in the right ventricle, but its ectopic expression throughout the heart prevents interventricular septum formation (Togi et al., 2006). These findings have led to the hypothesis that the interventricular septum forms at the anterior border of *Tbx5* expression, a region where *Hand1* and *Hand2* expression are excluded (Togi et al., 2006).

From the common atria, the atrial appendages balloon out from the dorsolateral walls to form the right and left atria, which are connected to both ventricles via the atrioventricular canal prior to valvulogenesis and septation (Moorman et al., 2003). The left atrial appendage connects with the pulmonary vein, and the right atrial appendage connects with the superior and inferior caval veins (Moorman et al., 2003). The atria become septated by growth and fusion of the muscular primary atrial septum and the two opposing endocardial cushions of the atrioventricular canal (Anderson et al., 2003).

The atrioventricular canal is found between the common atrium and the left ventricle. Expression of *Tbx2* is required to maintain the primitive myocardial phenotype until the atrioventricular canal is recruited for endocardial cushion development (Christoffels et al., 2004). The atrioventricular canal maintains the primitive myocardial phenotype until BMP2, expressed in the atrioventricular canal, and β-catenin signalling induce the underlying endocardial cells to undergo an epithelial-mesenchymal transition (EMT) and invade the cardiac jelly between the endocardial and myocardial layers to form the endocardial cushions (de Lange et al., 2004; Hurlstone et al., 2003; Keyes et al., 2003; Ma et al., 2005; Sugi et al., 2004). The location of cushion formation is determined by a negative feedback loop between Notch, Hey1 and Hey2 in the chambers and BMP2 and Tbx2 in the atrioventricular canal (Rutenberg et al., 2006; Yamada et al.,

2000). Mutual antagonism between the chamber factors and the primitive myocardium factors in the atrioventricular canal creates sharp borders between the regions, limiting the capacity for endocardial cushion formation to a small region. Two opposing endocardial cushions are induced in the atrioventricular canal and they grow towards one another and fuse to generate the atrioventricular septum.

The behaviour of cells within the endocardial cushions is regulated by *Tbx20*, which is expressed at high levels in the endocardial cushions until remodelling for valve formation is initiated (Shelton and Yutzey, 2007). *Tbx20* promotes proliferation of endocardial cushion cells and expression of matrix metalloproteases, which modify the extracellular matrix, necessary for expansion and fusion of the endocardial cushions to form the atrioventricular septum. To form valves within the atrioventricular septum, *Tbx20* expression is downregulated to decrease proliferation and permit extracellular matrix remodelling. The endocardial cushion mesenchyme differentiates to form cartilage, which creates the structure of the valve leaflets, and tendon cells, which hold the leaflets in place and provide elastic strength to the valve. Cartilaginous development is promoted by BMP2 signalling, while tendon development is promoted by FGF4 signalling (Lincoln et al., 2006). At this point, the atria and ventricles are aligned and connected, with valves regulating blood flow, and the pulmonary and systemic circulation pathways are separated.

1.2.5.3 Cardiac Conduction System

The conduction system carries the electrical impulse from the pacemaker cells in the sinoatrial node to the working myocytes, triggering their contraction and the overall synchronized contraction of the heart. The conduction system consists of the sinoatrial

node, which initiates the action potential, the atrioventricular node, which delays the action potential to allow ventricular filling prior to contraction, the insulated atrioventricular bundle, which propagates the action potential to the apex of the ventricles, and the Purkinje fiber network, which delivers the action potential to the working myocytes of the ventricles (Gourdie et al., 2003). The nodes, the atrioventricular bundle and their associated structures are known as the central conduction system, while the Purkinje network is known as the peripheral conduction system.

The conduction system consists of cells derived from a number of sources: cells which maintain the primitive cardiac phenotype, and differentiated ventricular myocytes. *Nkx2-5* is required for cardiac conduction system development, beyond its general requirement for cardiac development. Expression of *Nkx2-5* is upregulated in cells differentiating to conduction system fates, and in the absence of *Nkx2-5* the central and peripheral conduction systems are hypocellular, causing conduction abnormalities in the hearts (Jay et al., 2004b; Meysen et al., 2007; Thomas et al., 2001).

The precursors of the central conduction system are delineated by expression of the transcriptional repressor *Tbx3* as early as E8.5 (Hoogaars et al., 2004). In combination with *Tbx2,* which is expressed more broadly*, Tbx3* represses the chamber myocardium differentiation program (Christoffels et al., 2004; Hoogaars et al., 2004). *Tbx2* is required for preventing chamber differentiation in the central conduction system precursor cells, maintaining this population in an undifferentiated state until their differentiation is required (Christoffels et al., 2004; Harrelson et al., 2004). *Tbx5*, in addition to its role in atrial and left ventricular development, is expressed in and required

for normal development and functioning of the atrioventricular node and ventricular conduction system (Bruneau et al., 2001b; Moskowitz et al., 2004). Haploinsufficiency of human *Tbx5* causes Holt-Oram syndrome, which is characterized by atrial and ventricular septal defects, atrioventricular and bundle branch conduction defects, and limb abnormalities (Reviewed by Hatcher and McDermott, 2006). Conditional deletion of a single *Tbx5* allele in mouse reproduces the cardiac structural and conduction defects, and limb abnormalities of human Holt-Oram syndrome (Bruneau et al., 2001b), demonstrating the importance of the *Tbx5* gene in conduction system development.

The atrioventricular bundle and the peripheral conduction system cells are recruited from nearby myocyte populations prior to their terminal differentiation (Cheng et al., 1999; Gourdie et al., 1995). The ventricular myocytes are recruited to the peripheral conduction system by endothelin-1 in chick and endothelin-1 and neuregulin-1 in the mouse embryo (Patel and Kos, 2005; Rentschler et al., 2002; Takebayashi-Suzuki et al., 2000). Myocytes are only sensitive to induction for a brief period of time, which is determined by the temporal expression of endothelin receptors in chick (Kanzawa et al., 2002). Following recruitment to the conduction system, central conduction components, including the atrioventricular bundle, upregulate *Wnt11*, and peripheral conduction components upregulate *Wnt7a* (Bond et al., 2003).

1.3 Regulatory Element Identification with Bioinformatics

Regulatory elements are small regions of DNA that contain transcriptional regulatory information, detailing when and where a particular gene is to be expressed. Regulatory elements can be found in the proximal promoter, within introns, or downstream of the gene in intergenic regions. These elements may act as enhancers,

which act to upregulate expression under certain developmental or physiological conditions, or suppressors, which inhibit expression. The elements are constituted by binding sites for transcription factors, which activate or inhibit transcription. The transcription factors can act by directly influencing recruitment or activity of the transcriptional machinery, by recruiting co-regulator proteins, or by influencing genomic structure in the region.

Transcription factors are proteins that bind short DNA sequences and regulate transcription. Transcription factors can be categorized by the amino acid sequence and structure of their DNA binding domains, which determine the DNA sequences recognized. Transcription factors interact with DNA based on electrostatic and hydrophobic interactions and compatible shapes (Locker, 2001). Certain nucleotide residues will interact best with a given position of the DNA binding domain, while other positions may be more flexible in their nucleotide compatibility, leading to a flexible preferred binding site. The binding sites utilized by transcription factors can be influenced by several factors, including DNA methylation, availability of other transcription factors for combinatorial binding, and the chromatin accessibility (Locker, 2001).

The nucleotide sequence specificity of transcription factor binding sites have been defined using biochemical assays. They can be represented as probability weight matrices, which describe the likelihood of each nucleotide at each position in the binding site and are used when searching DNA sequence for putative binding sites. The probability weight matrices have largely been collected in two databases, *JASPAR* and *Transfac* (Matys et al., 2003; Vlieghe et al., 2006). The matrices are constructed from

experimental data and so they do not reflect all possible binding sites for an individual transcription factor, nor do they consider the influence of adjacent sequences, DNA modification or binding partners on binding site selection. Therefore, many recognition sites will be missed when a genomic sequence is surveyed with a consensus sequence, and many non-functional sites will be identified. An additional limitation is that only a small number of transcription factors have been investigated, so the binding preferences of most transcription factors, including many tissue and development specific factors, are unknown and can only be loosely inferred from other factors within the same family.

Regulatory elements are typically identified functionally by using reporter gene assays with a large genomic region containing the transcription start site, promoter and putative regulatory regions driving a reporter gene, and carrying out a deletion analysis on the genomic region to identify a minimum region which is responsible for an expression pattern, as has been done for *Drosophila Hand* and mouse *Nkx2-5* (Han and Olson, 2005; Schwartz and Olson, 1999). The availability of fully sequenced genomes for a variety of organisms covering large portions of the eukaryotic phylogenetic tree has created an opportunity for *in silico* searches for regulatory elements, allowing researchers to initially narrow a large genomic region with computational analysis that looks for evolutionary conservation and presence of putative transcription factor binding sites. After the region is narrowed, regulatory activity still must be verified with reporter assays.

Prediction of regulatory elements with bioinformatics tools relies on several key principles . First, an element that has a conserved function will likely have a conserved sequence as it is under selection against change. Second, regulatory elements exert

effects on gene expression through the transcription factors that bind them. These proteins recognize short, degenerate DNA sequences, which can be identified within genomic sequences.

Several approaches have combined the principles described above to identify putative regulatory elements. The comparative genomics approach, which identifies sequences conserved between distantly related species through alignments or dot-plot comparisons, has been used to identify several regulatory elements. Programs like *ECR Browser* permit the user to browse syntenic regions of multiple genomes and identify evolutionarily conserved regions (http://ecrbrowser.dcode.org/) (Ovcharenko et al., 2004b). By combining comparative genomics and reporter assays, Sabherwal et al (2007) identified regulatory elements within a 200 kb deletion found in patients with Léri-Weill dyschondrosteosis that did not have mutations in the *SHOX* gene, which typically causes this syndrome. Large-scale studies with comparative genomics have identified general non-coding elements conserved between humans and bony fishes (Venkatesh et al., 2006), and non-coding elements conserved in human and pufferfish in the vicinity of developmentally important genes, which have transcriptional enhancer activity when tested in a reporter assay in developing zebrafish embryos (Woolfe et al., 2005).

The comparative genomics approach has been combined with a search for putative transcription factor binding sites or short overrepresented sequences that could be new putative binding sites through programs like *ConSite* (http://www.phylofoot.org/consite), *rVista* (http://rvista.dcode.org), and *TOUCAN2* (http://www.esat.kuleuven.ac.be/~saerts/software/toucan.php) (Aerts et al., 2005; Loots

and Ovcharenko, 2004; Sandelin et al., 2004). This approach is suited for studying regulation of a single gene that is present among a group of diverse species. Long-range tissue-specific regulatory elements have been identified for *Sox9* using comparison between human and *Fugu rubripes* (Bagheri-Fam et al., 2006), and enhancers regulating *Isl1* expression in motor and sensory neurons (Uemura et al., 2005).

To identify regulatory elements for a certain tissue, developmental event or physiological response, a group of co-regulated genes is identified and entered into a program like *TOUCAN2* (http://www.esat.kuleuven.ac.be/~saerts/software/toucan.php) or *Creme2.0* (http://creme.dcode.org/), which searches for elements present in the genomic regions around all or most of these genes (Aerts et al., 2005; Sharan et al., 2004). This approach has been successfully used to identify putative regulatory elements for a set of cell-cycle regulated genes (Sharan et al., 2004).

1.4 The *Hand1* Gene

The *Hand1* gene encodes a basic helix-loop-helix transcription factor that is essential for placental, yolk sac and heart development (Cross et al., 1995; Firulli et al., 1998; Hollenberg et al., 1995; Morikawa and Cserjesi, 2004; Riley et al., 1998; Riley et al., 2000). The *Hand1* gene has two exons, with 5' and 3' untranslated regions, and a ~1.5 kb intron. *Hand1* is expressed in the developing heart, trophoblast giant cells and extraembryonic membranes, and neural crest derivatives (Cserjesi et al., 1995). In the heart, the *Hand1* expression domain is tightly defined, limited to the ventral side of the linear heart tube, the middle portion along the anterior-posterior axis, and is upregulated on the left side of the heart tube as looping is initiated (Biben and Harvey, 1997; Srivastava et al., 1997). Additional cardiac expression is found in the distal portion of the

outflow tract (Biben and Harvey, 1997; Srivastava et al., 1997).

1.4.1 **Hand1** *and Mouse Heart Development*

Hand1 mutant mouse embryos arrest at E7.5 due to placental insufficiency, but also have delayed heart development (Firulli et al., 1998; Riley et al., 1998). Using tetraploid aggregation, which combines wild type tetraploid (4n) cells and mutant embryos or embryonic stem cells to rescue placental defects and permit further embryonic development, it was demonstrated that *Hand1* mutant hearts arrest at the linear heart tube stage, and appear to lack differentiation of chamber myocardium (Riley et al., 1998). Therefore, the delay in cardiac development seen in *Hand1* mutants is not simply secondary to general embryonic delay due to placental insufficiency, but rather reflects a specific role of *Hand1* in heart development. Intriguingly, the *Hand1* expression domain is complementary to the domain of secondary heart field contribution as shown with *Isl1-Cre* lineage tracing (Cai et al., 2003). These results support the theory that *Hand1*is not simply required for the left ventricular identity, which it is broadly used as a marker for, but also for early patterning or morphogenesis and possibly for recruiting or patterning secondary heart field derivatives.

The function of *Hand1* after looping morphogenesis has been investigated using various techniques. In chimeric embryos generated by combining ROSA26 embryos with *Hand1*$^{-/-}$ embryonic stem cells, rescue of the cardiac arrest only occurs when wild type cells occupy the outer curvature of the left ventricle indicating that *Hand1* is required in cells within this specific domain (Riley et al., 2000). Increasing the level of *Hand1* expression in the endogenous cardiac expression domain prevents myocardial differentiation and promoted proliferation, causing expansion of the heart tube, with an

elongated outflow tract which affects alignment of the looped heart, and impaired differentiation and expansion of the left ventricle (Risebro et al., 2006). Expression of *Hand1* throughout the heart under control of the *MLC2V* promoter prevents formation of the interventricular septum (Togi et al., 2004), and Cre-mediated deletion of *Hand1* shortly after the linear heart tube stage, leads to defects in ventricular development, valvulogenesis, interventricular septum development and outflow tract development (McFadden et al., 2005).

Collectively these data suggest that *Hand1* has two roles in cardiac development, one at the linear heart tube stage which is necessary for looping and chamber morphogenesis, and one in the looped heart which regulates ventricular growth and differentiation, interventricular septum formation, valvulogenesis and outflow tract development.

1.4.2 Hand1 *Through Evolution*

Hand1 is a member of the *Hand* subfamily of basic helix-loop-helix transcription factors, and is closely related to *Hand2* (Cross et al., 1995; Srivastava et al., 1995). *Hand1* and *Hand2* genes are present in human, mouse, chick and *Xenopus* (Cross et al., 1995; Hollenberg et al., 1995; Knofler et al., 1998; Russell et al., 1997; Russell et al., 1998; Sparrow et al., 1998; Srivastava et al., 1995). These organisms all have three or more cardiac chambers, and it has been argued that the *Xenopus* ventricle functionally separates systemic and pulmonary circulation (Angell et al., 1966; Victor et al., 1999), suggesting that the presence of two *Hand* genes arose when the systemic and pulmonary circulation were separated. In *Drosophila*, which have a simple linear cardiac tube, and zebrafish, which have an atrium and a single ventricle, there is only one *Hand* gene

(Kolsch and Paululat, 2002). A phylogenetic tree constructed with the basic helix-loop-helix domain protein sequence suggests that the the ancestral *Hand* gene underwent duplication and divergence to generate *Hand1* and *Hand2*, and the *Hand2* genes are more similar to the ancestral *Hand* gene, as represented by *Drosophila Hand*. (Kolsch and Paululat, 2002; Moore et al., 2000; Yelon et al., 2000). A single *Hand* gene is also present in the ascidian *Ciona intestinalis*, which has a simple heart tube that, as in higher organisms, develops from bilateral precursors that migrate to the midline to fuse and form a tube (Davidson, 2007; Satou et al., 2003; Satou et al., 2004).

The genomic structures of *Hand1* and *Hand2* have been conserved in human, mouse, chick, *Xenopus*, fish and *Ciona intestinalis*, with two exons and an intron of variable size. *Drosophila Hand* has four exons and three introns, suggesting that there has been reorganization of the gene in *Drosophila* (Kolsch and Paululat, 2002). Expression of the *Hand* genes in cardiac tissues has been conserved, with *Hand* marking all cardiac precursor and differentiated cardiac cells in *Ciona* and *Drosophila* (Kolsch and Paululat, 2002; Satou et al., 2004). In higher organisms, expression of *Hand1* and *Hand2* become more localized, ultimately being restricted to the left and right ventricular outer curvatures of the mammalian heart.

Differences in *Hand* genes have arisen in the higher organisms. In mouse, *Hand1*is expressed in the left ventricle, and *Hand2* is expressed in the right ventricle of the mouse heart (Cserjesi et al., 1995; Srivastava et al., 1995). In chick, *Hand1* and *Hand2* are expressed throughout the heart (Srivastava et al., 1995). Independent knockdown of *Hand1* or *Hand2* in the chick heart had no effect, but a double knockdown caused arrest at cardiac looping, suggesting that the two genes are redundant or can

compensate for one another (Srivastava et al., 1995). It is possible that *Hand1* and *Hand2* have overlapping or compensatory functions, and that these are only revealed in chick due to their overlapping expression patterns. The ultimate test of this theory would be to replace one gene with the other in mouse, and observe embryonic development, but this has not been done. The ability of ectopic expression of both *Hand1* and *Hand2* to interfere with interventricular septum development suggests that they do have overlapping functions (Togi et al., 2004; Togi et al., 2006). However, the phenotype of *Hand1* mutant mouse embryos, which presumably have normal *Hand2* expression throughout the linear heart tube, argues that they do have distinct, non-overlapping, functions (Riley et al., 1998; Riley et al., 2000).

1.4.3 Hand1 *and Cardiac Transcription Factors*

In the linear heart tube, when the *Hand1* expression domain is first defined, most other cardiac transcription factors are expressed more broadly in the heart. For example, *Nkx2-5, Gata4* and *Tbx20* are expressed throughout the entire linear heart tube (Heikinheimo et al., 1994; Komuro and Izumo, 1993; Lints et al., 1993; Takeuchi et al., 2005). *Tbx5* is expressed in a posterior-anterior gradient covering the posterior half of the heart and overlaps the *Hand1* expression domain (Bruneau et al., 1999). *Tbx2* is expressed throughout the heart tube before being restricted to regions retaining the primitive phenotype (Christoffels et al., 2004; Yamada et al., 2000). *Irx4* is expressed in the ventricular region, which overlaps the *Hand1* expression domain (Bao et al., 1999). As no other cardiac transcription factor has a similar expression pattern, the *Hand1* domain is likely defined through combinatorial regulation by these factors, signalling molecules and perhaps undiscovered cardiac transcription factors.

There are a few insights into the regulation of *Hand1* gene transcription based on mutant analysis, as summarized in Table 1.2. In *Nkx2-5* mutant mice, which arrest at the looping stage and lack the left ventricle, *Hand1* is expressed on the ventral side of the heart tube, but fails to be upregulated on the left side (Biben and Harvey, 1997; Tanaka et al., 1999). *Irx4*, the ventricular determinant gene, is required for strong expression of *Hand1* in the looped heart, although the general pattern is unchanged in *Irx4* mutant hearts (Bruneau et al., 2001a). *Hand1* expression in *Irx4* mutant hearts has not been reported at the linear heart tube stage, but based on the findings in the looped heart, it is likely expressed in the correct pattern but at a lower level. *Tbx5* regulates *Hand1* expression level in a dose-dependent manner, as revealed by a *Tbx5* allelic series, and ectopic *Tbx5* expression induces ectopic *Hand1* expression (Bruneau et al., 2001b; Mori et al., 2006; Takeuchi et al., 2003). Tbx5 and Tbx20 have been shown to functionally interact and compensate for one another's deficiencies, suggesting that Tbx20 may compensate for Tbx5 in *Tbx5*$^{-/-}$ hearts, allowing normal *Hand1* expression (Brown et al., 2005; Stennard et al., 2003).

Tbx20 is required for *Hand1* expression, but it is not clear if this effect is direct. *Tbx20* mutant embryos do not form the right ventricle and outflow tract, have decreased chamber myocardium gene expression and do not repress expression of *Tbx2* in the chamber myocardium (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005). Therefore, it is possible that the decrease in *Hand1* expression observed in *Tbx20* mutants is due to repression by *Tbx2*, which is deregulated. In *Tbx2* mutants, which do not have a constricted atrioventricular canal and die by E14.5, *Hand1* expression extends further into the atrioventricular region (Harrelson et al., 2004),

Table 1.2. Hand1 Expression in Cardiac Transcription Factor Mutant Embryos.

Transcription Factor	Expression	Hand1 Expression in mutants	References
Nkx2-5	*pan*-cardiac	Low level, not upregulated on left side	(Biben and Harvey, 1997; Lyons et al., 1995; Tanaka et al., 1999)
Gata4	*pan*-cardiac	No change	(Kuo et al., 1997; Molkentin et al., 1997; Peterkin et al., 2005)
FOG2	*pan*-cardiac	Decreased	(Crispino et al., 2001; Tevosian et al., 1999; Tevosian et al., 2000)
Mef2c	*pan*-cardiac	Delayed, decreased	(Edmondson et al., 1994; Vong et al., 2006)
FGF10	SHF	N/R	(Kelly et al., 2001; Marguerie et al., 2006)
Isl-1	SHF	No change	(Cai et al., 2003)
Hand1	LVt, AVC, OFT	No expression	(Cross et al., 1995; Cserjesi et al., 1995; Firulli et al., 1998; Hollenberg et al., 1995)
Hand2	RVt, Endocardium	No change	(Cross et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995; Srivastava et al., 1997)
Tbx1	PA	N/R	(Chapman et al., 1996; Xu et al., 2004)
Tbx2	Primitive myocardium	Expanded into AVC	(Christoffels et al., 2004; Harrelson et al., 2004)
Tbx3	Primitve myocardium; CCS	N/R	(Hoogaars et al., 2004)
Tbx5	LVt, At	Decreased	(Bruneau et al., 1999; Bruneau et al., 2001b; Mori et al., 2006)
Tbx20	*pan*-cardiac	Decreased	(Kraus et al., 2001; Singh et al., 2005; Takeuchi et al., 2005)
Irx4	LVt, RVt	Decreased	(Bao et al., 1999; Bruneau et al., 2001a)

Legend: SHF: Secondary Heart Field; N/R: Not Reported; LVt: Left Ventricle; AVC: Atrioventricular Canal; OFT: Outflow Tract; RVt: Right Ventricle; PA: Pharyngeal Arches; CCS: Central Conduction System; At: Atria

suggesting that *Tbx2* represses *Hand1* expression in the atrioventricular canal. As *Hand1* promotes cell proliferation (Risebro et al., 2006), it is likely that upregulated *Hand1* causes the expansion of the atrioventricular canal in the *Tbx2* mutants.

Gata4 mutant embryos exhibit cardia bifida due to a defect in ventral endoderm. When this defect is rescued with tetraploid complementation or chimera formation, the *Gata4* mutant hearts develop through looping but appear to have defective chamber morphogenesis (Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1997; Watt et al., 2004). In *Gata4* mutant embryos, *Hand1* is expressed at levels similar to wild type, but the expression pattern is not commented on. It has been suggested that *Gata6*, which is upregulated in *Gata4* mutant hearts, may compensate for the *Gata4* deficiency. Indeed, *Gata4;Gata6* compound heterozygotes do exhibit cardiac defects distinct from those seen in single mutants, which only show phenotypes as homozygotes, suggesting that there is some redundancy between the two genes and that heart development is sensitive to *Gata* gene dosage (Xin et al., 2006). Unfortunately, *Hand1* expression was not examined in the compound heterozygotes. However, *Hand1* expression is decreased in mutants deficient for *FOG2*, a *Gata4* cofactor, as well as in *Gata4* mutants that cannot interact with FOG2 (Crispino et al., 2001; Tevosian et al., 2000). Therefore, it is likely that *Gata4* regulates *Hand1*.

1.4.4 Hand Enhancers and Transcriptional Regulation

The expression patterns of the *Hand* genes are fascinating, with *Drosophila Hand* constituting the only known transcription factor expressed in every cardial cell, and with the vertebrate *Hand* genes exhibiting early chamber-specific expression in the heart as well as expression in other non-cardiac tissues. While studies have not been published

for *Hand1*, the transcriptional regulation of *Drosophila Hand* and mouse *Hand2* has been studied.

The *Drosophila Hand* gene is expressed in the contractile cardial cells and the non-contractile pericardial cells, in the circular visceral muscle precursors and in the lymph gland cells (Kolsch and Paululat, 2002). In the heart, the *Nkx2-5* homologue *tinman* and the *Gata4* homologue *pannier* and other cardiac genes are expressed in only a subset of cardial and pericardial cells (Han and Olson, 2005), making the *Hand* expression pattern unique. To understand where *Hand* fits into the cardiac development regulatory pathway, the genomic region was examined for enhancer activity, and a 513 bp enhancer was identified in intron 3 of the *Hand* gene that recapitulated cardiac and lymph gland expression (Han and Olson, 2005; Sellin et al., 2006). Through this enhancer, *Hand* is regulated by *tinman* and *pannier*, cardiac transcription factors, and *serpent*, a lymph gland transcription factor (Han and Olson, 2005).

Enhancers regulating expression of mouse *Hand2* in the heart and branchial arches have also been identified. A 1.5 kb upstream region was necessary and sufficient to recapitulate *Hand2* cardiac expression (McFadden et al., 2000). This enhancer contains separate elements for expression in the right ventricle and outflow tract. Comparison to the upstream region in human revealed broad conservation, but no increased conservation within the enhancer except for several putative GATA-binding sites which were necessary for enhancer activity, but were not dependent on the presence of GATA4 (McFadden et al., 2000). Branchial arch expression of *Hand2* is regulated by a 208 bp endothelin-1 and Dlx6 responsive enhancer at -6.5kb (Charite et al., 2001).

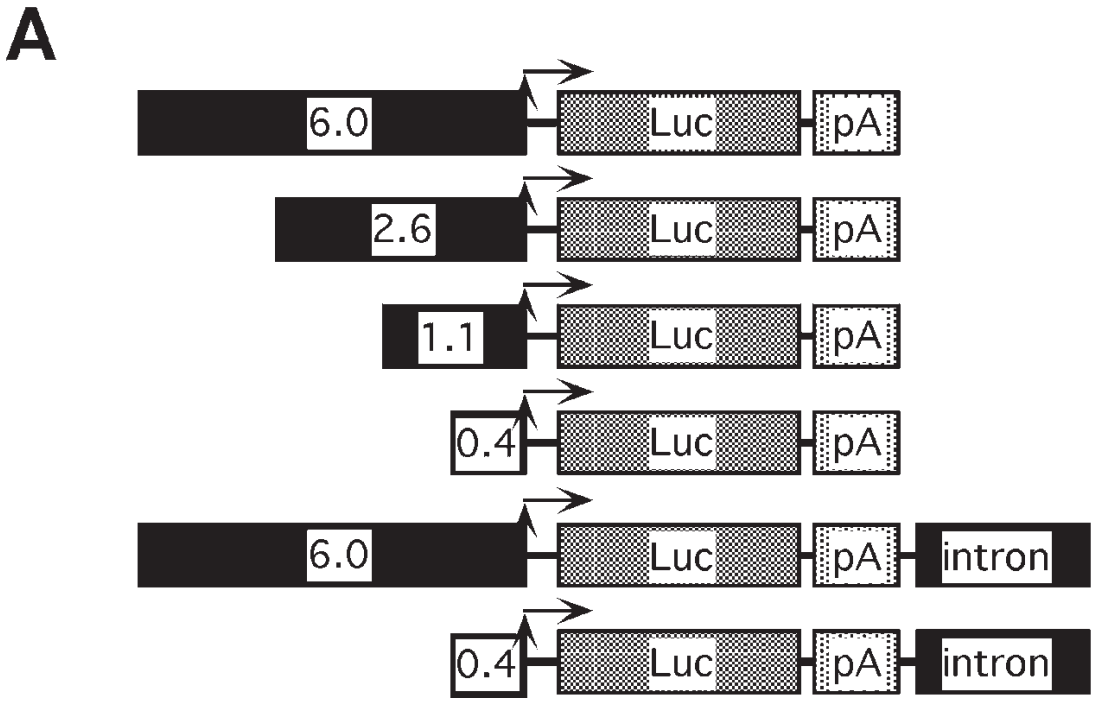
1.4.5 Transcriptional Regulation of the **Hand1** *Gene*

No studies have been published concerning the regulation of the *Hand1* gene. However, several studies conducted within the Cross laboratory form essential background to the studies in this thesis. Overall, they suggest that, like the enhancer in the *Drosophila hand* intron, the *Hand1* intron may act as a tissue-specific enhancer. To identify putative regulatory elements, reporter gene constructs were generated with 6.0 kb, 2.6 kb, 1.1kb or 0.4 kb of the 5' promoter (Fig 1.6A) (P. Riley, I. Scott, J.C. Cross, unpublished data). The *Hand1* intron and second exon were placed downstream of the poly-adenylation signal of the reporter gene in the 6.0 kb and 0.4 kb constructs. These constructs were transfected into two differentiating cell types normally expressing *Hand1* to test for tissue-specific enhancer activity: Rcho-1 cells, which model trophoblast development, and P19 cells, which model cardiomyocyte development (Fig 1.6B). In Rcho-1 cells, the greatest reporter activity was seen from the 6.0 kb construct, while addition of the intron had no effect on reporter activity. Expression from the 2.6 kb construct was lower than the 6.0 kb construct, suggesting that a regulatory element may be found between -2.6 kb and -6.0 kb. In P19 cells, expression increased from all constructs as differentiation progressed. Expression from the 0.4 kb construct, which could be considered a minimal promoter, or the 6.0 kb construct, is increased with addition of the intron, albeit to different degrees. Expression 6.0 kb + intron construct was more than the result of an additive interaction between the 6.0 kb construct and the 0.4 kb + intron construct, suggesting that the intron and the upstream region might act as a bipartite enhancer for *Hand1* cardiomyocyte expression.

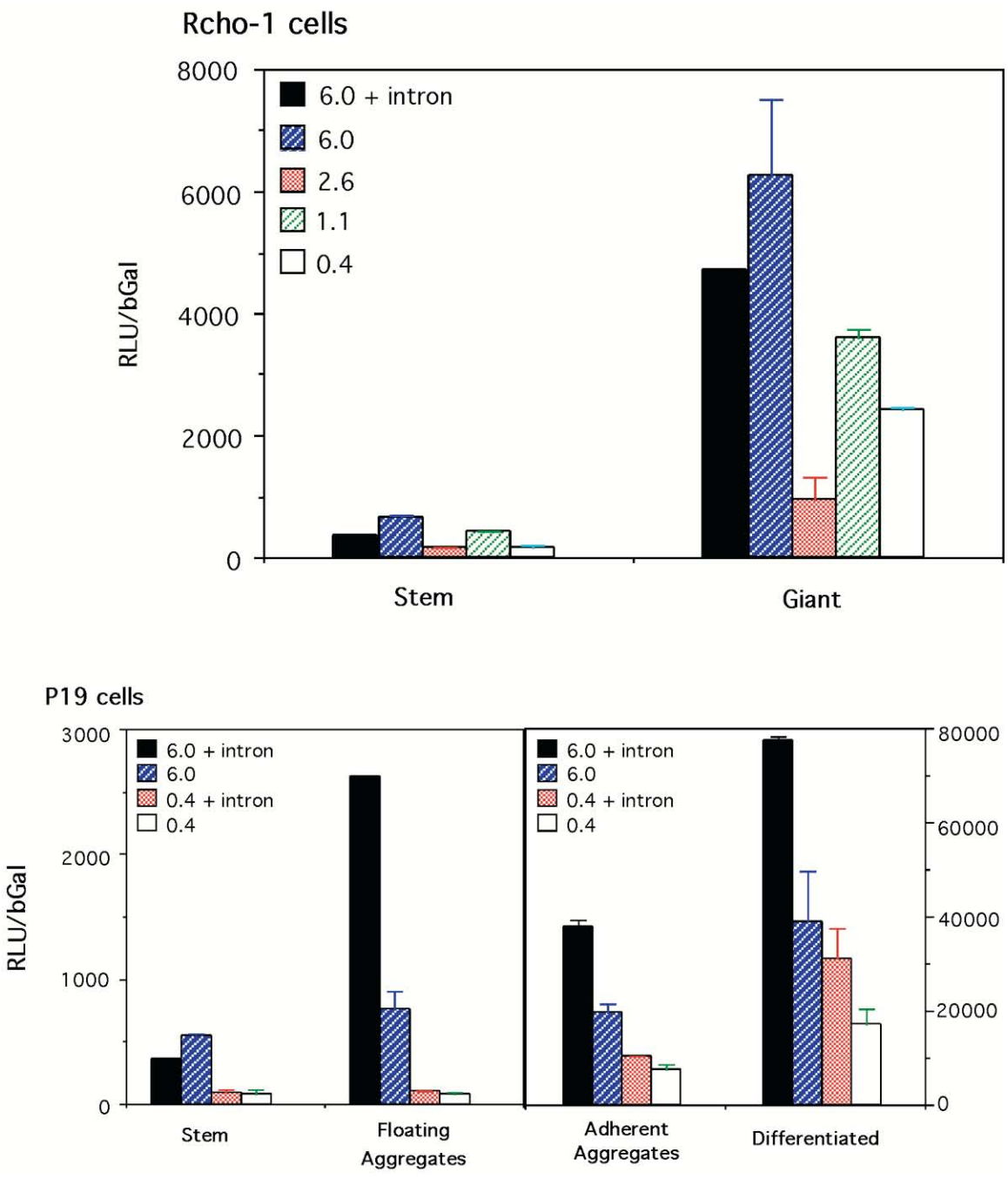
To test the hypothesis that the *Hand1* intron contains cardiac-specific enhancer

activity, the wild type *Hand1* allele was replaced with the *Hand1* cDNA in *Hand1*$^{+/-}$ embryonic stem (ES) cells to make *Hand1*$^{\Delta I/-}$ ES cells (X. Zhao and J.C. Cross, unpublished results). When differentiated, ES cells form embryoid bodies, which contain all embryonic cell types including cardiomyocytes, blood cells and neurons. The *Hand1*$^{\Delta I/-}$ cells had less *Hand1* mRNA than *Hand1*$^{+/-}$ cells (Fig 1.6C a) (Riley et al., 1998) (X. Zhao and J.C. Cross, unpublished results). Differentiated *Hand1*$^{+/-}$ ES cells express *Hand1* mRNA at a lower level than wild type ES cells, but they are normal with respect to development, so expression from a single allele is sufficient for development (X. Zhao and J.C. Cross, unpublished results). It is unclear whether the decrease in expression is specific to cardiomyocytes, with expression unchanged in other cell types, or is a global decrease in all cell types expressing *Hand1*. Differentiated embryoid bodies containing cardiomyocytes exhibit spontaneous beating, therefore the proportion of embryoid bodies exhibiting spontaneous beating is representative of the proportion of cardiomyocyte differentiation. *Hand1*$^{-/-}$ ES cells differentiate into cardiomyocytes at a ten-fold higher rate than wild type or *Hand1*$^{+/-}$ ES cells (I. Scott, X. Zhao and J.C. Cross, unpublished results). This appears to be due to a decrease in repression signalling that restricts the size of the cardiac field, based on chimeric embryoid bodies in which the presence of wild type cells suppresses the rate of cardiomyocyte differentiation of *Hand1*$^{-/-}$ ES cells (X. Zhao and J.C. Cross, unpublished results).

Figure 1.6. The *Hand1* intron contains a putative enhancer. A. *Hand1* promoter constructs. Fragments of the mouse 5' regulatory region drive expression of a reporter gene (luciferase), which is followed by a poly-adenylation signal. In some constructs, the intron and second exon were placed 3' of the poly-adenylation signal. B. *Hand1* promoter activity in trophoblast (Rcho-1) and cardiomyocyte (P19) cells. Rcho-1 cells were transfected using lipofectamine, and luciferase activity was assayed 48 hours after transfection in stem cells and differentiated giant cells. P19 cells were transfected with the CaPO4 method, and luciferase activity was assayed 48 hours after transfection in stem cells, and as floating aggregates (day 1 of differentiation), adherent aggregates (day 3) and as differentiated cells (day 8). Note that in Rcho-1 cells, the intron does not increase reporter activity, but that in P19 cells, the intron significantly increases reporter activity above levels achieved with the same 5' regulatory region. Reporter gene activity is represented as relative light units, normalized to beta-galactosidase (RLU/bGal). C. The *Hand1* intron was deleted using gene targeting in *Hand1+/-* embryonic stem (ES) cells to generate *Hand1*Δ*I/-* ES cells. (a)The *Hand1*Δ*I/-* and *Hand1+/-* ES cells were differentiated to embryoid bodies, which contain cardiomyocytes, and steady state *Hand1* mRNA levels were compared to differentiated *Hand1+/-* by Northern blot. (b) The *Hand1*+/+, +/-,Δ*I/-* and -/- ES cells were differentiated to embryoid bodies, and the percentage of the embryoid bodies exhibiting beating was compared. Embryoid bodies beat spontaneously when cardiomyocytes are present, therefore the proportion of beating bodies is representative of the relative levels of cardiomyocyte differentiation.

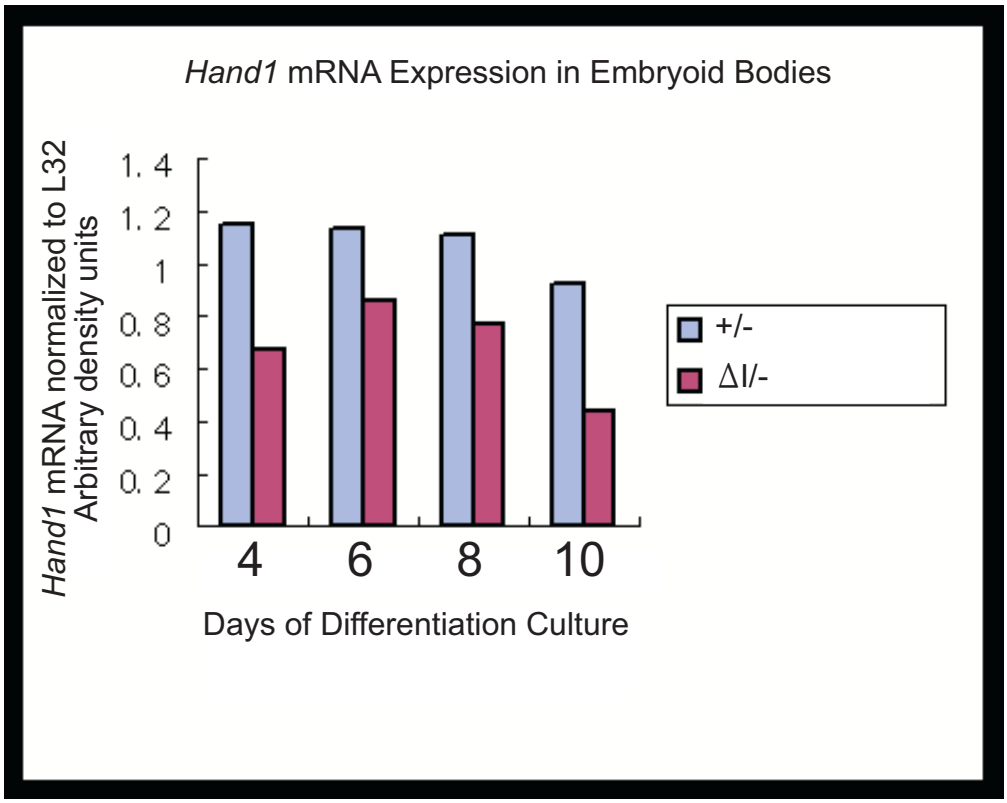


B

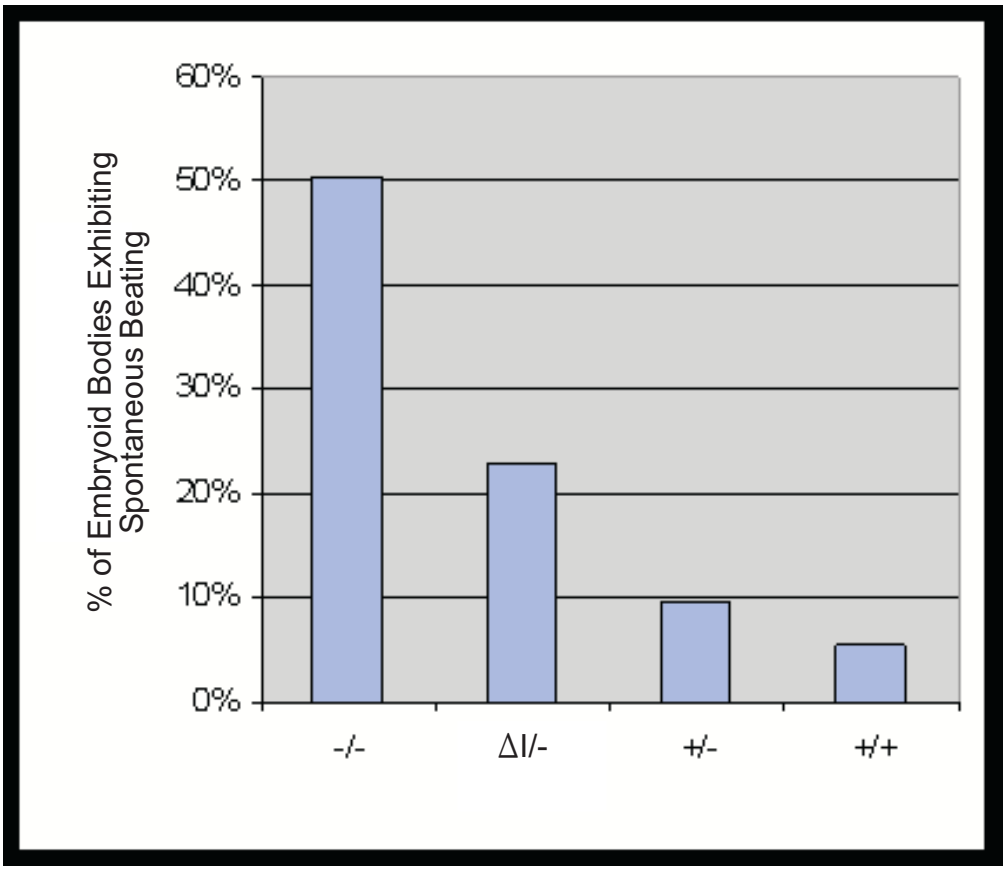

Rcho-1 cells
8000
6000
4000
2000
0
RLU/bGal
6.0 + intron
6.0
2.6
1.1
0.4
Stem
Giant
P19 cells
3000
2000
1000
0
RLU/bGal
6.0 + intron
6.0
0.4 + intron
0.4
80000
60000
40000
20000
0
Stem
Floating Aggregates
Adherent Aggregates
Differentiated

C

a


Hand1 mRNA Expression in Embryoid Bodies
Hand1 mRNA normalized to L32
Arbitrary density units
1. 4
1. 2
1
0. 8
0. 6
0. 4
0. 2
0
4
6
8
10
Days of Differentiation Culture
+/-
ΔI/-

b


% of Embryoid Bodies Exhibiting
Spontaneous Beating
60%
50%
40%
30%
20%
10%
0%
-/-
ΔI/-
+/-
+/+

When the *Hand1*$^{\Delta I/-}$ ES cells were compared to *Hand*$^{+/+}$, *Hand1*$^{+/-}$ and *Hand1*$^{-/-}$ ES cells after differentiation to embryoid bodies, it was determined that *Hand1*$^{\Delta I/-}$ exhibit a proportion of beating embryoid bodies intermediate to the *Hand1*$^{+/-}$ and *Hand1*$^{-/-}$ cells, demonstrating a functional effect of the decreased *Hand1* mRNA levels (Fig 1.6C b) (X. Zhao and J.C. Cross, unpublished results). These observations were consistent over three experimental repetitions.

To determine if 6 kb of 5' sequence, the intron and second exon with 3' untranslated region (UTR) are sufficient for Hand1 expression, transgenic embryos were generated with a reporter gene under the control of the 6 kb, intron and second exon (P. Riley and J.C. Cross, unpublished results). No reporter gene expression was observed in any tissue in three independent lines or 10 injected embryos. As several independent lines were assessed, it is unlikely that position-dependent silencing prevented reporter gene expression, and therefore 6 kb of 5' sequence, the intron and the second exon are not sufficient for *Hand1* expression. However, these regions may be necessary for *Hand1* expression. Additionally, a transgene utilizing up to 20 kb of upstream sequence was not sufficient for expression (A. Firulli, personal communication). Therefore, the transgenic approach to identifying regulatory regions that are sufficient for *Hand1* expression has not been successful to date, and new approaches are required.

1.4.6 Overall Rationale for Thesis Work

The *Hand1* gene is expressed in and is critical for trophoblast, yolk sac and cardiac development in mouse (Firulli et al., 1998; Morikawa and Cserjesi, 2004; Riley et al., 1998; Riley et al., 2000). The *Hand1* cardiac expression pattern is particularly interesting due to its novel pattern established early in heart development (Biben and

Harvey, 1997; Srivastava et al., 1997). Expression in multiple tissues is often regulated by modular enhancers, like the cardiac and branchial arch enhancers of *Hand2* (Charite et al., 2001; McFadden et al., 2000). As described, previous results in the lab suggest that the *Hand1* intron may contain tissue-specific enhancer activity (P. Riley, I. Scott, X. Zhao, unpublished results).

The hypothesis investigated in this thesis was that the intron contains a cardiac-specific enhancer element, and that deletion of the intron would disrupt only the cardiac expression domain. I utilized bioinformatics to study conservation and putative transcription factor binding sites in the intron. Based on this information, I proceeded to compare expression of cardiac transcription factors to that of *Hand1*, to identify putative regulators of *Hand1*. Finally, the *Hand1* intron was deleted, and the effect on *Hand1* expression was examined.

Chapter Two: Materials and Methods

2.1 *In situ* Hybridization probes

Digoxygenin-labeled probes for whole mount *in situ* hybridization were generated with an RNA transcription kit according to manufacturer's instructions (Roche). Probes were generated as follows:

- *Hand1* (Cross et al., 1995), plasmid 6-13, linearize with SalI, transcribe with T7
- *Hand2* (Cross et al., 1995), plasmid 6-26, linearize with BamHI, transcribe with T3
- *Irx4* (Bruneau et al., 2000), plasmid 19-59, linearize with XbaI, transcribe with T3
- *Tbx2* (Bollag et al., 1994), plasmid 20-5, linearize with EcoRV, transcribe with T7
- *Tbx5* (Bruneau et al., 1999), plasmid 17-15, linearize with SpeI, transcribe with T7
- *Tbx20*, (Carson et al., 2000) plasmid 20-1, linearize with NotI, transcribe with T7
- *α-cardiac actin* (X. Zhao), plasmid 20-38, linearize with SalI, transcribe with T7

2.2 RNA Expression Analysis-

2.2.1 Whole Mount in situ Hybridization

Whole mount *in situ* hybridization was performed according to the protocol published by Conlon and Rossant (1992). Methanol/hydrogen peroxidase bleaching was omitted, as it was unnecessary for these samples. Throughout this protocol, samples were rocked on a nutator, or rotated in the hybridization oven. All solutions used prior to RNase treatment are treated with diethyl pyrocarbonate (DEPC, *Sigma, D-5758*) or DEPC-treated water.

Briefly, tissue for whole mount *in situ* hybridization was collected from timed matings of CD1 mice or $Hand1^{+/-}$ x $Hand1^{+/\Delta I}$ mice. Following dissection, tissues were fixed in 4% paraformaldehyde (PFA) in 1 X PBS overnight at 4ºC, washed three times in

1 X PBT, then transferred through a 25%, 50% and 75% methanol/PBT gradient to 100% methanol. Samples are stored in 100% methanol at -20ºC until use. Samples are rehydrated through a methanol/PBT gradient to 1XPBT, treated with 20 µg/µl proteinase K (*Roche, 03 115 879 001*) in PBT (E8.5: 3 minutes; E10.5: 4 minutes), washed in PBT, and re-fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in PBS for 20 minutes. The samples are washed in PBT three times, then twice in hybridization buffer (50% formamide, 0.75M NaCl, 1XPE, 100 µg/ml yeast tRNA (*Roche, 10 109 495 001*), 0.05% heparin, 0.1% bovine serum albumin (*Sigma, A4503*) and 1% sodium dodecyl sulfate). Samples are pre-hybridized in hybridization buffer at 63ºC for 3 hours, then the hybridization buffer is replaced and digoxygenin-labeled probe is added (0.5 – 2.0 µg/ml), and samples are incubated at 63ºC overnight.

Samples are washed according to protocol and RNase treated with RNase T1 (*Roche, 109 193*) (Conlon and Rossant, 1992), and blocked prior to antibody incubation with 10% heat inactivated goat serum in Tris-buffered saline with 0.1% Tween-20 (TBST) for one hour. At this stage, 0.5 mg/ml levamisole was included in all solutions to inhibit endogenous alkaline phosphatase activity. Alkaline phosphatase conjugated anti-digoxygenin antibody is blocked against acetone-prepared embryo powder (see Conlon and Rossant, 1992 for instructions), then incubated with samples overnight at 4ºC. The following morning, samples are washed in TBST prior to alkaline phosphatase activation in NTMT (0.1M NaCl, 0.1M Tris, pH 9.5, 0.05M $MgCl_2$, 0.1% Tween-20) and addition of colourimetric substrate (4.5µl/ml NBT, 3.5 µl/mlBCIP, Promega). Colour is developed at room temperature, protected from light. Colour reaction was terminated with PBT washes and 0.1M glycine pH 2.2 with 0.1% Tween-20, followed by TBST

washes. Embryos were cleared in glycerol, photographed and stored at 4ºC.

2.2.2 In Situ Hybridization on Tissue Sections

In situ hybridization on tissue sections was carried out to detect the expression of mRNA for *Hand1* (Cross et al., 1995), *Tbx2* (Bollag et al., 1994), and α-cardiac actin. Embryos for section *in situ* hybridization were dissected at E8.25 to E8.5, fixed in 4% PFA for four hours at 4ºC, rinsed in 1X PBS, and cryoprotected through a sucrose gradient prior to embedding in OCT. Transverse sections, 10 μm thick, were cut on a cryostat and mounted on Super Frost Plus slides (*VWR*).

In situ hybridization on tissue sections was performed as described by Simmons et al (2007). Briefly, slides are rehydrated in PBS and fixed in 4% PFA for 15 minutes, treated with 10 mg/ml proteinase K for 5 minutes and refixed in 4% PFA for 5 minutes. Slides are acetylated for 10 minutes (0.25% acetic anhydride, *Sigma*) to reduce background signal, washed and hybridized overnight in a humidified chamber with probe in hybridization buffer (1X Salts, 50% formamide, 5% dextran sulphate, 1 mg/ml yeast tRNA, 1X Denhardt's). Probe is diluted 1:2000 in hybridization buffer from synthesis reaction when applied to slides. The next day, slides are washed to remove unbound and non-specifically bound probe prior to blocking, as described in Simmons et al (2007). Alkaline phosphatise-conjugated anti-digoxygenin antibody is applied to slides after blocking, and slides are incubated at 4ºC in a humidified chamber overnight. Slides are washed, alkaline phosphatise is activated and colour development proceeds at room temperature in the dark. Staining is stopped with 1X PBS and 0.1M glycine-HCl pH 2.2 prior to photographing. Slides were photographed without counterstaining.

2.2.3 Northern Analysis

To characterize quantitative changes in gene expression, RNA was collected from E10.5 conceptuses from *Hand1*$^{+/-}$ x *Hand1*$^{+/\Delta I}$ matings. Implantation sites were dissected to separate the embryonic (embryo, yolk sac) and extraembryonic (decidua, placenta and parietal trophoblast giant cells) tissues. RNA was extracted by homogenizing each tissue sample in 2 ml of Trizol (*Invitrogen*) and processed according to the manufacturer's instructions. A tail sample was reserved for genotyping.

Messenger RNA was separated by electrophoresis on a standard agarose/formaldehyde gel as described for Northern blotting in Sambrook et al (1989). Briefly, gel boxes for RNA gels were pretreated with hydrogen peroxide and rinsed with RNase-free water prior to use. RNA was run on an RNA gel (1X MOPS, 4% formaldehyde, 1% agarose) in 1X MOPS buffer. RNA samples were dried down to 4.5 ul, 10 ul sample buffer was added (1X MOPS, 4% formaldehyde, 70% formamide;), samples were heat denatured (65ºC, 10 minutes), cooled on ice, and loading dye was added (3 ul: 50% glycerol, 1mM EDTA, bromophenol blue, xylene cyanol, 1 ug/ul ethidium bromide). The gel ran at 80V for 3.5 hours, and was then photographed. The gel was washed with RNase-free water, transferred to a nylon membrane overnight by capillary transfer, then cross-linked to the membrane with the Stratalinker.

Northern blots were probed for *Hand1* mRNA according to standard Cross lab protocol. The *Hand1* probe was released from plasmid 6-13 (Cross et al., 1995) by digesting with EcoRI. The probe fragment was labelled with ^{32}P (50 ul: 50 ng DNA, 5 ul 10X RPLB buffer, 2.5 ul random hexamers, 2 ul bovine serum albumin (10 ul/ug), 2 ul Klenow fragment (9 unites/ul), 5 ul dCTP) for 3 hours, isolated on a MicroSpin S-200

HR column (GE Healthcare). The Northern blots were pre-hybridized in Church buffer (0.02 g/ml bovine serum albumin, 0.5M $NaPO_4$, 7% SDS, 1mM EDTA), and labelled probe was added to 10^6 counts/ml. Blots were hybridized overnight at 65ºC, then washed twice (2x SSC, 0.1% SDS) at room temperature before exposing to film.

2.3 Bioinformatics

2.3.1 Sequences

All sequences, unless otherwise specified, were downloaded from the Ensembl database (www.ensembl.org). Genomic regions, cDNAs, introns and protein sequences were downloaded. Sequence accession numbers are listed in Table 2.1.

2.3.2 Sequence Analysis

Phylogenetic analysis was carried out with ClustalW (http://bips.u-strasbg.fr/fr/Documentation/ClustalX) (Thompson et al., 1994) and Phylip (http://evolution.genetics.washington.edu/phylip.html), which were downloaded for execution on a local computer. Multiple alignments were constructed with ClustalW, then used directly to generate trees with the Phylip subprogram *drawgram*, or a bootstrapped tree was generated with *seqboot, dnapars* or *protpars*, *consense* and *drawgram*. *Ciona intestinalis* was used as an outgroup species, as it is the most primitive organism considered.

To identify conserved regulatory regions, pair-wise comparisons were carried out with zPicture (http://zpicture.dcode.org) (Ovcharenko et al., 2004a) and rVista (http://rvista.dcode.org) (Loots and Ovcharenko, 2004). All transcription factor binding site motifs available in rVista were used to search for conserved binding sites.

Table 2.1. *Hand* gene family sequence accession numbers from Ensembl.

Gene	Accession number
Mus musculus (mouse) *Hand1*	ENSMUSG00000037335
Mus musculus (mouse) *Hand2*	ENSMUSG00000038193
Homo sapiens (human) *Hand1*	ENSG00000113196
Homo sapiens (human) *Hand2*	ENSG00000164107
Rattus norvegicus (rat) *Hand1*	ENSRNOG00000002582
Rattus norvegicus (rat) *Hand2*	ENSRNOG00000022069
Xenopus tropicalis (frog) *Hand1*	ENSXETG00000008479
Xenopus tropicalis (frog) *Hand2*	ENSXETG00000016908
Gallus gallus (chick) *Hand1*	ENSGALG00000012831 [1]
Gallus gallus (chick) *Hand2*	ENSGALG00000010757
Danio rerio (zebrafish) *hand2*	ENSDARG00000008305
Fugu rubripes (fugu fish) *hand*	NEWSINFRUG00000127883
Drosophila melanogaster (fruit fly) *hand*	CG18144
Ciona intestinalis (sea squirt) *hand*	ENSCING00000005750
Bos taurus (cow) *Hand1*	ENSBTAG00000002335
Canis familiaris (dog) *Hand1*	ENSCAFG00000017641

1: This annotation is no longer in the Ensembl database. There is no annotation for a *Gallus gallus Hand1* gene as of February 20, 2007.

2.4 Animals

All animal work was carried out in compliance with the University of Calgary Animal Care Committee guidelines.

For wild type embryos used for whole mount and section *in situ* hybridization, CD1 (Charles River) mice were crossed and pregnant females were dissected at various times on E8.5. To acquire embryos at early heart stages on E8.0 and to minimize stage variability between litters, female mice were placed in the cage with the male mouse for 3 hours (7 am – 10 am), and cages were placed under a sheet for darkness to encourage breeding.

The *Hand1*$^{\Delta I}$ allele was generated by replacing the *Hand1* gene with the *Hand1* cDNA (X. Zhao and J.C. Cross, unpublished). The strategy is outlined in Figure 2.1. A floxed puromycin selection cassette was placed into a XhoI site in the 3' untranslated region of the cDNA. The *Hand1* gene was targeted in R1 embryonic stem cells through electroporation. To confirm targeting, Southern blots were generated with ES cell genomic DNA digested with BglII, and probed with an upstream region external to the targeting construct (Fig 2.1). Chimeric mice were generated by aggregating targeted ES cells with C57/BL6 embryos. Chimeric males were bred to 129 female mice, and germline transmission of the *Hand1*$^{\Delta I}$ allele was obtained. The floxed puromycin selection cassette was then removed by crossing the *Hand1*$^{\Delta I/+}$ animals to Mox2-Cre mice, which express Cre throughout the epiblast (Tallquist and Soriano, 2000). Following Cre-mediated deletion of the selection cassette, the *Hand1*$^{+/\Delta I}$ mice were bred onto CD1 and 129 backgrounds for further analysis. Work described in this thesis utilized the *Hand1*$^{+/\Delta I}$ mice on the CD1 background. Backcrossing onto the 129

background is ongoing. The *Hand1* null mouse line was previously described, and is on the CD1 background (Riley et al., 1998).

Hand1$^{+/\Delta I}$ and *Hand1*$^{+/-}$ mouse lines were intercrossed and litters went to term to determine if all genotypes were viable. To study embryonic lethality, the same crosses were performed and dissected at E10.5 and E8.5. Yolk sacs were collected for genotyping, and embryos and implantation sites were saved for whole mount *in situ* hybridization.

2.5 Genotyping

The *Hand1* wildtype and *Hand1* null alleles are detected by PCR: wild type forward primer (JCC 136): 5'-CCACTAGGATCGCACGTGCA-3'; null forward primer (JCC 126): 5'-GCAAAGCTGCTATTGGCCGC-3'; common reverse primer (JCC 179): 5'-AAGCAGAGGTGCTGCGCAAG-3', PCR program: 94C 1 minute, 56C 1 minute, 72C 1 minute, 30 cycles. (Riley et al., 1998). The *Hand1*$^{\Delta I}$ allele was also detected with JCC 136 and JCC 179, producing a band 59 bp larger due to the presence of the recombined loxP site in the 3' UTR (X. Zhao and J.C. Cross, unpublished).

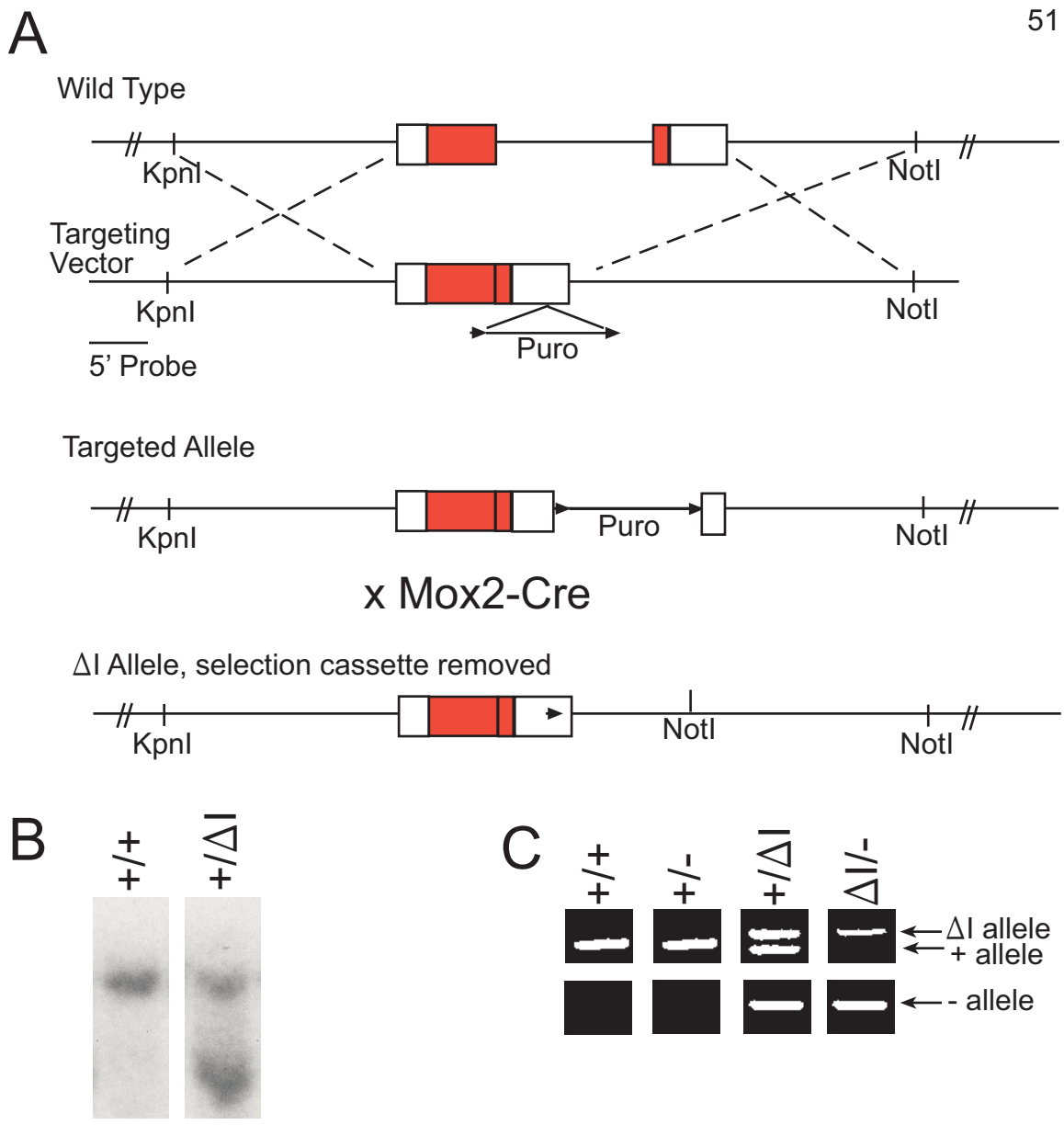


Figure 2.1. Generation of the intron mutant (ΔI) allele. (A) The wild type *Hand1* allele contains two exons (untranslated region (UTR): clear; coding region: red) and a large (1.5kb) exon. The targeting vector contains 5' and 3' homologous sequence, and the *Hand1* gene is replaced with the *Hand1* cDNA. A loxP-flanked puromyocin selection cassette was inserted into the 3' UTR. R1 embryonic stem cells were electroporated with the targeting vector (linearized at KpnI and NotI), and selected for recombination with puromyocin. (B) Puromyocin resistant colonies were screened for homologous recombination by digesting DNA with BglII, and probing southern blots with a 5' probe which was external to the linearized targeting vector. Targeted cells were used to make chimeric mice, which were screened for germline transmission of the ΔI allele with PCR (C). The floxed puromyocin cassette was removed by crossing +/ΔI mice to Mox2-Cre mice.

Chapter Three: Results

3.1 Bioinformatics

Previous experimental results from the Cross laboratory suggested that the *Hand1* intron contains tissue-specific enhancer activity. I utilized bioinformatics tools to characterize the intron sequences of *Hand* gene family members, with the goal of identifying evolutionarily conserved regions and putative transcription factor binding sites that may contribute to the hypothesized enhancer activity. Three mammalian species, mouse, rat and human, one avian species, *Gallus gallus*, one amphibian species, *Xenopus tropicalis*, two fish species, *Danio rerio* and *Fugu rubripes*, one insect species, *Drosophila melanogaster*, and one primitive chordate, *Ciona intestinalis,* were included in this analysis. These species were selected as they mark important points of cardiac evolution, have at least one *Hand* gene, and they provide broad and fine coverage of evolution. *Fugu* was included because its genome is severely compacted, up to an eight-fold compaction in comparison to the human genome. Therefore, non-coding sequences conserved between *Fugu* and higher species are likely to share functionality (Boffelli et al., 2004; McLysaght et al., 2000).

To understand the evolutionary history of the *Hand* gene family, a phylogenetic tree was constructed from the predicted whole protein sequences of the *Hand* gene family. The predicted protein sequences were aligned with ClustalW (Thompson et al., 1994), then processed with the PHYLIP software package to generate phylogenetic trees (http://evolution.genetics.washington.edu/phylip.html). Trees were generated with and without rooting to *Ciona intestinalis*, the most primitive organism considered, and with and without bootstrapping. Bootstrapping creates many trees by sub-sampling the

aligned sequences, which are then examined for consensus, adding statistical strength to a tree. From this bootstrapped tree, shown in Figure 3.1, several conclusions can be drawn. First, the *Hand* gene family can be divided into two subfamilies, *Hand1* and *Hand2*, with the single *Hand* genes divided between the subfamilies. Second, the *Danio* and *Ciona Hand* genes are more similar to the *Hand2* subfamily, while the *Drosophila* and *Fugu Hand* genes are more similar to the *Hand1* subfamily. This differs from a previous interpretation, which was based the basic helix-loop-helix domain of the *Hand* proteins only (Kolsch and Paululat, 2002), compared with my analysis of the entire protein sequence. With this understanding of relationships within the *Hand* family, and the established interest in the *Hand1* cardiac expression pattern, a search for putative cardiac regulatory regions was undertaken.

Hand genes have a conserved genomic structure of two exons and a single intron, with the exception of *Drosophila*, which has four exons, two small introns and one large intron. In all mammals, *Xenopus*, *Fugu* and *Drosophila,* the introns are over 1000 bp (Table 3.1). *Gallus, Danio, Ciona,* and two of the *Drosophila Hand* introns are less than 1000 bp. The relatively large size of the intron in *Fugu* is suggestive of functionality conserved with higher vertebrates. When intron sizes are overlaid on the unrooted, bootstrapped phylogenetic tree generated from protein sequences, it can be observed that the single *Hand* genes with the small introns segregate to the *Hand2* subfamily, while the single *Hand* genes with larger introns are grouped with the *Hand1* subfamily. In the future, it might be interesting to include *Hand* genes from more species to this analysis to see if these observations hold true for a larger data set.

To identify putative regulatory elements within the *Hand* intron, a comparative

genomics approach was employed. As the *Fugu* genome is considerably compacted, the retention of a large intron suggests that functional non-coding sequence may be conserved within introns. The *Fugu* intron sequence was compared to the mouse *Hand1* intron sequence by ClustalW alignment. Some short regions of similarity were identified, but similarity was too low for analysis with combination alignment and transcription factor binding site search programs like zPicture or rVista (Loots and Ovcharenko, 2004; Ovcharenko et al., 2004a). Comparison of the *Hand1* intron sequences from mouse against those from human, rat, dog and cow with rVista identified an evolutionarily conserved region at the 3' end of the intron, with several conserved and aligned putative transcription factor sites in those regions (Fig. 3.2). Comparison of the mouse *Hand1* intron to the mouse *Hand2* intron, the human *Hand2* intron, and the chick and frog *Hand1* introns yielded no significant similarity, and therefore the conserved regions identified are specific to the mammalian *Hand1* introns included in this analysis. The conserved and aligned putative transcription factor binding sites identified by rVista were not for cardiac transcription factors, but as few models are available for cardiac specific transcription factor binding sites, cardiac specific transcription factor binding sites may be present, but undetected. The alternative is that cardiac transcription factor binding sites do exist, but that their positions are not precisely conserved. Consistent with this proposal, examination of the mouse *Hand1* intron sequences individually revealed clustering of putative cardiac transcription factor binding sites, including sites for Nkx, Gata, Tbx and Irx factors (Fig 3.2B d). Based on the clustering of cardiac transcription factor binding sites, as well as the unpublished data indicating that the intron has transcriptional activity in transfected cardiomyocytes (P. Riley, I. Scott, J.C. Cross,

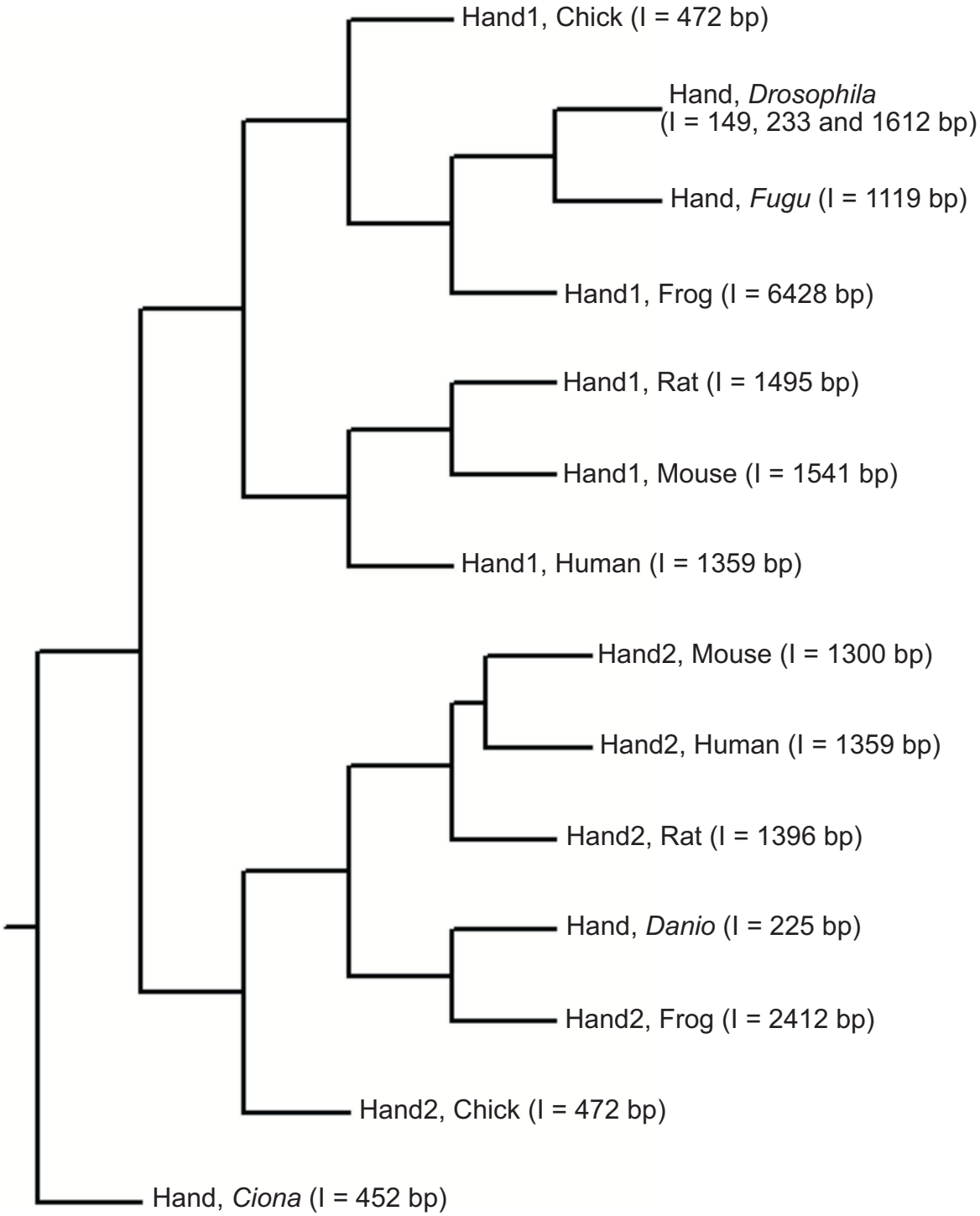


Figure 3.1. Phylogenetic tree, constructed with *Hand* protein sequences. Aligned with ClustalW, bootstrapped and consensus tree constructed with Phylip. Intron sizes, in base pairs (bp), are noted beside the gene name and organism.

Table 3.1. Cardiac structure, *Hand* genes and intron sizes.

Species	**Cardiac Structure**	**Hand Genes**	**Intron Length** (kb)
Human	4 chambers	*Hand1*	1,555
		Hand2	1,359
Mouse	4 chambers	*Hand1*	1,537
		Hand2	1,300
Rat	4 chambers	*Hand1*	1,495
		Hand2	3; 62,385; 1,396
Chicken	4 chambers	*Hand1*	4; 473
		Hand2	2; 4; 429; 472
Frog (*X.tropicalis)*	3 chambers	*Hand1*	6,428
		Hand2	2,421
Zebrafish	2 chambers	*Hand*	225
Fugufish	2 chambers	*Hand*	1,119
Drosophila	Linear Tube	*Hand*	149; 233; 1,612

unpublished data), we tentatively concluded that the *Hand1* intron may contain a regulatory element.

3.2 Comparison of Cardiac Expression Domains between *Hand1* and Other Cardiac Transcription Factors

To determine which cardiac transcription factors are expressed in a manner consistent with a role in regulation of *Hand1*, a comparative expression analysis was carried out. Transcription factors were selected for study based on the identification of putative binding sites in the intron, restricted expression within the heart, roles in cardiac patterning, and evidence from gene mutants suggesting that they may regulate *Hand1*. Whole mount *in situ* hybridization was used to detect expression of *Hand1, Hand2, Irx4, Tbx2, Tbx5* and *Tbx20* in E8.5 mouse embryos during early heart development. Expression was compared between embryos of similar heart looping stages (Biben and Harvey, 1997), rather than somite number, as heart looping stage is not strictly related to somite number. mRNA expression patterns are summarized in Figure 3.6.

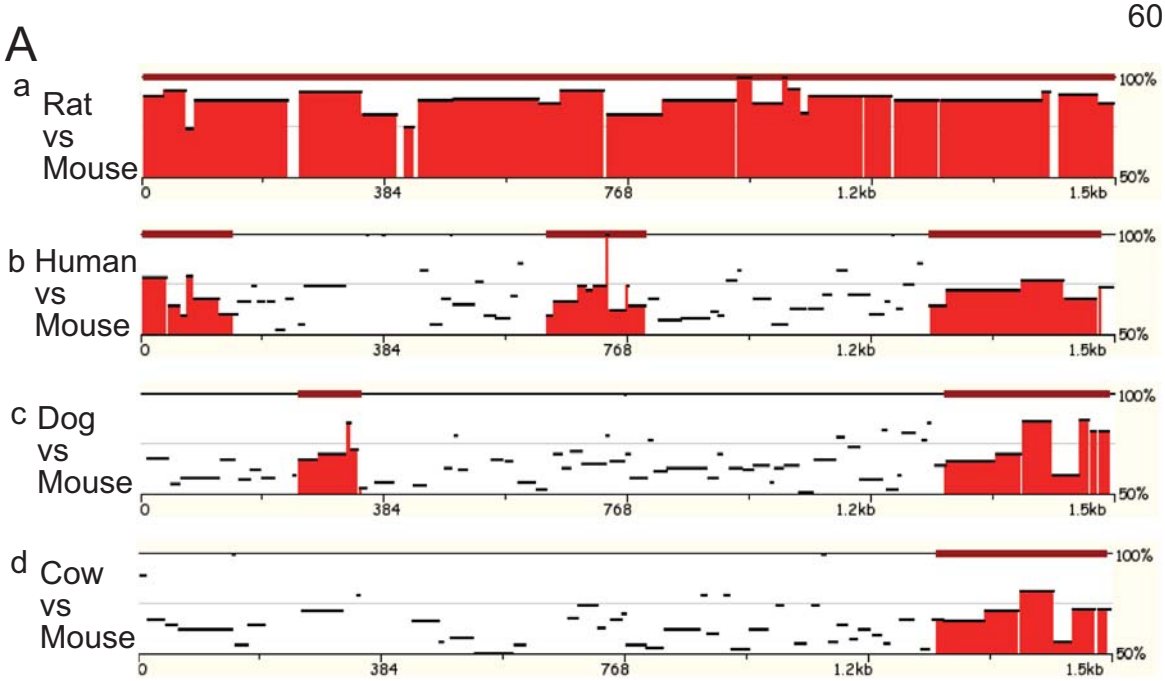
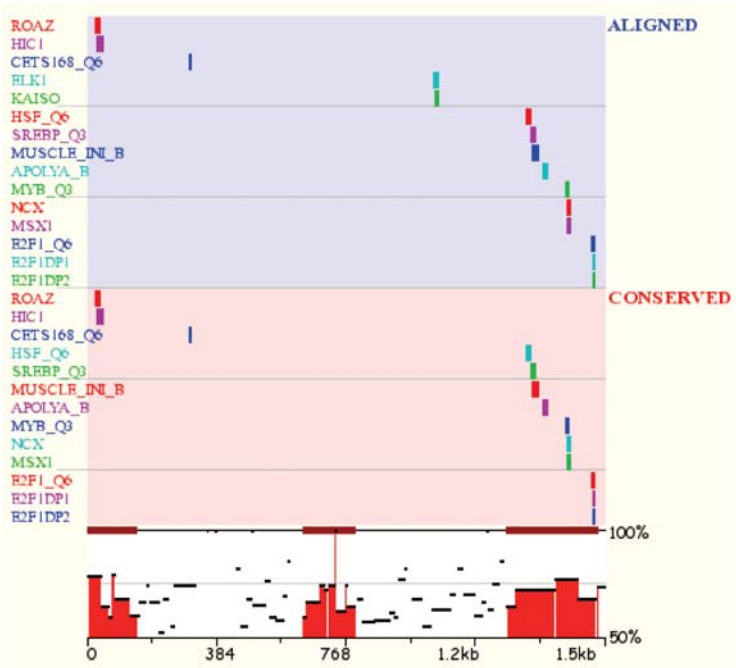


Figure 3.2. *Hand1* intron analysis with zPicture. (A) *Hand1* introns from human, rat, cow and dog were compared to the mouse *Hand1* intron using zPicture to identify conserved regions. The line above each comparison is marked with boxes to indicate regions of 100 bp or more with similarity greater than 70%. The 5' end of the sequences is to the left. (a) The rat intron, which is closely related to mouse, has extensive conservation throughout the intron. The human intron (b) has three conserved regions, the dog intron (c) has two conserved regions, and the cow intron (d) has one conserved region. Notably, the 3' conserved region is present in all organisms. No similarity was identified between mouse *Hand1* and *Hand2* introns (data not shown).
(B) Results from zPicture were forwarded to rVista for identification of conserved putative transcription factor binding sites. As in (A), the lower sequence in each comparison is the mouse *Hand1* intron. The entire database of transcription factor binding sites was searched against the zPicture alignments. Several putative transcription factor binding sites were conserved within and between the comparisons. (a) Mouse-human comparison. (b) Mouse-cow comparison. (c) Mouse-dog comparison. (d) Manual identification of cardiac transcription factor binding sites, in same scale as zPicture alignments.

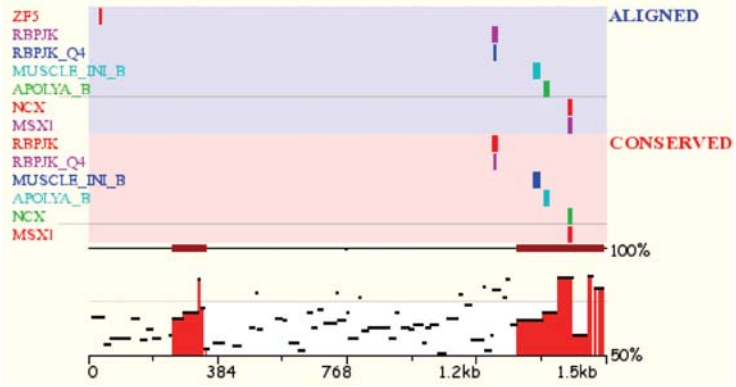
B

a

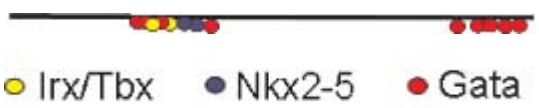


b

c



d Mouse *Hand1* Intron



3.2.1 Hand1 Cardiac Expression

Hand1 is expressed in the developing myocardial layer of the heart from the cardiac crescent stage onward. At all stages, *Hand1* expression can be detected in the pericardium. For clarity, the pericardium was removed from the embryo prior to *in situ* hybridization, with small portions of the pericardium remaining at the anterior and posterior poles of the heart. *Hand1* is expressed in the posterior portion of the linear heart tube, but does not extend posterior from the heart tube into the sinus venosus (Fig 3.3). As the heart initiates looping and rotates leftward, *Hand1* is expressed in the atrioventricular canal and the outer curvature of the future left ventricle. Expression within the future left ventricle does not extend to the interventricular groove, where the interventricular septum will form. In the looped heart, *Hand1* is still expressed in the atrioventricular canal and outer curvature of the left ventricle, and expression will be initiated in the outflow tract. In the left ventricle, *Hand1* mRNA is expressed only in the region which is derived from the primary heart field. Notably, the *Hand1* mRNA expression domain does not follow chamber myocardium boundaries, as it extends from the left ventricle into the atrioventricular canal, suggesting that it is not simply a chamber myocardium gene expressed in the left ventricle.

3.2.2 **Hand2** *Cardiac Expression*

Hand2 mRNA is initially detected in the endocardial layer of the developing heart, and is later detected in the myocardial layer Saga et al (1999). Consistent with this, *Hand2* mRNA was detected in the the heart, with staining appearing to be internal to the heart tube, suggestive of endocardial expression (Fig 3.3). *Hand2* expression has been detected in the myocardium of the looping heart tube by McFadden et al (2000).

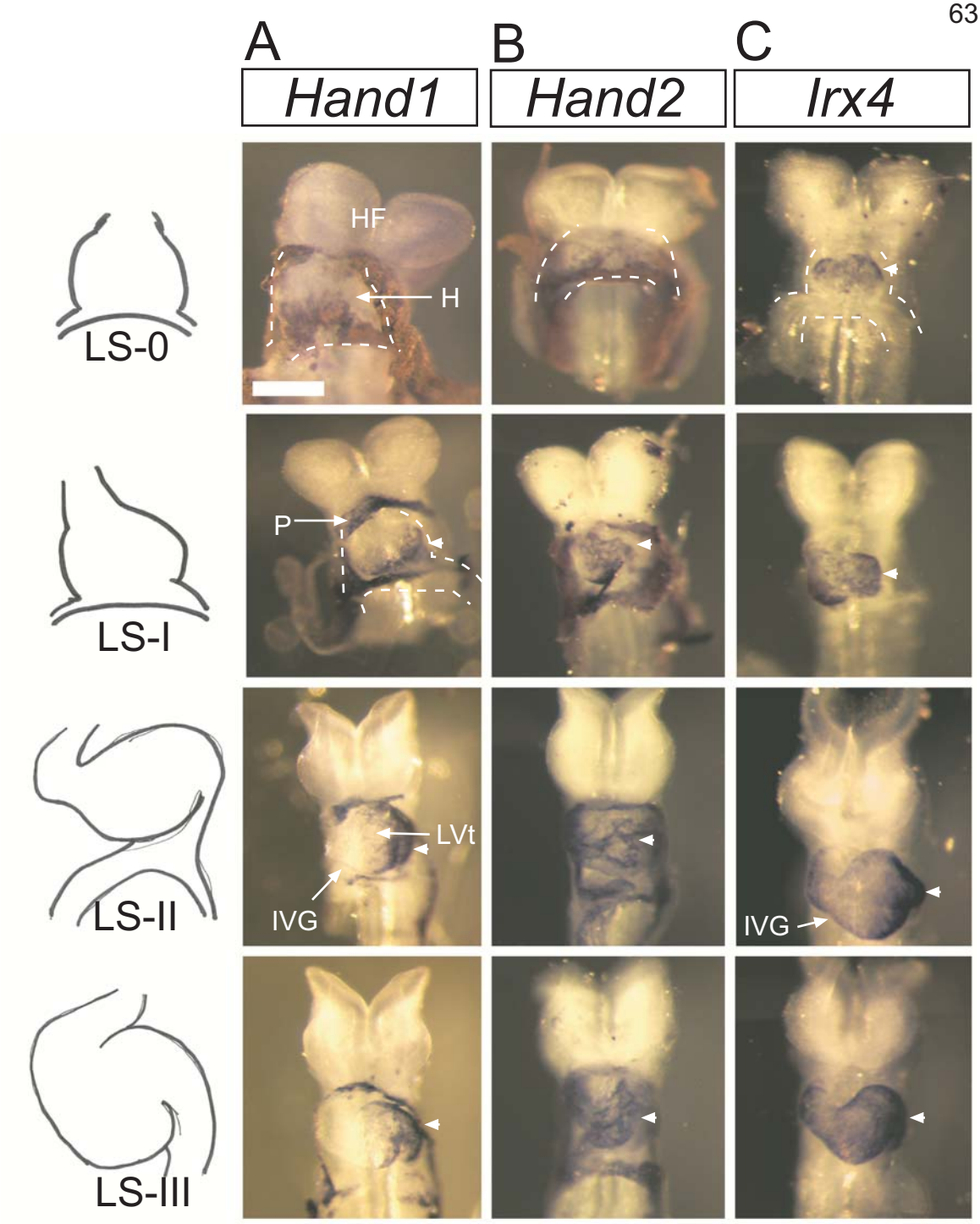


Figure 3.3. Comparison of *Hand1, Hand2* and *Irx4* mRNA expression patterns as detected by *in situ* hybridization during different cardiac looping stages (LS), as defined in Figure 1.1 and described by Biben and Harvey (1997). (E8.0-8.5). A schematic of heart development is provided along the left hand side. (A) *Hand1* expression. (B) *Hand2* expression. (C) *Irx4* expression. Scale bar is 0.25 mm. Head folds (HF), heart (H), pericardium (P), interventricular groove (IVG). Arrowheads indicate cardiac expression patterns.

However, in my studies, *Hand2* mRNA was not detected in the myocardium before E9.5. After E9.5, *Hand2* mRNA is detected in the myocardium of the right ventricle and outflow tract (data not shown). In my study, *Hand2* mRNA was detected by *in situ* hybridization, whereas the McFadden et al (2000) study was identifying modular enhancers for the *Hand2* gene, using a putative enhancer driving expression of *lacZ* in transgenic mice. This is an artificial system, and expression may be ectopic, or it may be more sensitive system, allowing detection of lower mRNA expression levels. As *Hand1* is expressed in the myocardium in the developing heart, it is unlikely that *Hand2* directly regulates *Hand1* expression.

3.2.3 **Irx4** *Cardiac Expression*

Irx4 is expressed in the myocardial layer of the developing heart (Bao et al., 1999). In the linear heart tube, *Irx4* mRNA is detected in the middle region of the heart tube (Fig 3.3). This domain extends anterior to the expression domain of *Hand1*, but may overlap with that of *Hand1*. This region expands as heart development proceeds, encompassing the left and right ventricles, the interventricular groove (Fig 3.3B), and extending into the atrioventricular canal (data not shown, and Bao et al., 1999). The extension of *Irx4* mRNA into the atrioventricular canal in the looped heart is similar to that of *Hand1*. *Irx4* expression fully encompasses the *Hand1* left ventricular expression domain, ending at a similar position at the venous pole of the heart, and extending beyond *Hand1* expression towards the arterial pole.

3.2.4 **Tbx2** *Cardiac Expression*

Expression of *Tbx2* mRNA, which encodes a transcriptional repressor involved in cardiac patterning, was not detectable by whole mount *in situ* hybridization before

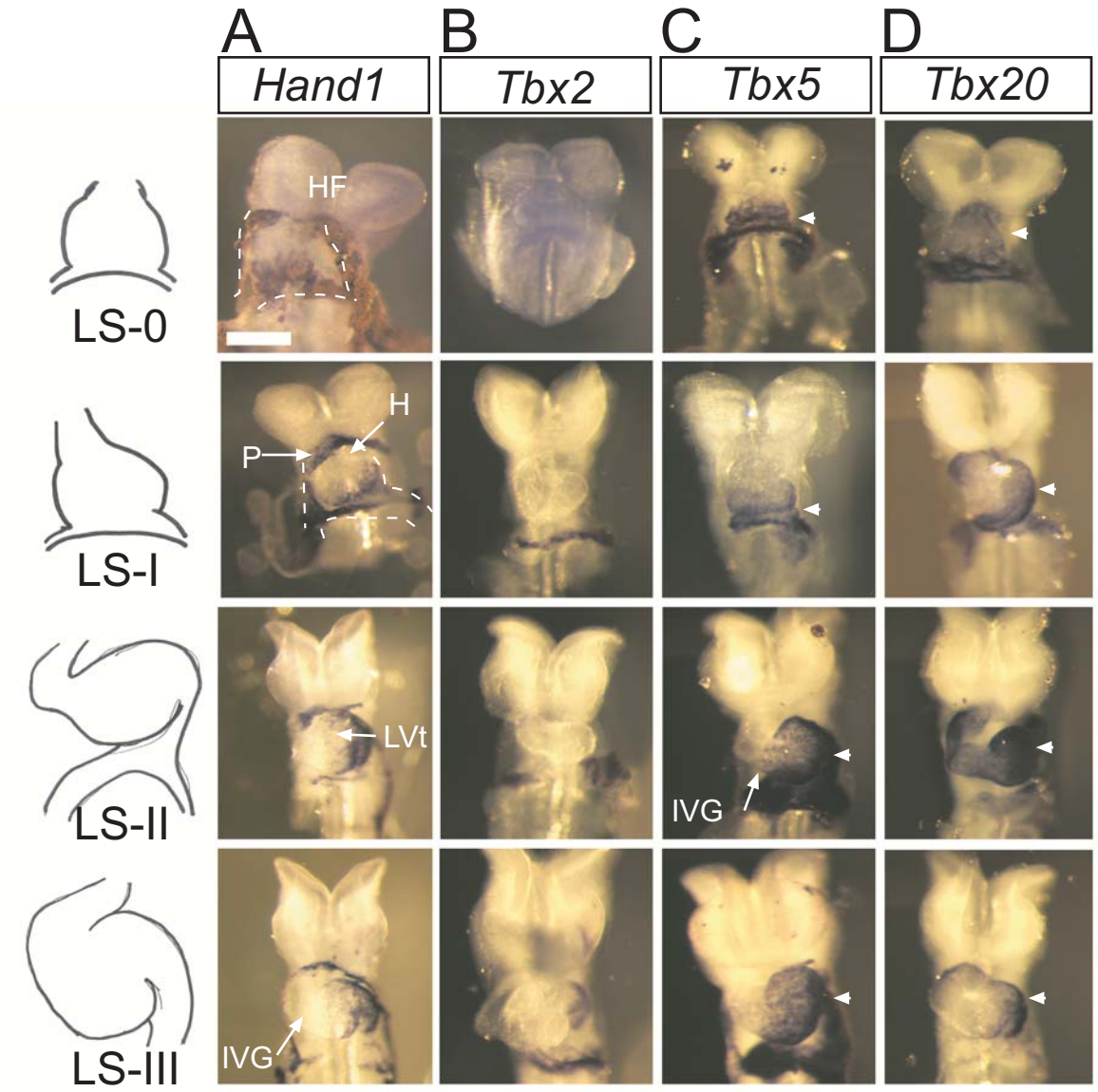
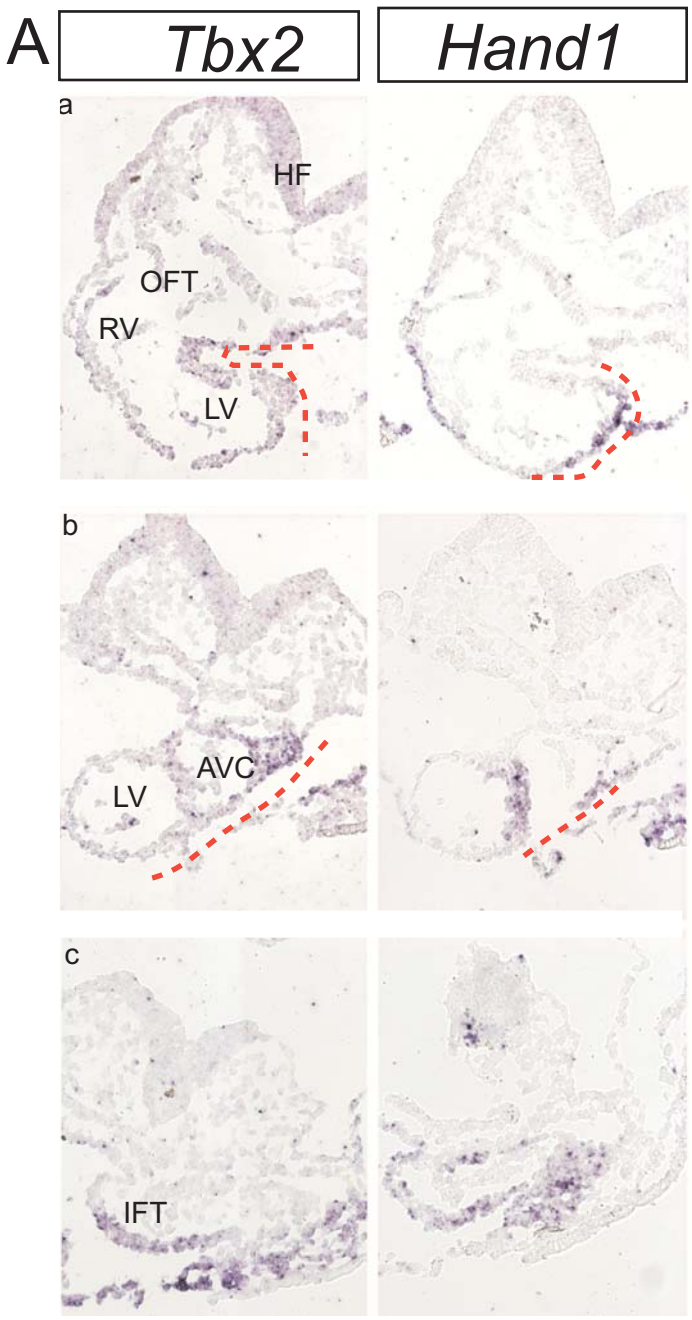


Figure 3.4. Comparison of *Hand1, Tbx2, Tbx5* and *Tbx20* mRNA expression patterns as detected by *in situ* hybridization during cardiac looping (E8.0-8.5). A schematic of heart development is provided along the left side. (a) *Hand1* mRNA expression. (b) *Tbx2* mRNA expression. (c) *Tbx5* mRNA expression. (d) *Tbx20* mRNA expression. Scale bar is 0.25 mm. Headfolds (HF), heart (H), pericardium (P), left ventricle (LVt), interventricular groove (IVG). Arrowheads mark cardiac mRNA expression domains.



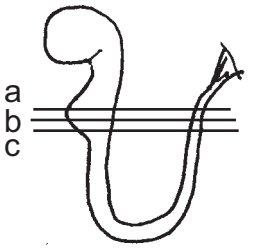
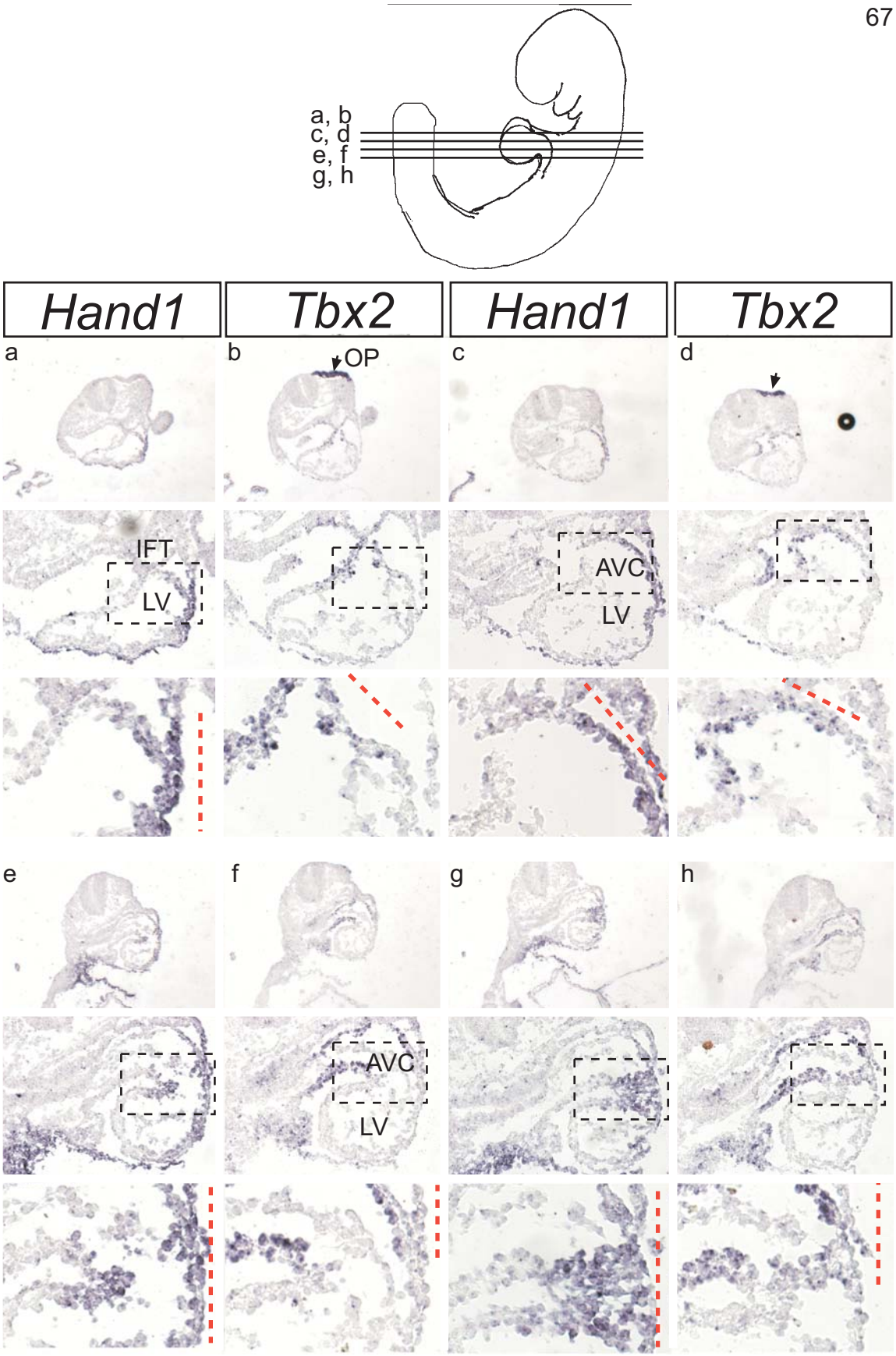


Figure 3.5. *Hand1* and *Tbx2* mRNA expression in the looping stage and looped heart, as detected with *in situ* hybridization on transverse cryosections. Adjacent transverse sections were probed for *Hand1* and *Tbx2* mRNA. The extent of mRNA expression is indicated by dotted lines. (A) E8.25 hearts, looping stage. *Hand1* and *Tbx2* overlap in the inflow region, 10x magnification. (B) E8.5 hearts, looped. Sections are shown at low (4x) and high (10x) magnification, and the region where *Hand1* and *Tbx2* expression intersect in the atrioventricular canal and left ventricle is expanded, as marked by the boxes. Arrowheads mark detection of *Tbx2* mRNA in the otic pits. LV, primitive left ventricle; RV, primitive right ventricle; IFT, inflow tract; AVC, atrioventricular canal; OFT, outflow tract; HF, head folds; OP, otic pit.

B


a, b
c, d
e, f
g, h
Hand1
Tbx2
Hand1
Tbx2
a
b
OP
c
d
IFT
LV
AVC
LV
e
f
g
h
AVC
LV

looping stage 3 (LS-III) (Fig 3.4b). *Tbx2* has been detected by others prior to this stage (Christoffels et al., 2004; Harrelson et al., 2004), so I utilized *in situ* hybridization on tissue sections, which is more sensitive than whole mount *in situ* hybridization, to compare *Hand1* and *Tbx2* expression. Adjacent transverse sections of embryos with looping stage and looped hearts were probed for *Hand1* and *Tbx2* expression. The extent of *Tbx2* and *Hand1* mRNa expression is marked on the sections, for comparison. In looping hearts (Fig 3.5 A), *Tbx2* mRNA is detected in the inflow region, but not the future ventricle. *Hand1* and *Tbx2* mRNA expression overlap in the inflow region, but not in the future ventricular region. In looped hearts (Fig 3.5B), *Tbx2* mRNA is detected in the inflow region, the atrioventricular canal, but does not extend into the future ventricle. *Tbx2* mRNA is also detected in the otic pits (arrowheads in Fig 3.5B b, d), confirming that signal is specific to *Tbx2*. *Hand1* mRNA is detected in the atrioventricular canal and the left ventricle, but does not extend into the right half of the unseptated ventricle, which will form the right ventricle. The *Hand1* and *Tbx2* mRNA expression domains appeared to overlap by several cells in the atrioventricular canal. Notably, *Hand1* mRNA is detected in the inner curvature of the atrioventricular canal and left ventricle, where it has not been previously documented, and this domain is reciprocal to the detected *Tbx2* domain (Fig 3.5). Therefore, *Hand1* and *Tbx2* overlap to varying degrees in different regions of the heart.

3.2.5 Tbx5 *Cardiac Expression*

Tbx5 mRNA expression is detected in the posterior portion of the linear heart tube and in the sinus venosus (Fig 3.4c). During looping, this expression pattern is maintained, with *Tbx5* mRNA detected in the inflow region, the atria, the atrioventricular

canal and the left ventricle. Expression does not extend to the future right ventricle. The *Tbx5* mRNA expression domain fully encompasses the *Hand1* expression domain, and has similar limits at the transition to the right ventricle.

3.2.6 Tbx20 *Cardiac Expression*

Tbx20 mRNA expression is detected throughout the heart, from the sinus venosus to the outflow tract at all stages examined (Fig 3.4d). Strongest expression is observed at the anterior and posterior ends of the tube, as observed in the LS-I and LS-III embryos. The *Tbx20* mRNA expression domain fully encompasses the *Hand1* expression domain, but does not share any common borders.

3.3 Effect of Removing the *Hand1* intron on Embryonic Survival and *Hand1* Expression

Previous results within the Cross lab suggested that the *Hand1* intron may act as a regulatory element, and may act specifically on cardiac *Hand1* expression. To determine if the *Hand1* intron is necessary for or influences *Hand1* transcription, gene targeting was used to create an allele lacking the intron ($Hand1^{\Delta I}$) in ES cells (Fig 2.1). The targeting vector contained a puromycin-resistance gene selection cassette, flanked by loxP sites. Chimeras were made with the $Hand1^{+/\Delta I}$ ES cells, and they were bred to 129Sv/J female mice for germline transmission of the $Hand1^{\Delta I}$ allele. These mice were then bred to *Mox2*-Cre mice, for removal of the floxed puromycin-resistance cassette. Following removal of the puromyocin-resistance cassette, the mice were backcrossed onto the 129SvJ background for colony expansion and also outcrossed onto the outbred CD1 strain background for initial analysis because of their larger litter sizes. My analysis was carried out on the outbred CD1 background.

3.3.1 Lethality Associated with the **Hand1$^{\Delta I}$** *Allele*

To determine if the *Hand1ΔI* allele is lethal, we bred *Hand1$^{\Delta I/+}$* mice to *Hand1$^{+/-}$* mice, to generate +/+, +/-, +/ΔI and ΔI /- embryos. At E8.5 and E10.5, all genotypes are recovered at approximately the expected ratios. At weaning, three weeks after birth, there are fewer *Hand1$^{\Delta I/-}$* pups than expected (Table 3.1). Additionally, three *Hand1$^{\Delta I/-}$* pups died shortly after birth and one *Hand1$^{\Delta I/-}$* animal died between birth and weaning. Chi-square analysis confirms that the genotype ratios are significantly different than expected at weaning ($P<0.05$), but not at E8.5 or E10.5.

Embryos were examined for morphological defects. At E8.5, *Hand1$^{-/-}$* embryos have arrested heart looping and extensive yolk sac hemorrhaging, and are dying (Riley et al., 1998). By contrast, ΔI /- embryos, appeared to be normal. At E10.5, ΔI /- implantations and embryos ranged from normal appearance and size, to slightly smaller with slight aberrations in cardiac morphology (Fig 3.6). Of 11 ΔI /- embryos, 3 appeared slightly abnormal. Abnormal hearts contained two ventricles, the interventricular groove, and an outflow tract that appeared largely normal. In comparison to +/+ (Fig 3.6 a), the atrioventricular canal in two ΔI /- embryos appears to be less constricted (Fig 3.6 b, d). In one ΔI /- embryo, the inflow and outflow poles of the heart were separated, and the heart appeared small for the embryo (Fig 3.6 c). With the exception of this embryo (Fig 3.6 c'), the ΔI /- hearts appear normal from the right side.

Table 3.1. Genotyping results of *Hand1*$^{\Delta I/+}$ x *Hand1*$^{+/-}$ crosses.

	Litters	Total	+/+	+/-	'+/ΔI	ΔI/-	Resorption	X^2	Significant (p<0.05)
Expected			25%	25%	25%	25%			
E8.5	6	74	28.4%	17.6%	31.1%	20.3%	2.7%	3.778	No
E10.5	6	80	21.3%	32.5%	17.5%	26.3%	2.5%	4.145	No
Weaning	10	103	20.4%	31.1%	36.9%	11.7%		15	Yes
Dead pups		4				100.0%			

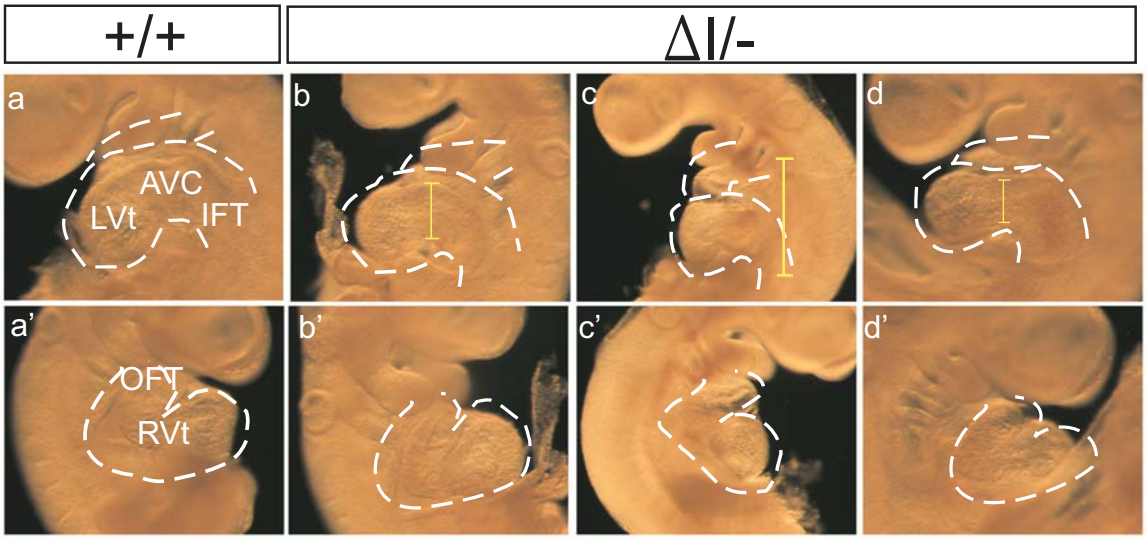


Figure 3.6. Abnormalities in E10.5 ΔI/- hearts. Embryos are shown from the left and right sides. Note restriction of atrioventricular canal in (a), decreased restriction in b. Note distance between inflow and outflow tracts in (c, c'), denoted with yellow bar, compared to (a). Note ventrally extended and poorly restricted atrioventricular canal in (d), denoted with yellow bar. Inflow tract (IFT), atrioventricular canal (AVC), left ventricle (LVt), right ventricle (RVt), outflow tract (OFT).

3.3.2 Hand1 Expression in Hand1$^{\Delta I /-}$ Embryos and Implantation Sites at E8.5 and E10.5.

To determine if the *Hand1*$^{\Delta I}$ allele affected *Hand1* mRNA expression level at the level of the whole embryo and implantation site, timed matings between *Hand1*$^{+/-}$ and *Hand1*$^{+/\Delta I}$ mice, and embryos and implantation sites were collected at E10.5. RNA was collected from the placenta/decidua, and the embryos and used to prepare Northern blots (Fig 3.7). On the placenta blot (Fig 3.7B), a band was present at approximately 2000 bp, where *Hand1* mRNA is expected. Compared to +/+, expression was lower in +/- embryos, and yet +/- embryos develop normally. Expression in ΔI/- embryos was variable with some samples showing expression similar to +/-, and some showing lower expression compared to +/-. We were unable to detect a *Hand1* mRNA band on the embryonic blot (Fig 3.7A).

To determine if the *Hand1* spatial pattern of expression was disrupted, embryos were collected at E8.5 and E10.5 from *Hand1*$^{\Delta I /+}$ x *Hand1*$^{+/-}$ crosses, and *Hand1* mRNA expression was detected by using *in situ* hybridization. Expression was compared to that of +/+ and +/- embryos, as +/- embryos develop normally (Riley et al., 1998). Two examples are shown of ΔI/- embryos to demonstrate the range of expression observed.

In +/+ cardiac crescent stage embryos, *Hand1* mRNA was detected in the cardiac crescent, extraembryonic membranes, and the allantois (Fig 3.8). This pattern of expression was observed in +/ΔI and ΔI/- embryos, with no sites of ectopic expression (Fig 3.8). In +/+ linear heart tube stage embryos, *Hand1* mRNA was detected in the linear heart tube, in a small anterior-posterior restricted domain, in the pericardium, which was removed for photography, in the extraembryonic membranes, and in the

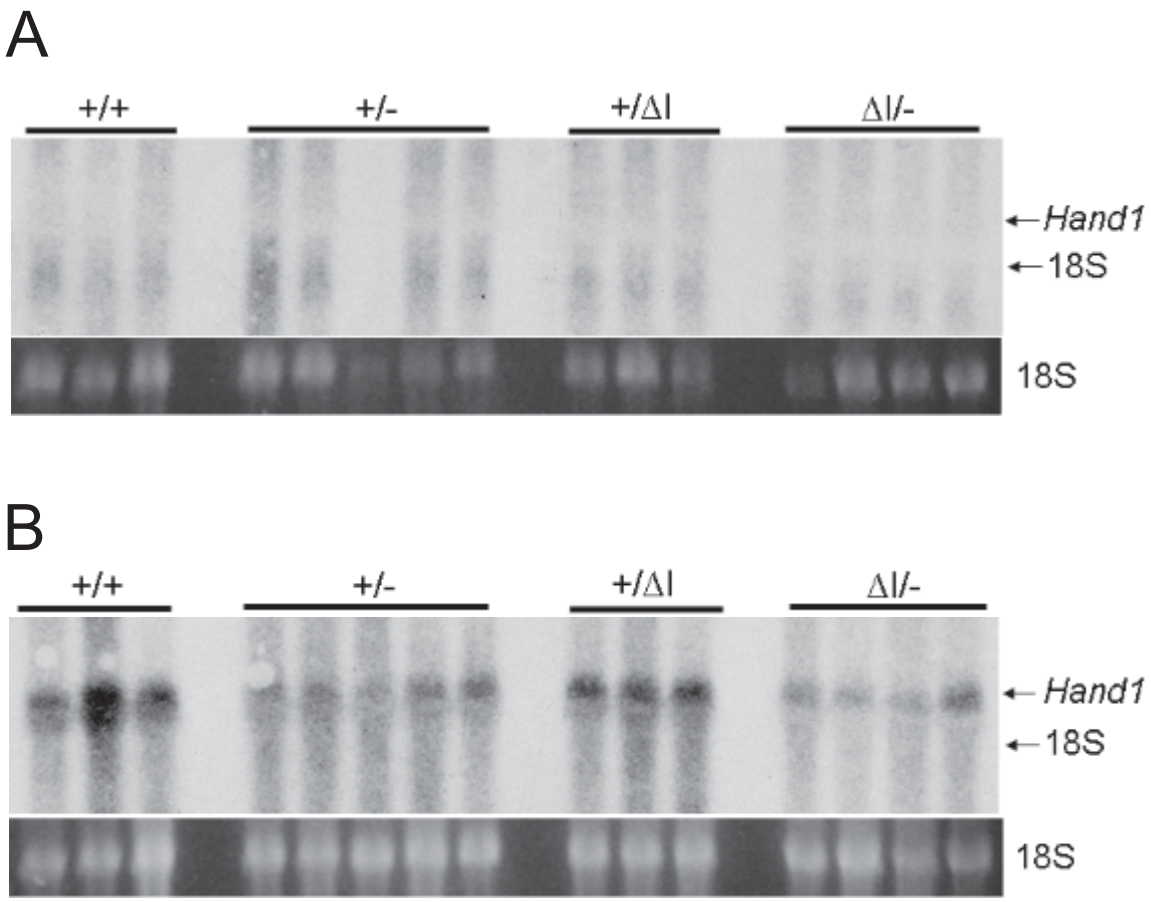


Figure 3.7. Northern blot analysis of *Hand1* mRNA expression in E10.5 embryos and implantation sites from *Hand1+/- x Hand+/*ΔI crosses. Each lane represents an Individual embryo (A) or implantation site (B) (decidua, placenta and parietal trophoblast giant cells). Blots were probed with a *Hand1* probe. 18S RNA is shown below the Northern blot for normalization.

allantois (Fig 3.8). This pattern of expression is unchanged in +/ΔI and ΔI/- embryos, with no sites of ectopic expression (Fig 3.8).

As the *Hand1* intron was predicted to contain a cardiac-specific enhancer, *Hand1* cardiac expression was closely examined at E8.5 (Fig 3.9). In +/+ and ΔI/- cardiac crescent stage embryos, *Hand1* mRNA was detected in the cardiac crescent, although it was barely detectable in the ΔI/- embryo (Fig 3.9b). In the linear heart tube, expression in the ΔI/- embryo (Fig 3.9d) appeared to occupy a similar domain to that in the +/+ embryo (Fig 3.9c). In ΔI/- looping stage embryos (Fig 3.9f, h), *Hand1* mRNA expression was present on the ventral surface, although it was barely detectable. As the expression pattern in the ΔI/- looped heart (Fig 3.9j) was unchanged from +/+ (Fig 3.9i), encompassing the inflow tract, the atrioventricular canal, and the left ventricle, it is unlikely that the expression pattern in looping hearts is altered from +/+ (Fig 3.11e, g).

In E10.5 +/+ embryos, *Hand1* mRNA was detected in the outer curvature of the left ventricle (Fig 3.8). *Hand1* mRNA was difficult to detect in some ΔI/- embryos at E10.5, but in embryos where *Hand1* mRNA was detected, the expression pattern was unchanged, and no ectopic expression was observed. In addition to expression in the heart and implantation site, *Hand1* is expressed in E10.5 embryos in the optic and otic

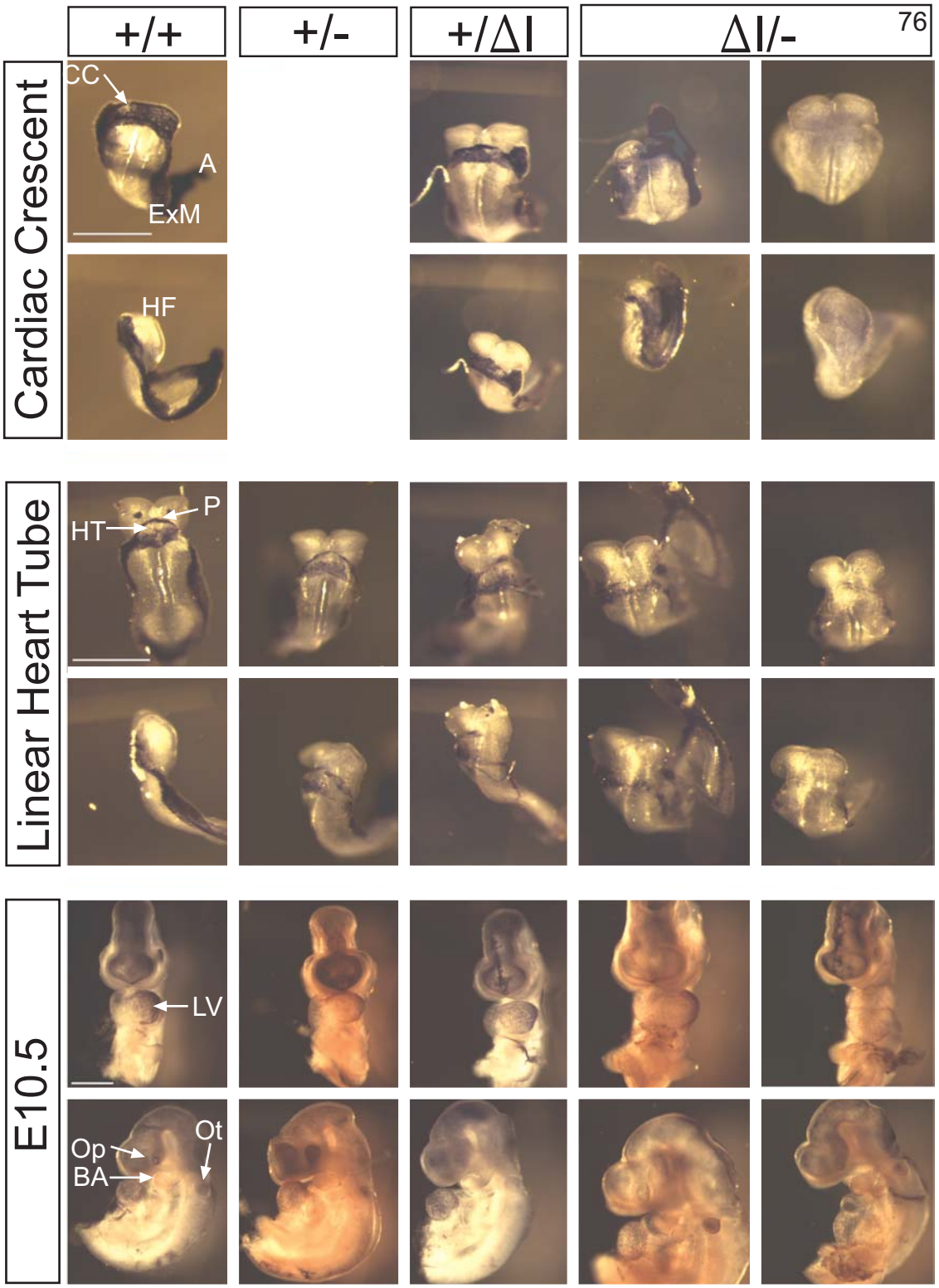


Figure 3.8. *Hand1* expression in ΔI/- embryos and littermates at E8.5 and E10.5. Embryos are shown in ventral and left side views. Two examples of *Hand1* expression in ΔI/- embryos are provided at each stage, to demonstrate the observed variability. Scale bars are 1 mm. CC, cardiac crescent; HT, heart tube; LV, left ventricle; P, pericardium; HF, head folds; A, allantois; ExM, extraembryonic membranes; BA, branchial arches; Op, optic pit; Ot, otic pit.

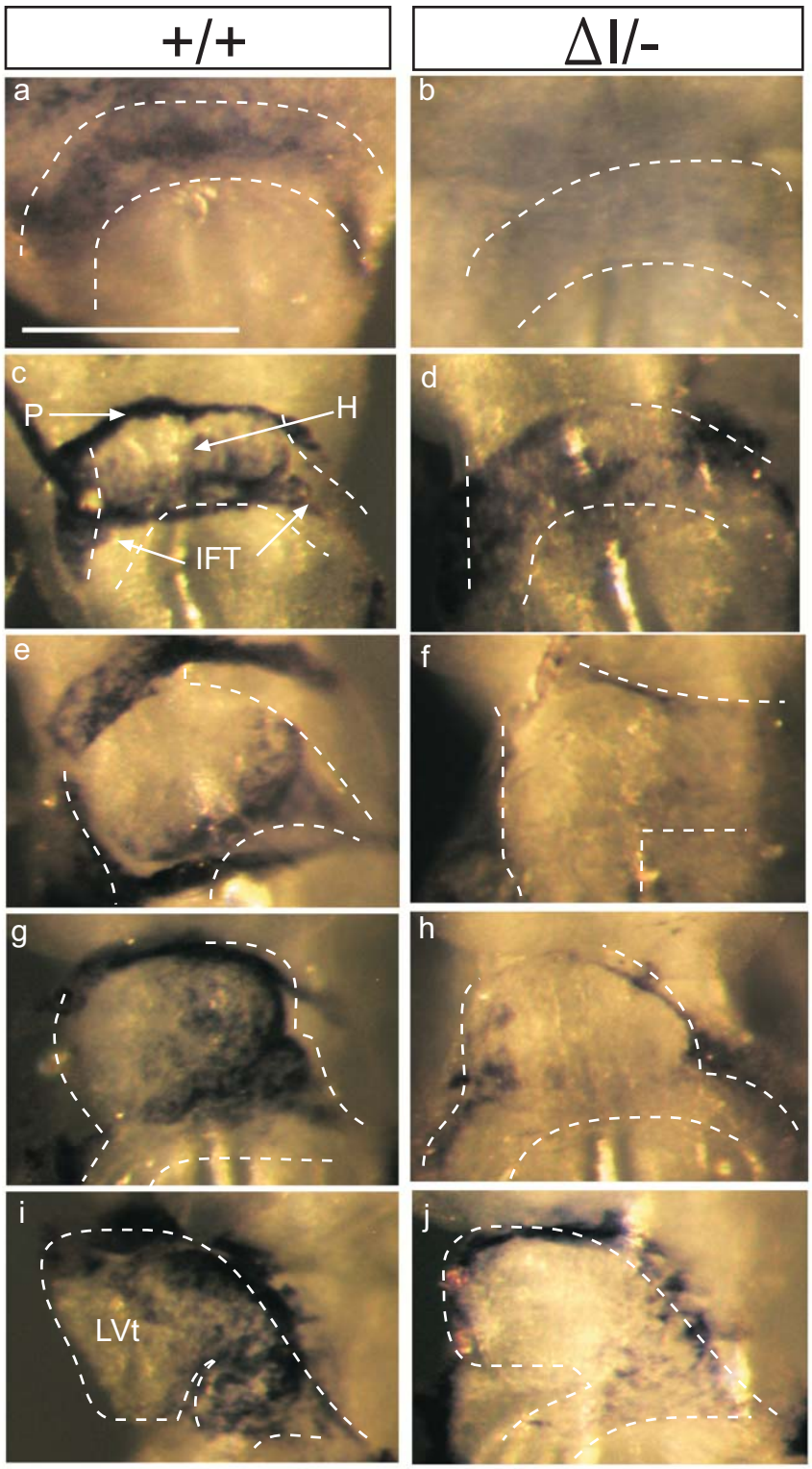


Figure 3.9. *Hand1* expression in +/+ and ΔI/- embryos during cardiac looping. *Hand1* mRNA detected by *in situ* hybridization, ventral views of stage matched embryos shown. Headfold is at the top of the image. *Hand1* expression in ΔI/- is variably downregulated: b, f, h and j exhibit less staining than +/+ controls, while d exhibits staining similar to c. Scale bar is 0.5 mm. Pericardium (P), heart (H), inflow tract (IFT), left ventricle (LVt).

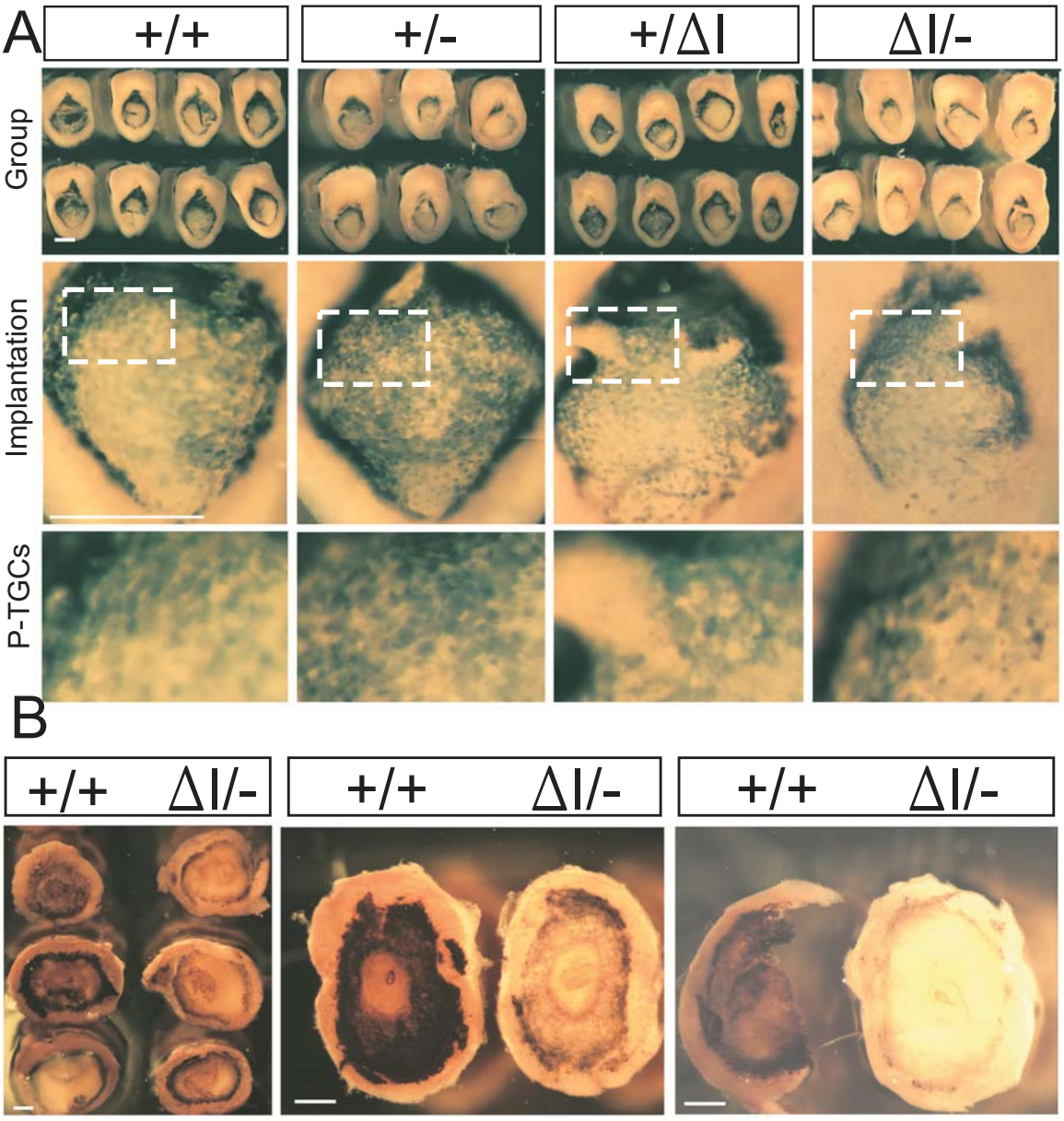


Figure 3.10. *Hand1* expression in the implantation site. (A) E8.5 implantation sites, bisected sagitally and processed by *in situ* hybridization for *Hand1* mRNA. In groups for an overall impression, single implantation sites and parietal trophoblast giant cells (P-TGCs) for detail. Mesometrial pole is at the top of each image, anti-mesometrial pole is at the bottom of each image. (B) E10.5 implantation sites, bisected transversely to show the basal surface of the placenta with a ring of P-TGCs around the perimeter, processed by *in situ* hybridization for *Hand1* mRNA. Groups of implantation halves are shown at low magnification, and two pairs of +/+ and ΔI/- halves at higher magnification. Scale bars are 1 mm.

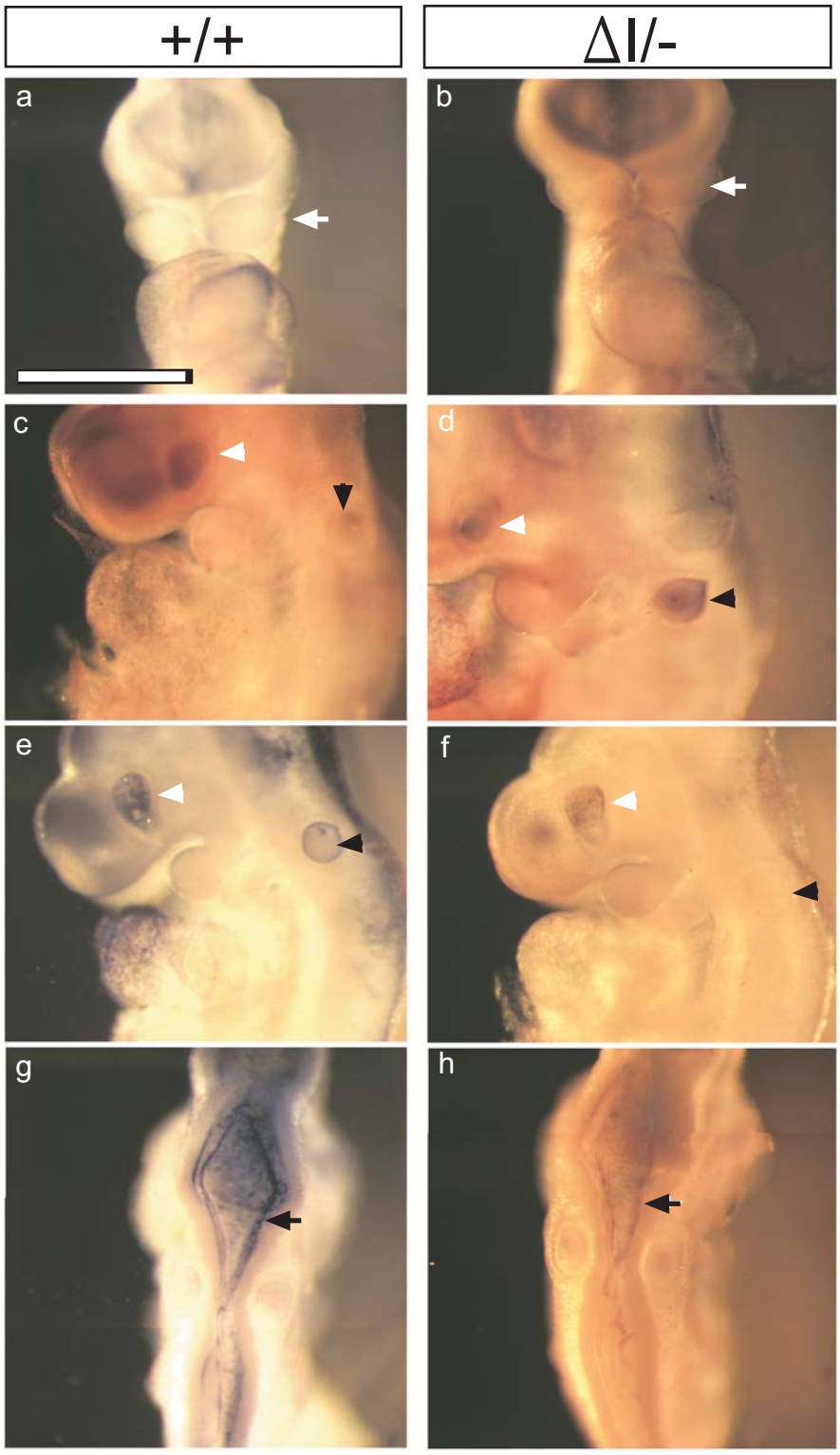


Figure 3.11. Extracardiac *Hand1* mRNA expression at E10.5 in +/+ and ΔI/- embryos. Expression in branchial arches (a, b), otic and optic pits (c-f), and dorsal neural tube (g, h). Branchial arches (white arrow), optic pit (white arrowhead), otic pit (black arrowhead), dorsal neural tube (black arrow). Scale bar is 1 mm.

pits, branchial arches, and the dorsal neural tube (Cserjesi et al., 1995; Riley et al., 2000). In the current studies, *Hand1*mRNA was not detectable in the branchial arches of either +/+ or -/ΔI embryos (Fig 3.11a, b). *Hand1* mRNA was detected in the optic and otic pits of ΔI/- embryos (Fig 3.11d, f), and in the dorsal neural tube (Fig 3.11g, h).

Hand1 mRNA was expressed in parietal trophoblast giant cells (parietal-TGCs) lining the E8.5 and E10.5 implantation sites, and on the basal surface of the E10.5 placenta (Fig 3.11). *Hand1* mRNA was detected in parietal-TGCs of +/ΔI and ΔI/- implantation sites at E8.5 and E10.5.

3.3.3 Intensity of Hand1 *mRNA signal in ΔI/- Embryos and Implantation Sites.*

In situ hybridization is not a quantitative analysis of mRNA expression level. However, if decreased intensity is observed, it could be due to fewer cells expressing the mRNA, or less mRNA expressed by each cell. In ΔI/- embryos, I observed variably decreased staining intensity in all tissues. Some embryos exhibited staining intensity similar to +/+, while staining in others was barely detectable. For example, in the heart at E8.5 (Fig 3.9), the signal intensity of one embryo (d) was similar to +/+ (c), two were decreased from +/+ (b, j compare to a, i), and two ΔI/- embryos had almost undetectable signal in the heart (f, h). In Figure 3.9j, signal appeared to occupy a similar region of the heart as in +/+ (i), and in Figure 3.9b, signal appeared to encompass a similarly sized crescent compared to +/+ (a). In E8.5 implantation sites, when a high magnification of parietal-TGCs is compared (Fig 3.10A, lower images), signal appeared to be less intense in each cell. Therefore, it is possible that removing the *Hand1*intron has decreased *Hand1* expression in all *Hand1* expressing cells, and it is not a cardiac-specific enhancer. Northern blotting studies detected variably decreased *Hand1* mRNA levels in ΔI/-

embryos compared to +/-, supporting the *in situ* hybridization observation of decreased signal intensity (Fig 3.7B).

Chapter Four: Discussion and Future Directions

The overall hypothesis of this project was that the intron of the *Hand1* gene contains a cardiac enhancer element. The specific aims were to identify putative transcriptional regulators of the *Hand1* gene, and to test the effect of deleting the intron from the endogenous gene on gene expression. In this section, I will discuss the role of *Hand1* in heart development, propose a model for patterning the cardiac *Hand1* expression domain, and discuss my findings in the $Hand1^{\Delta I}$ mouse line. Finally, I will propose future directions for the study of *Hand1* transcriptional regulation.

4.1 *Hand1* and the Developing Heart

The unique cardiac expression pattern and the striking phenotype of *Hand1* have suggested a key role for *Hand1* in regulating heart development. A primary role has been proposed for *Hand1* in patterning at the linear heart tube stage, which is largely supported by the arrest of heart development at the linear heart tube stage in $Hand1^{-/-}$ embryos (Firulli et al., 1998; Riley et al., 1998), and observation of defects in cardiomyocyte differentiation *in vitro* from $Hand1^{-/-}$ embryonic stem cells (X. Zhao, G. Teng, and J.C. Cross unpublished data). A second role has been proposed in chamber ballooning and morphogenesis later in heart development, based on the effects of misexpression of *Hand1* in the heart, preventing formation of the interventricular septum and driving cell proliferation (McFadden et al., 2005; Risebro et al., 2006; Togi et al., 2004) as well as chimera experiments indicating a specific cell autonomous function for Hand1 in the outer curvature of the left ventricle (Riley et al., 2000). Although it has been suggested that *Hand1* and *Hand2* are partially redundant based on analysis of compound mutant embryos with cardiac-specific deletion of *Hand1* (McFadden et al., 2005), the cardiac

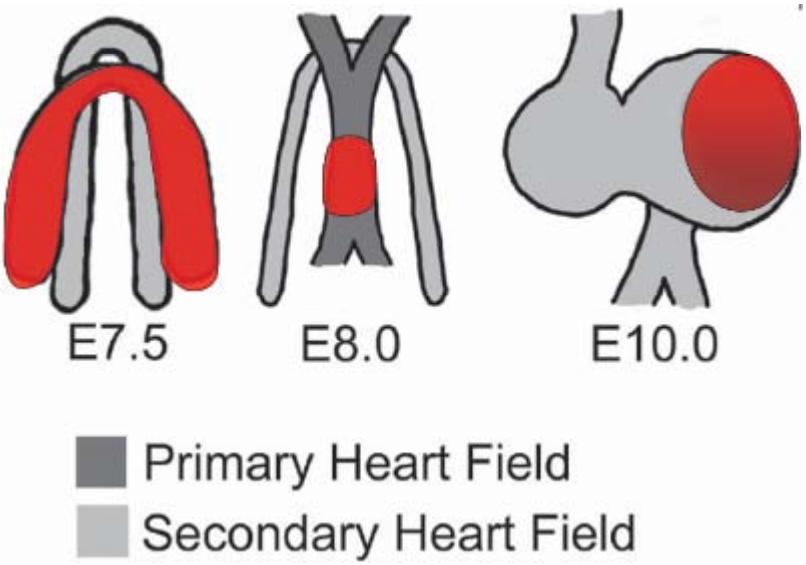
arrest in *Hand1*$^{-/-}$ embryos suggests that either *Hand1* has a distinct role at the linear heart tube stage, or *Hand2* is not expressed in the correct domain to compensate for the loss of *Hand1*.

With the *Hand1*$^{\Delta I}$ mouse model, *Hand1* expression appeared to be variably downregulated below levels observed in *Hand1*$^{+/-}$ embryos at E8.5 in embryos and extraembryonic tissues, with no observed effect on early cardiac development. This suggests that low levels of *Hand1* are sufficient for early cardiac development. At E10.5, *Hand1* mRNA expression in *Hand1*$^{\Delta I/-}$ embryos also appeared variably downregulated. Subtle abnormalities were observed in some E10.5 *Hand1*$^{\Delta I/-}$ hearts, but cardiac morphogenesis appeared grossly normal in most *Hand1*$^{\Delta I/-}$ hearts at this stage. Downregulation could be directly due to the loss of the intron, or epigenetic changes on the Es cell-derived chromosome, which carries the *Hand1*$^{\Delta I}$ allele. Phenotypic and gene expression variability may be due to expression of genetic background modifiers, or variable expression of compensating factors. A study by McFadden et al (2005) suggests that compensatory activity may exist between *Hand1* and *Hand2*. Variability in *Hand2* expression could lead to variable levels of rescue or compensation. However, it is unlikely that *Hand2* compensates for the low expression level at E8.5, as it is expressed in the endocardium, not the myocardium, at that stage.

4.2 Transcriptional Regulation of the *Hand1* Gene in the Early Mouse Heart

The *Hand1* gene exhibits a novel cardiac expression pattern, with restriction in all three body axes at a time when most other cardiac transcription factors are expressed more broadly. The *Hand1* mRNA expression domain does not follow known boundaries in the heart, as illustrated in Figure 4.1. In the left ventricle, *Hand1* mRNA is expressed

A



B

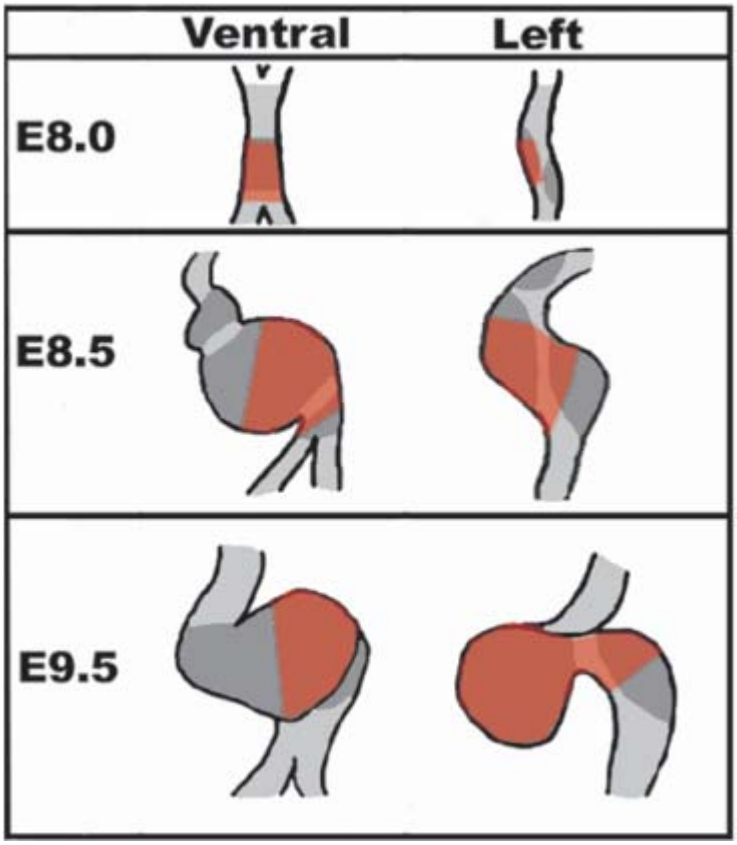


Figure 4.1. Summary of *Hand1*mRNA expression with reference to concepts of cardiac development. A. *Hand1* mRNA is detected in the primaruy cardiac crescent, a subpopulation of the primary heart tube, and the primary heart field-derived outer curvature of the left ventricle, where secondary heart field derivatives are not found. B. *Hand1* mRNA does not follow chamber myocardium boundaries. *Hand1* mRNa extends beyond the ventricular zone into the atrioventricular canal, which is a region of retained primitive myocardium. Therefore, the concepts of heart fields, and chamber myocardium are not sufficient to explain the *Hand1* mRNa expression domain.

only in the region which is derived from the primary heart field. The *Hand1* mRNA expression domain does not follow chamber myocardium boundaries, as it extends from the left ventricle into the atrioventricular canal, suggesting that it is not simply a chamber myocardium gene expressed in the left ventricle. Therefore, regulation of the *Hand1* mRNA expression domain must require additional patterning information beyond chamber myocardium patterning.

As the *Hand1* expression pattern is unique among known cardiac transcription factors, and does not obey the boundaries of chamber myocardium, it is likely regulated combinatorially, with several factors defining the expression borders. A summary of the cardiac transcription factor mRNA expression patterns is presented in Figure 4.2. The atrial and ventricular transcriptional activator *Tbx5,* the ventricular transcriptional activator *Irx4,* and the transcriptional regulator *Tbx20* likely contribute to regulation of the *Hand1* expression domain. Expression of *Tbx5* and *Irx4* partially overlap in the linear heart tube, although *Hand1* expression is not restricted to this region of overlap. The anterior boundary of *Tbx5* mRNA expression in the linear heart tube is similar to that of *Hand1*, but *Irx4* and *Hand1* do not share a common border. Additionally, misexpression of *Tbx5* in the right ventricle of the heart causes ectopic expression of *Hand1* (Takeuchi et al., 2003). Expression of *Tbx20* is broad, and is key in restricting *Tbx2* expression to retained primitive myocardium. Given the demonstrated role of *Tbx20* in patterning the heart tube and its expression overlap with *Hand1*, *Tbx20* could also provide patterning information to *Hand1*. Therefore, the transcriptional activators *Tbx5, Tbx20* and *Irx4* likely promote *Hand1* expression, but activation by these factors is not sufficient to explain the restricted expression pattern of *Hand1*, and other factors must be considered.

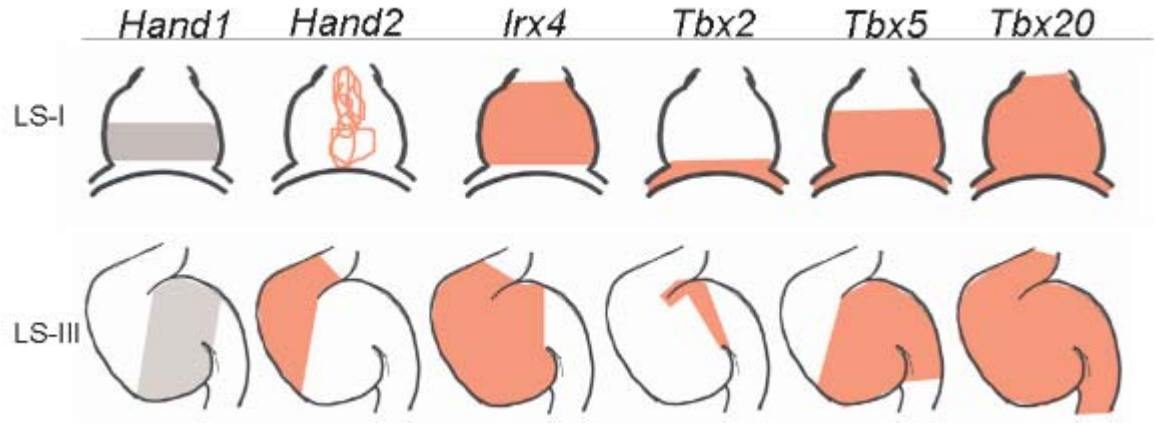


Figure 4.2. Summary of cardiac transcription factor mRNA expression patterns in the mouse E8.0-9.5 looping heart tube. *Hand1* mRNa expression is indicated in grey, others are indicated in red.

The transcriptional repressor *Tbx2* is known to repress expression of chamber myocardium genes, including *ANF* and *Cx43*, with a strictly complementary expression pattern between *Tbx2* and its targets. Using section *in situ* hybridization on serial histological sections, it was determined that the mRNA expression patterns of *Hand1* and *Tbx2* overlap in the atrioventricular canal. Tbx2 is a transcriptional repressor. Therefore, if *Hand1* is a target of *Tbx2*, the T-box binding sites in *Hand1* regulatory regions may have higher affinity for *Tbx5* or *Tbx20*, allowing activation in the presence of *Tbx2*. Alternatively, other transcription factors or co-regulators may be involved that are not yet identified, and their expression pattern and transcriptional regulatory activity would explain the expression overlap that is unusual for a putative *Tbx2* target gene.

In addition to the above transcription factors, regulation of *Hand1* expression may involve intercellular signalling. Anterior-posterior patterning information is provided by retinoic acid signalling. Retinoic acid from the posterior mesoderm participates in cardiac anterior-posterior specification, and anterior-posterior determination involves retinoic acid from the anterior lateral mesoderm (Hochgreb et al., 2003). Retinoic acid signalling is known to regulate *Hand1* expression in chick limb buds (Fernandez-Teran et al., 2003), *Hand2* expression in rat embryonic cardiomyocytes (Li and Li, 2006), and cooperates with Hedgehog signalling to pattern the anterior-posterior axis of the *Drosophila* dorsal vessel (Liu et al., 2006). Therefore, retinoic acid signalling may directly regulate the anterior-posterior boundaries of *Hand1* expression, or it may act indirectly through *Tbx5* or other factors.

Hand1 expression is upregulated on the left side of the linear heart tube as looping is initiated. This could be due to the leftward rotation of the heart tube as looping is

initiated (Voronov et al., 2004), causing the expression domain to shift with the heart tube, or it could actually involve upregulation in response to left-right patterning. The pre-cardiac mesoderm receives left-right patterning information while it is in the anterior lateral plate mesoderm, which receives left-right signal information from the embryonic node. The asymmetrically expressed transcription factor *Pitx2* is expressed in mesoderm on the left side of the embryo, where it determines the left side identity through currently unidentified targets (Ramsdell, 2005; Shiratori and Hamada, 2006). *Hand1* could be regulated by Pitx2 or other left-right laterality signals, if its expression is truly left-right asymmetrical.

Hand1 mRNA is expressed on the ventral side of the heart tube, but not the dorsal side. Little is known about dorsal-ventral patterning in the heart. Theoretically though, dorsal ventral patterning could be accomplished by defining dorsal-ventral in the heart tube, or by defining medial-lateral in the lateral plate mesoderm prior to heart tube formation, where the medial tissue forms the dorsal region of the heart tube and the lateral tissue forms the ventral region. An inhibitory signal from a dorsal tissue, possibly the dorsal mesocardium, the neural tube or the notochord, could inhibit *Hand1* expression in the dorsal portion of the heart tube. Alternatively, a medially originating signal could inhibit expression of *Hand1*in medial cells or a laterally-originating signal could promote *Hand1* expression in lateral cells prior to heart tube formation.

4.3 A Transcriptional Regulatory Element in the *Hand1* Intron

Based on previous results within the lab suggesting that the intron acts as a tissue-specific regulatory element (P. Riley, I. Scott and X. Zhao, unpublished results), and the discovery of an enhancer element in intron 3 of the *Drosophila Hand* gene, we

hypothesized that removal of the intron would cause tissue-specific changes in *Hand1* expression. Using bioinformatics tools to study the *Hand1* intron, I observed that in the *Hand* family, large introns are associated with *Hand1* genes in species that have two *Hand* genes and the single *Hand* genes in those species in which it is more similar to *Hand1* genes than *Hand2*. This, in combination with the relatively large size of the intron in *Fugu*, suggests that the *Hand* intron may contain regulatory information. Additionally, a region in the 3' end of the intron is conserved between *Hand1* genes in mammals, and putative transcription factor binding sites are conserved within this region, suggesting that regulatory information may reside there.

There is a severe limitation to the use of bioinformatics analysis to identify putative regulatory elements in some tissues, especially the heart, as the analysis depends on experimental description of transcription factor binding sites to generate matrices. Although many transcription factor binding matrices have been generated, there are very few for known cardiac transcription factors, especially chamber-specific transcription factors like the T-box proteins. This complicates regulatory element prediction, as a strongly conserved site may appear to have very few, irrelevant, conserved binding sites. Additionally, not all conserved sites will have regulatory activity, while other regulatory elements will not be conserved. Therefore, while bioinformatics prediction can help narrow the search for a conserved regulatory element within a large genomic sequence, activity must be determined experimentally.

A standard approach for localizing and characterizing enhancer elements is to generate transgenic mice with reporter gene expression driven by putative enhancer elements and a minimal promoter. Using the transgenic approach, 6 kb of upstream

sequence and the *Hand1* intron were shown to be insufficient for reporter gene expression (P. Riley and J.C. Cross, unpublished data), and 20 kb of upstream sequence was also insufficient for reporter gene expression (A. Firulli, personal communication). As these regions were not sufficient to recapitulate the *Hand1* expression pattern, it was concluded that more genomic DNA was required. As a large region was already investigated, considerable effort would likely be required to identify the regions sufficient for *Hand1* expression. Therefore, the necessity of the *Hand1* intron for the *Hand1* expression pattern *in vivo* was investigated, with the $Hand1^{\Delta I}$ mouse line.

Another common approach for studying enhancer elements is to generate reporter constructs with the putative enhancer elements driving expression, and transfect these into cell culture models. Minimal regions sufficient for gene expression can be identified and mutated with relative ease to thoroughly study an enhancer element, and specific elements could then be removed from the endogenous gene for verification of regulatory activity. This approach can provide insight into on/off elements and expression level regulation, but as the cell culture system does not recapitulate embryonic or tissue patterning, it cannot provide insight into spatial regulation. Regulation of the *Hand1* expression pattern is interesting due to its novel spatial pattern and therefore studying regulatory elements in a transfected cell culture system would not significantly increase our understanding of *Hand1* regulation.

Consequently, the most appropriate approach for studying *Hand1* regulation and enhancer activity in the *Hand1* intron was the knockout model. If enhancer activity is present in the *Hand1* intron, removing the intron by replacing the *Hand1* gene with the *Hand1* cDNA should alter the *Hand1* expression pattern. To determine if removing the

Hand1 intron affected *Hand1* expression, *Hand1*$^{\Delta I/+}$ mice were bred to *Hand1*$^{+/-}$ mice. There is no expression from the null allele, so *Hand1*$^{\Delta I/-}$ animals will only have expression from the ΔI allele, thereby demonstrating any changes in spatial or temporal patterning. The *Hand1* spatial expression pattern was unchanged in *Hand1*$^{\Delta I/-}$ embryos and implantation sites, compared to their littermates, and therefore it was concluded that the *Hand1* intron is not necessary for correct spatial patterning of *Hand1* gene expression.

However, the *Hand1* intron does appear to contribute to full expression levels of the *Hand1* gene. Signal intensity from the *in situ* hybridization was variably decreased in *Hand1*$^{\Delta I/-}$ embryos and implantation sites to levels below that seen in *Hand1*$^{+/+}$ or *Hand1*$^{+/-}$ littermates. Northern analysis of mRNA from *Hand1*$^{\Delta I/-}$ decidua and placentas indicates that *Hand1* mRNa is expressed at variably decreased levels. Therefore, the *Hand1* intron may participate in regulating *Hand1* expression levels.

At E10.5, some *Hand1*$^{\Delta I/-}$ embryos had subtle, variable cardiac defects, suggesting that *Hand1* expression levels are important in cardiac morphogenesis, consistent with *Hand* misexpression studies which show that expression of *Hand1* or *Hand2* drives cardiac cell proliferation in E10.5 hearts (Risebro et al., 2006; Togi et al., 2004; Togi et al., 2006). The studies described here were carried out on a mixed outbred genetic background and therefore some phenotype and gene expression variability could be due to variation in other genes in the genetic background. Backcrossing the *Hand1*$^{\Delta I}$ mice onto the inbred 129SvJ strain would limit the background variation, and allow a more careful characterization of the *Hand1*$^{\Delta I}$ phenotype.

Based on the *in vitro* experiments, loss of the *Hand1* intron was expected to eliminate or strongly reduce cardiac *Hand1* expression, and have no effect on trophoblast

expression. This would have provided us with a model in which we could study the role of *Hand1* in cardiac development without the complication of the trophoblast-related lethality. The lack of effect of the Δ*I* allele on the expression domain can be explained several ways. First, the *Hand1* intron may be part of a bipartite regulatory element, with one portion residing in the 5' promoter region, which is sufficient for the expression pattern, while the intron portion regulates expression level. This is compatible with the transfection construct results, which showed increased reporter expression in cardiac cells as differentiation progressed (P. Riley, I. Scott and J.C. Cross, unpublished results). Second, increased expression in transfection assays associated with the intron constructs may have been driven by elements in the second exon or 3' untranslated region, which were included in the intron constructs, and were not affected in the $Hand1^{\Delta I}$ allele. Third, the *in vitro* effect of the *Hand1* intron may be unrelated to *in vivo* regulation of *Hand1* mRNA expression, and the *in vitro* results may be an artefact of the transfection constructs or the test system.

Decreased expression throughout the conceptus from the Δ*I* allele could be explained in several ways. The *Hand1* intron may regulate transcription level, as a conserved region was observed in the intron and transcription factor binding sites contained in this region could regulate transcription level, but it is important to rule out other possibilities. First, as the puromycin selection cassette from the targeting vector was removed using Cre-loxP technology, the decreased expression levels are not due to selection cassette-related expression inhibition, which has been observed when selection cassettes are not removed (Pham et al., 1996). Second, the loxP site that remains following puromycin cassette removal is located in the 3' untranslated region (UTR). No

putative transcription factor binding sites are disrupted or created by the loxP insertion. Therefore it is unlikely that decreased mRNA expression is related to the loxP site. Third, removing the intron may have disrupted pre-mRNA processing, as expression of some transcripts is intron-dependent (Nott et al., 2003). However, as *Hand1* can be expressed from a cDNA in transfected cells, a requirement of the presence of an intron for high transcript levels may be dependent on promoter and genomic context. Alternately, *Hand1* mRNA levels may appear lower due to higher transcript turnover, rather than decreased transcription. Transcript stability is often regulated by sequence elements in the 3' UTR, and their disruption can alter transcript stability, leading to drastic changes in steady-state mRNA levels (Eberhardt et al., 2007). The single loxP site remaining in the 3' UTR does not introduce or interrupt known UTR regulatory elements, as revealed by a UTR element BLAST search (http://www.ba.itb.cnr.it/BIG/Blast/BlastUTR.html, data not shown) (Mignone et al., 2005). Therefore, the loxP site in the 3' UTR of the *Hand1*$^{\Delta I}$ allele does not likely disrupt *Hand1* transcript stability, and decreased expression is likely a genuine effect of deleting the *Hand1* intron.

4.4 Future directions

Characterization of the *Hand1*$^{\Delta I}$ mouse line is currently incomplete. During the initial characterization, three main observations were made: there are fewer *Hand1*$^{\Delta I/-}$ animals after birth than expected, the *Hand1* expression pattern is normal but expression appears to be variably downregulated, and there is a subtle, variable defect in some E10.5 *Hand1*$^{\Delta I/-}$ embryos. These initial observations have been made on the outbred CD1 background.

4.4.1 Complete Characterization of Hand1$^{\Delta I/-}$ *Mouse Line*

The phenotype observed in *Hand1*$^{\Delta I/-}$ animals was variable. As well, *Hand1* mRNA expression level appeared to be variable based on differences in mRNA staining intensity. The variability in phenotype and expression could be caused by the outbred genetic background of the CD1 strain. Backcrossing the line onto different inbred backgrounds would allow distinction between the effect of background genetics and the loss of the *Hand1* intron on *Hand1* expression. The *Hand1*$^{\Delta I}$ mouse line is currently being backcrossed onto the 129Sv background for this purpose. Additionally, northern blotting to assess *Hand1* mRNA expression in E10.5 *Hand1*$^{\Delta I/-}$ embryos should be repeated.

Analysis at E10.5 and E18.5, just prior to birth, will reveal cardiac defects during chamber morphogenesis, and following septation and valvulogenesis. These animals are likely dying perinatally, as animals with significant cardiac defects, like *Tbx5*$^{+/-}$ mice, can survive *in utero* if their cardiac development does not arrest prior to chamber morphogenesis and looping, but then die perinatally as their circulatory system is insufficient (Bruneau et al., 2001b). Analysis at E18.5 would determine if some *Hand1*$^{\Delta I/-}$ embryos die before or after birth, and will reveal defects in chamber septation, valvulogenesis, and blood vessel septation and alignment with chambers. Marker expression for chamber and valve development, and development of the cardiac conduction system, should be analyzed at E10.5 and E18.5, to complement the structural observations. In addition to analysis at E10.5 and E18.5 to characterize structural defects, cardiac function should be analyzed in these mice after birth.

In addition to the heart, *Hand1* mRNA is expressed during development in the

trophoblast, yolk sac, branchial arches, a subset of neural crest derivatives, and the limb bud (Cross et al., 1995; Cserjesi et al., 1995; Fernandez-Teran et al., 2003; Hollenberg et al., 1995). The *Hand1* mRNA expression pattern in heart and trophoblast was unchanged in *Hand1*$^{\Delta I/-}$ embryos compared to *Hand1*$^{+/+}$ embryos at E8.5 and E10.5. Expression was not detected in the branchial arches or limb buds, and the neural crest derivatives were not examined. Gross development of the placenta, branchial arches and limb buds should be studied histologically in *Hand1*$^{\Delta I/-}$ embryos to verify that development is unaffected.

4.4.2 Identification of Key Tissue-Specific Regulatory Elements for Hand1

Regulation of gene expression patterns in different tissues is often modular, with different enhancer elements regulating expression in different tissues. Two enhancer elements have been identified for the *Hand2* gene, regulating expression in the branchial arches and in the right ventricle (McFadden et al., 2000; Yanagisawa et al., 2003). Expression of *Nkx2-5* is regulated by multiple modular enhancers, with multiple enhancers for *pan*-cardiac expression, and further enhancers for extra-cardiac expression (Chi et al., 2005). Modular regulation of gene expression patterns is not a phenomenon unique to the heart: *Sox9* expression is regulated by separate enhancers for inner ear and neural crest, neuroectoderm, ventral spinal cord and hindbrain, and testes (Bagheri-Fam et al., 2006).

The work described in this thesis has demonstrated that the *Hand1* intron does not contain a spatial or temporal enhancer that is completely essential for *Hand1* expression. This finding does not make the regulation of *Hand1* expression in different tissues any less interesting. Unfortunately, as discussed previously, the transgenic approach of integrating a reporter gene under control of putative regulatory elements has not

identified regulatory regions sufficient for expression, even with quite large constructs. While this approach could be expanded, starting with a bacterial artificial chromosome, then using deletions to identify the regulatory region, other approaches may be more expeditious for identifying *Hand1* regulatory elements.

First, the second exon, specifically the 3' untranslated region (UTR), should be investigated for enhancer activity. In the transfection experiments conducted by Paul Riley and Ian Scott, the 'intron' constructs contained the intron and second exon. Therefore, constructs should be made with 6 kb of upstream sequence, and the intron, the second exon or just the 3' UTR. This would allow dissection of enhancer activity from the previous 'intron' constructs.

Second, the future analysis of constructs for enhancer activity needs to be expanded from trophoblast (Rcho-1) and cardiac (P19) cells. *Hand1* is also expressed in limb buds and neural crest derivatives. One approach would be to simply include cell lines which represent limb bud and neural crest cells, or to transfect explanted limb buds or whole embryos. The disadvantage of using tissue culture cell lines is that they do not completely represent the situation in the embryo, and there is no way to assess a change in spatial expression patterning. To overcome this, a technique has been developed to electroporate embryos *in vivo* to evaluate spatial expression of a reporter construct (Fukuchi-Shimogori and Grove, 2001). This approach has been used to study brain development and neuronal migration (Fukuchi-Shimogori and Grove, 2001; Nakahira and Yuasa, 2005), but has not been applied to early developmental stages, when the *Hand1* mRNA expression pattern is most interesting.

An alternate approach for spatial evaluation of reporter construct activity in early

embryos is embryo culture and electroporation. Post-implantation mouse embryos can be cultured *in vitro* for up to two days (Tam, 1998), and can be manipulated by electroporation of reporter constructs or RNAi knockdown constructs (Calegari et al., 2004) while in culture, as routinely practiced with chick embryos. With this approach, putative regulatory elements can be quickly assayed for activity without the complicating factors of integration site, copy number and other transgenesis-related issues. When an element with regulatory activity is identified and characterized, it could be co-transfected with RNAi constructs to knock down expression of a putative upstream regulator, quickly integrating the element and gene into the larger network of transcriptional regulation. This approach has several advantages: it is a quick assay, it provides information on spatial patterning, and it provides information on regulation of *Hand1* expression in all embryonic tissues. Unfortunately, this approach is not suitable for study of placental development. Transfection of differentiated trophoblast stem cells in culture, like Rcho-1 or ROSA-26 cells, would be the best quick assay to complement the embryo culture and electroporation approach, although it lacks spatial information (Sahgal et al., 2006; Tanaka et al., 1998).

Third, the intron may be part of a bi-partite enhancer. Strong enhancement of reporter activity was observed when the intron and second exon were combined with 6 kb of 5' proximal promoter sequence in transfected P19-derived cardiomyocytes. A bi-partite enhancer might be located in the intron and proximal promoter sequence, and deletion of the intron may not be sufficient to disrupt the gene expression pattern. Therefore, I suggest that the 5' proximal promoter be deleted in conjunction with the intron, to determine if these regions form a bipartite enhancer. This could be done in the

endogenous gene, or in the context of a bacterial artificial chromosome that recapitulates the *Hand1* expression pattern.

Fourth, biochemical approaches may provide insight into the transcriptional regulation of *Hand1*. Using genomic footprinting, regions bound by transcription factors near *Hand1* can be identified in different cell or tissue types. Comparing the sequences identified through footprinting with known transcription factor binding sites from TRANSFAC or JASPAR (Matys et al., 2003; Vlieghe et al., 2006), a set of transcription factors which may be responsible for the footprints can be identified. Binding to the footprinted regions can be verified and investigated through electrophoretic mobility shift assays.

4.5 Conclusions

The *Hand1* gene presents a fascinating, but difficult problem in gene regulation and heart development. It has an early role in patterning the developing heart, and exhibits a unique expression pattern at a time when other known cardiac transcription factors are expressed more broadly. Understanding regulation of *Hand1* expression would improve our understanding of early cardiac patterning. While the *Hand1* intron does not contain a critical cardiac enhancer as was initially hypothesized, further study should identify the enhancer. When the *Hand1* cardiac enhancer is identified, the network of upstream regulators can be dissected through site-directed mutagenesis of the enhancer, and enhancer assays in culture embryos combined with RNAi-based gene knockdown.

The $Hand1^{\Delta I/-}$ mice generated in this study can be used to study the effect of

downregulation of *Hand1* expression on heart development. As misexpression or overexpression of *Hand1* have provided insight into cardiac patterning and proliferation, I expect that the *Hand1*$^{\Delta I/-}$ mice will provide further insight into cardiac development.

References

Aerts, S., Van Loo, P., Thijs, G., Mayer, H., de Martin, R., Moreau, Y. and De Moor, B. (2005). TOUCAN 2: the all-inclusive open source workbench for regulatory sequence analysis. *Nucleic Acids Res* **33**, W393-6.

Anderson, R. H., Webb, S., Brown, N. A., Lamers, W. and Moorman, A. (2003). Development of the heart: (2) Septation of the atriums and ventricles. *Heart* **89**, 949-58.

Angell, C. S., Hipona, F. A. and Novick, A. (1966). Angiocardiographic studies of the anuran double circulation. *Anat Rec* **156**, 41-53.

Auda-Boucher, G., Bernard, B., Fontaine-Perus, J., Rouaud, T., Mericksay, M. and Gardahaut, M.-F. (2000). Staging of the commitment of murine cardiac cell progenitors. *Developmental Biology* **225**, 214-25.

Bagheri-Fam, S., Barrionuevo, F., Dohrmann, U., Gunther, T., Schule, R., Kemler, R., Mallo, M., Kanzler, B. and Scherer, G. (2006). Long-range upstream and downstream enhancers control distinct subsets of the complex spatiotemporal Sox9 expression pattern. *Dev Biol* **291**, 382-97.

Bao, Z. Z., Bruneau, B. G., Seidman, J. G., Seidman, C. E. and Cepko, C. L. (1999). Regulation of chamber-specific gene expression in the developing heart by Irx4. *Science* **283**, 1161-4.

Biben, C. and Harvey, R. P. (1997). Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene eHand during murine heart development. *Genes Dev* **11**, 1357-69.

Bishopric, N. H. (2005). Evolution of the heart from bacteria to man. *Ann N Y Acad Sci* **1047**, 13-29.

Bodmer, R. and Venkatesh, T. V. (1998). Heart development in Drosophila and vertebrates: conservation of molecular mechanisms. *Dev Genet* **22**, 181-6.

Boffelli, D., Nobrega, M. A. and Rubin, E. M. (2004). Comparative genomics at the vertebrate extremes. *Nat Rev Genet* **5**, 456-65.

Bollag, R. J., Siegfried, Z., Cebra-Thomas, J. A., Garvey, N., Davison, E. M. and Silver, L. M. (1994). An ancient family of embryonically expressed mouse genes sharing a conserved protein motif with the T locus. *Nat Genet* **7**, 383-9.

Bond, J., Sedmera, D., Jourdan, J., Zhang, Y., Eisenberg, C. A., Eisenberg, L. M. and Gourdie, R. G. (2003). Wnt11 and Wnt7a are up-regulated in association with differentiation of cardiac conduction cells in vitro and in vivo. *Dev Dyn* **227**, 536-43.

Brown, D. D., Martz, S. N., Binder, O., Goetz, S. C., Price, B. M., Smith, J. C. and Conlon, F. L. (2005). Tbx5 and Tbx20 act synergistically to control vertebrate heart morphogenesis. *Development* **132**, 553-563.

Bruneau, B. G., Bao, Z. Z., Fatkin, D., Xavier-Neto, J., Georgakopoulos, D., Maguire, C. T., Berul, C. I., Kass, D. A., Kuroski-de Bold, M. L., de Bold, A. J. et al. (2001a). Cardiomyopathy in Irx4-deficient mice is preceded by abnormal ventricular gene expression. *Mol Cell Biol* **21**, 1730-6.

Bruneau, B. G., Bao, Z. Z., Tanaka, M., Schott, J. J., Izumo, S., Cepko, C. L., Seidman, J. G. and Seidman, C. E. (2000). Cardiac expression of the ventricle-specific homeobox gene Irx4 is modulated by Nkx2-5 and dHand. *Dev Biol* **217**, 266-77.

Bruneau, B. G., Logan, M., Davis, N., Levi, T., Tabin, C. J., Seidman, J. G. and

Seidman, C. E. (1999). Chamber-specific cardiac expression of Tbx5 and heart defects in Holt-Oram syndrome. *Dev Biol* **211**, 100-8.

Bruneau, B. G., Nemer, G., Schmitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman, C. E. et al. (2001b). A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106**, 709-21.

Cai, C. L., Liang, X., Shi, Y., Chu, P. H., Pfaff, S. L., Chen, J. and Evans, S. (2003). Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev Cell* **5**, 877-89.

Cai, C. L., Zhou, W., Yang, L., Bu, L., Qyang, Y., Zhang, X., Li, X., Rosenfeld, M. G., Chen, J. and Evans, S. (2005). T-box genes coordinate regional rates of proliferation and regional specification during cardiogenesis. *Development* **132**, 2475-87.

Calegari, F., Marzesco, A. M., Kittler, R., Buchholz, F. and Huttner, W. B. (2004). Tissue-specific RNA interference in post-implantation mouse embryos using directional electroporation and whole embryo culture. *Differentiation* **72**, 92-102.

Carroll, S. B., Grenier, J. K. and Weatherbee, S. D. (2001). From DNA to diversity : molecular genetics and the evolution of animal design. Malden, MA: Blackwell Science.

Carson, C. T., Kinzler, E. R. and Parr, B. A. (2000). Tbx12, a novel T-box gene, is expressed during early stages of heart and retinal development. *Mech Dev* **96**, 137-40.

Chapman, D. L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S. I., Gibson-Brown, J. J., Cebra-Thomas, J., Bollag, R. J., Silver, L. M. and Papaioannou, V. E. (1996). Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. *Dev Dyn* **206**, 379-90.

Charite, J., McFadden, D. G., Merlo, G., Levi, G., Clouthier, D. E., Yanagisawa, M., Richardson, J. A. and Olson, E. N. (2001). Role of Dlx6 in regulation of an endothelin-1-dependent, dHAND branchial arch enhancer. *Genes Dev* **15**, 3039-49.

Cheng, G., Litchenberg, W. H., Cole, G. J., Mikawa, T., Thompson, R. P. and Gourdie, R. G. (1999). Development of the cardiac conduction system involves recruitment within a multipotent cardiomyogenic lineage. *Development* **126**, 5041-9.

Chi, X., Chatterjee, P. K., Wilson, W., 3rd, Zhang, S. X., Demayo, F. J. and Schwartz, R. J. (2005). Complex cardiac Nkx2-5 gene expression activated by noggin-sensitive enhancers followed by chamber-specific modules. *Proc Natl Acad Sci U S A* **102**, 13490-5.

Christoffels, V. M., Habets, P. E., Franco, D., Campione, M., de Jong, F., Lamers, W. H., Bao, Z.-Z., Palmer, S., Biben, C., Harvey, R. P. et al. (2000). Chamber formation and morphogenesis in the developing mammalian heart. *Developmental Biology* **223**, 266-78.

Christoffels, V. M., Hoogaars, W. M., Tessari, A., Clout, D. E., Moorman, A. F. and Campione, M. (2004). T-box transcription factor Tbx2 represses differentiation and formation of the cardiac chambers. *Dev Dyn* **229**, 763-70.

Conlon, R. A. and Rossant, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. *Development* **116**, 357-68.

Corfas, G., Rosen, K. M., Aratake, H., Krauss, R. and Fischbach, G. D. (1995). Differential expression of ARIA isoforms in the rat brain. *Neuron* **14**, 103-15.

Crispino, J. D., Lodish, M. B., Thurberg, B. L., Litovsky, S. H., Collins, T.,

Molkentin, J. D. and Orkin, S. H. (2001). Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. *Genes Dev* **15**, 839-44.

Cross, J. C., Flannery, M. L., Blanar, M. A., Steingrimsson, E., Jenkins, N. A., Copeland, N. G., Rutter, W. J. and Werb, Z. (1995). Hxt encodes a basic helix-loop-helix transcription factor that regulates trophoblast cell development. *Development* **121**, 2513-23.

Cserjesi, P., Brown, D., Lyons, G. E. and Olson, E. N. (1995). Expression of the novel basic helix-loop-helix gene eHAND in neural crest derivatives and extraembryonic membranes during mouse development. *Dev Biol* **170**, 664-78.

Davidson, B. (2007). Ciona intestinalis as a model for cardiac development. *Semin Cell Dev Biol* **18**, 16-26.

de la Cruz, M., Sanchez-Gomez, C. and Palomino, M. (1989). The primitive cardiac regions in the straight tube heart (stage 9) and their anatomical expression in the mature heart: an experimental study in the chick embryo. *J Anat.* **165**, 121–131.

de la Cruz, M. V., Sanchez Gomez, C., Arteaga, M. M. and Arguello, C. (1977). Experimental study of the development of the truncus and the conus in the chick embryo. *J Anat* **123**, 661-86.

de Lange, F. J., Moorman, A. F., Anderson, R. H., Manner, J., Soufan, A. T., de Gier-de Vries, C., Schneider, M. D., Webb, S., van den Hoff, M. J. and Christoffels, V. M. (2004). Lineage and morphogenetic analysis of the cardiac valves. *Circ Res* **95**, 645-54.

Dodou, E., Verzi, M. P., Anderson, J. P., Xu, S. M. and Black, B. L. (2004). Mef2c is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development. *Development* **131**, 3931-42.

Eberhardt, W., Doller, A., Akool, E. S. and Pfeilschifter, J. (2007). Modulation of mRNA stability as a novel therapeutic approach. *Pharmacol Ther*.

Edmondson, D. G., Lyons, G. E., Martin, J. F. and Olson, E. N. (1994). Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* **120**, 1251-63.

Fernandez-Teran, M., Piedra, M. E., Rodriguez-Rey, J. C., Talamillo, A. and Ros, M. A. (2003). Expression and regulation of eHAND during limb development. *Dev Dyn* **226**, 690-701.

Finelli, P., Pincelli, A., Russo, S., Bonati, M., Recalcati, M., Masciadri, M., Giardino, D., Cavagnini, F. and Larizza, L. (2007). Disruption of Friend of GATA 2 gene (FOG-2) by a de novo t(8;10) chromosomal translocation is associated with heart defects and gonadal dysgenesis. *Clin Genet* **71**, 195-204.

Firulli, A. B., McFadden, D. G., Lin, Q., Srivastava, D. and Olson, E. N. (1998). Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nat Genet* **18**, 266-70.

Franco, D., Meilhac, S. M., Christoffels, V. M., Kispert, A., Buckingham, M. and Kelly, R. G. (2006). Left and right ventricular contributions to the formation of the interventricular septum in the mouse heart. *Dev Biol*.

Fukuchi-Shimogori, T. and Grove, E. A. (2001). Neocortex patterning by the secreted signaling molecule FGF8. *Science* **294**, 1071-4.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-4.

Gourdie, R. G., Harris, B. S., Bond, J., Justus, C., Hewett, K. W., O'Brien, T. X., Thompson, R. P. and Sedmera, D. (2003). Development of the cardiac pacemaking and conduction system. *Birth Defects Res C Embryo Today* **69**, 46-57.

Gourdie, R. G., Mima, T., Thompson, R. P. and Mikawa, T. (1995). Terminal diversification of the myocyte lineage generates Purkinje fibers of the cardiac conduction system. *Development* **121**, 1423-31.

Habets, P. E., Moorman, A. F., Clout, D. E., van Roon, M. A., Lingbeek, M., van Lohuizen, M., Campione, M. and Christoffels, V. M. (2002). Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. *Genes Dev* **16**, 1234-46.

Han, Z. and Olson, E. N. (2005). Hand is a direct target of Tinman and GATA factors during Drosophila cardiogenesis and hematopoiesis. *Development* **132**, 3525-36.

Harrelson, Z., Kelly, R. G., Goldin, S. N., Gibson-Brown, J. J., Bollag, R. J., Silver, L. M. and Papaioannou, V. E. (2004). Tbx2 is essential for patterning the atrioventricular canal and for morphogenesis of the outflow tract during heart development. *Development* **131**, 5041-52.

Hatcher, C. J. and McDermott, D. A. (2006). Using the TBX5 transcription factor to grow and sculpt the heart. *Am J Med Genet A*.

Haun, C., Alexander, J., Stainier, D. Y. and Okkema, P. G. (1998). Rescue of Caenorhabditis elegans pharyngeal development by a vertebrate heart specification gene. *Proc Natl Acad Sci U S A* **95**, 5072-5.

Heikinheimo, M., Scandrett, J. M. and Wilson, D. B. (1994). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev Biol* **164**, 361-73.

Hochgreb, T., Linhares, V. L., Menezes, D. C., Sampaio, A. C., Yan, C. Y., Cardoso, W. V., Rosenthal, N. and Xavier-Neto, J. (2003). A caudorostral wave of RALDH2 conveys anteroposterior information to the cardiac field. *Development* **130**, 5363-74.

Hollenberg, S. M., Sternglanz, R., Cheng, P. F. and Weintraub, H. (1995). Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. *Molecular and Cellular Biology* **15**, 3813-22.

Hoogaars, W. M., Tessari, A., Moorman, A. F., de Boer, P. A., Hagoort, J., Soufan, A. T., Campione, M. and Christoffels, V. M. (2004). The transcriptional repressor Tbx3 delineates the developing central conduction system of the heart. *Cardiovasc Res* **62**, 489-99.

Hurlstone, A. F., Haramis, A. P., Wienholds, E., Begthel, H., Korving, J., Van Eeden, F., Cuppen, E., Zivkovic, D., Plasterk, R. H. and Clevers, H. (2003). The Wnt/beta-catenin pathway regulates cardiac valve formation. *Nature* **425**, 633-7.

Ilagan, R., Abu-Issa, R., Brown, D., Yang, Y. P., Jiao, K., Schwartz, R. J., Klingensmith, J. and Meyers, E. N. (2006). Fgf8 is required for anterior heart field development. *Development* **133**, 2435-45.

Jay, P. Y., Harris, B. S., Buerger, A., Rozhitskaya, O., Maguire, C. T., Barbosky, L. A., McCusty, E., Berul, C. I., O'Brien T, X., Gourdie, R. G. et al. (2004a). Function

follows form: cardiac conduction system defects in Nkx2-5 mutation. *Anat Rec* **280A**, 966-72.

Jay, P. Y., Harris, B. S., Maguire, C. T., Buerger, A., Wakimoto, H., Tanaka, M., Kupershmidt, S., Roden, D. M., Schultheiss, T. M., O'Brien, T. X. et al. (2004b). Nkx2-5 mutation causes anatomic hypoplasia of the cardiac conduction system. *J Clin Invest* **113**, 1130-7.

Kanzawa, N., Poma, C. P., Takebayashi-Suzuki, K., Diaz, K. G., Layliev, J. and Mikawa, T. (2002). Competency of embryonic cardiomyocytes to undergo Purkinje fiber differentiation is regulated by endothelin receptor expression. *Development* **129**, 3185-94.

Kardong, K. V. (2005). An introduction to biological evolution. Boston: McGraw-Hill Higher Education.

Kelly, R. G., Brown, N. A. and Buckingham, M. E. (2001). The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev Cell* **1**, 435-40.

Kelly, R. G. and Papaioannou, V. E. (2007). Visualization of outflow tract development in the absence of Tbx1 using an FgF10 enhancer trap transgene. *Dev Dyn* **236**, 821-8.

Keyes, W. M., Logan, C., Parker, E. and Sanders, E. J. (2003). Expression and function of bone morphogenetic proteins in the development of the embryonic endocardial cushions. *Anat Embryol (Berl)* **207**, 135-47.

Knofler, M., Meinhardt, G., Vasicek, R., Husslein, P. and Egarter, C. (1998). Molecular cloning of the human Hand1 gene/cDNA and its tissue-restricted expression in cytotrophoblastic cells and heart. *Gene* **224**, 77-86.

Kolsch, V. and Paululat, A. (2002). The highly conserved cardiogenic bHLH factor Hand is specifically expressed in circular visceral muscle progenitor cells and in all cell types of the dorsal vessel during Drosophila embryogenesis. *Development Genes and Evolution* **212**, 473-85.

Komuro, I. and Izumo, S. (1993). Csx: a murine homeobox-containing gene specifically expressed in the developing heart. *Proc Natl Acad Sci U S A* **90**, 8145-9.

Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E. and Theill, L. E. (1996). Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc Natl Acad Sci U S A* **93**, 4833-8.

Kraus, F., Haenig, B. and Kispert, A. (2001). Cloning and expression analysis of the mouse T-box gene tbx20. *Mech Dev* **100**, 87-91.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev* **11**, 1048-60.

Lamers, W. H. and Moorman, A. F. (2002). Cardiac septation: a late contribution of the embryonic primary myocardium to heart morphogenesis. *Circ Res* **91**, 93-103.

Lavine, K. J., Yu, K., White, A. C., Zhang, X., Smith, C., Partanen, J. and Ornitz, D. M. (2005). Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. *Dev Cell* **8**, 85-95.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-8.

Li, W. and Li, Y. (2006). Regulation of dHAND protein expression by all-trans retinoic acid through ET-1/ETAR signaling in H9c2 cells. *J Cell Biochem*.

Liberatore, C. M., Searcy-Schrick, R. D. and Yutzey, K. E. (2000). Ventricular expression of tbx5 inhibits normal heart chamber development. *Dev Biol* **223**, 169-80.

Lincoln, J., Alfieri, C. M. and Yutzey, K. E. (2006). BMP and FGF regulatory pathways control cell lineage diversification of heart valve precursor cells. *Developmental Biology* **292**, 290-302.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I. and Harvey, R. P. (1993). Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* **119**, 419-31.

Liu, J., Qian, L., Wessells, R. J., Bidet, Y., Jagla, K. and Bodmer, R. (2006). Hedgehog and RAS pathways cooperate in the anterior-posterior specification and positioning of cardiac progenitor cells. *Dev Biol* **290**, 373-85.

Locker, J. (2001). Transcription factors. Oxford
San Diego, CA: BIOS ;
Academic Press
ebrary Inc.

Loots, G. G. and Ovcharenko, I. (2004). rVISTA 2.0: evolutionary analysis of transcription factor binding sites. *Nucleic Acids Res* **32**, W217-21.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L. and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev* **9**, 1654-66.

Ma, L., Lu, M. F., Schwartz, R. J. and Martin, J. F. (2005). Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development* **132**, 5601-11.

Marguerie, A., Bajolle, F., Zaffran, S., Brown, N. A., Dickson, C., Buckingham, M. E. and Kelly, R. G. (2006). Congenital heart defects in Fgfr2-IIIb and Fgf10 mutant mice. *Cardiovasc Res* **71**, 50-60.

Matys, V., Fricke, E., Geffers, R., Gossling, E., Haubrock, M., Hehl, R., Hornischer, K., Karas, D., Kel, A. E., Kel-Margoulis, O. V. et al. (2003). TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res* **31**, 374-8.

McFadden, D. G., Barbosa, A. C., Richardson, J. A., Schneider, M. D., Srivastava, D. and Olson, E. N. (2005). The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. *Development* **132**, 189-201.

McFadden, D. G., Charite, J., Richardson, J. A., Srivastava, D., Firulli, A. B. and Olson, E. N. (2000). A GATA-dependent right ventricular enhancer controls dHAND transcription in the developing heart. *Development* **127**, 5331-41.

McLysaght, A., Enright, A. J., Skrabanek, L. and Wolfe, K. H. (2000). Estimation of synteny conservation and genome compaction between pufferfish (Fugu) and human. *Yeast* **17**, 22-36.

Meilhac, S. M., Esner, M., Kelly, R. G., Nicolas, J. F. and Buckingham, M. E. (2004a). The clonal origin of myocardial cells in different regions of the embryonic mouse heart. *Dev Cell* **6**, 685-98.

Meilhac, S. M., Esner, M., Kerszberg, M., Moss, J. E. and Buckingham, M. E.

(2004b). Oriented clonal cell growth in the developing mouse myocardium underlies cardiac morphogenesis. *J Cell Biol* **164**, 97-109.
Meyer, D. and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-90.
Meysen, S., Marger, L., Hewett, K. W., Jarry-Guichard, T., Agarkova, I., Chauvin, J. P., Perriard, J. C., Izumo, S., Gourdie, R. G., Mangoni, M. E. et al. (2007). Nkx2.5 cell-autonomous gene function is required for the postnatal formation of the peripheral ventricular conduction system. *Dev Biol* **303**, 740-53.
Mignone, F., Grillo, G., Licciulli, F., Iacono, M., Liuni, S., Kersey, P. J., Duarte, J., Saccone, C. and Pesole, G. (2005). UTRdb and UTRsite: a collection of sequences and regulatory motifs of the untranslated regions of eukaryotic mRNAs. *Nucleic Acids Res* **33**, D141-6.
Mjaatvedt, C. H., Nakaoka, T., Moreno-Rodriguez, R., Norris, R. A., Kern, M. J., Eisenberg, C. A., Turner, D. and Markwald, R. R. (2001). The outflow tract of the heart is recruited from a novel heart-forming field. *Dev Biol* **238**, 97-109.
Mohun, T. J., Leong, L. M., Weninger, W. J. and Sparrow, D. B. (2000). The morphology of heart development in Xenopus laevis. *Dev Biol* **218**, 74-88.
Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev* **11**, 1061-72.
Mommersteeg, M. T., Hoogaars, W. M., Prall, O. W., de Gier-de Vries, C., Wiese, C., Clout, D. E., Papaioannou, V. E., Brown, N. A., Harvey, R. P., Moorman, A. F. et al. (2007). Molecular pathway for the localized formation of the sinoatrial node. *Circ Res* **100**, 354-62.
Montgomery, M. O., Litvin, J., Gonzalez-Sanchez, A. and Bader, D. (1994). Staging of commitment and differentiation of avian cardiac myocytes. *Dev Biol* **164**, 63-71.
Moore, A. W., Barbel, S., Jan, L. Y. and Jan, Y. N. (2000). A genomewide survey of basic helix-loop-helix factors in Drosophila. *Proc Natl Acad Sci U S A* **97**, 10436-41.
Moorman, A., Webb, S., Brown, N. A., Lamers, W. and Anderson, R. H. (2003). Development of the heart: (1) formation of the cardiac chambers and arterial trunks. *Heart* **89**, 806-14.
Moorman, A. F., de Jong, F., Denyn, M. M. and Lamers, W. H. (1998). Development of the cardiac conduction system. *Circ Res* **82**, 629-44.
Moorman, A. F., Schumacher, C. A., de Boer, P. A., Hagoort, J., Bezstarosti, K., van den Hoff, M. J., Wagenaar, G. T., Lamers, J. M., Wuytack, F., Christoffels, V. M. et al. (2000). Presence of functional sarcoplasmic reticulum in the developing heart and its confinement to chamber myocardium. *Dev Biol* **223**, 279-90.
Mori, A. D., Zhu, Y., Vahora, I., Nieman, B., Koshiba-Takeuchi, K., Davidson, L., Pizard, A., Seidman, J. G., Seidman, C. E., Chen, X. J. et al. (2006). Tbx5-dependent rheostatic control of cardiac gene expression and morphogenesis. *Dev Biol* **297**, 566-86.
Morikawa, Y. and Cserjesi, P. (2004). Extra-embryonic vasculature development is regulated by the transcription factor HAND1. *Development* **131**, 2195-204.
Moskowitz, I. P., Pizard, A., Patel, V. V., Bruneau, B. G., Kim, J. B., Kupershmidt, S., Roden, D., Berul, C. I., Seidman, C. E. and Seidman, J. G. (2004). The T-Box transcription factor Tbx5 is required for the patterning and maturation of the murine

cardiac conduction system. *Development* **131**, 4107-16.
Nakahira, E. and Yuasa, S. (2005). Neuronal generation, migration, and differentiation in the mouse hippocampal primoridium as revealed by enhanced green fluorescent protein gene transfer by means of in utero electroporation. *J Comp Neurol* **483**, 329-40.
Narita, N., Bielinska, M. and Wilson, D. B. (1997). Cardiomyocyte differentiation by GATA-4-deficient embryonic stem cells. *Development* **124**, 3755-64.
Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P. and Dolle, P. (2001). Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. *Development* **128**, 1019-31.
Nott, A., Meislin, S. H. and Moore, M. J. (2003). A quantitative analysis of intron effects on mammalian gene expression. *Rna* **9**, 607-17.
Olson, E. N. (2006). Gene regulatory networks in the evolution and development of the heart. *Science* **313**, 1922-7.
Ovcharenko, I., Loots, G. G., Hardison, R. C., Miller, W. and Stubbs, L. (2004a). zPicture: dynamic alignment and visualization tool for analyzing conservation profiles. *Genome Res* **14**, 472-7.
Ovcharenko, I., Nobrega, M. A., Loots, G. G. and Stubbs, L. (2004b). ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. *Nucleic Acids Res* **32**, W280-6.
Pashmforoush, M., Lu, J. T., Chen, H., Amand, T. S., Kondo, R., Pradervand, S., Evans, S. M., Clark, B., Feramisco, J. R., Giles, W. et al. (2004). Nkx2-5 pathways and congenital heart disease; loss of ventricular myocyte lineage specification leads to progressive cardiomyopathy and complete heart block. *Cell* **117**, 373-86.
Patel, R. and Kos, L. (2005). Endothelin-1 and Neuregulin-1 convert embryonic cardiomyocytes into cells of the conduction system in the mouse. *Dev Dyn* **233**, 20-8.
Patwardhan, V., Fernandez, S., Montgomery, M. and Litvin, J. (2000). The rostro-caudal position of cardiac myocytes affect their fate. *Dev Dyn* **218**, 123-35.
Peterkin, T., Gibson, A., Loose, M. and Patient, R. (2005). The roles of GATA-4, -5 and -6 in vertebrate heart development. *Semin Cell Dev Biol* **16**, 83-94.
Pham, C. T., MacIvor, D. M., Hug, B. A., Heusel, J. W. and Ley, T. J. (1996). Long-range disruption of gene expression by a selectable marker cassette. *Proc Natl Acad Sci U S A* **93**, 13090-5.
Ramsdell, A. F. (2005). Left-right asymmetry and congenital cardiac defects: getting to the heart of the matter in vertebrate left-right axis determination. *Dev Biol* **288**, 1-20.
Rentschler, S., Zander, J., Meyers, K., France, D., Levine, R., Porter, G., Rivkees, S. A., Morley, G. E. and Fishman, G. I. (2002). Neuregulin-1 promotes formation of the murine cardiac conduction system. *Proc Natl Acad Sci U S A* **99**, 10464-9.
Riley, P., Anson-Cartwright, L. and Cross, J. C. (1998). The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nature Genetics* **18**, 271-5.
Riley, P. R., Gertsenstein, M., Dawson, K. and Cross, J. C. (2000). Early exclusion of hand1-deficient cells from distinct regions of the left ventricular myocardium in chimeric mouse embryos. *Developmental Biology* **227**, 156-68.
Risebro, C. A., Smart, N., Dupays, L., Breckenridge, R., Mohun, T. J. and Riley, P. R. (2006). Hand1 regulates cardiomyocyte proliferation versus differentiation in the

developing heart. *Development* **133**, 4595-606.
Russell, M. W., Baker, P. and Izumo, S. (1997). Cloning, chromosomal mapping, and expression of the human eHAND gene. *Mammalian Genome* **8**, 863-5.
Russell, M. W., Kemp, P., Wang, L., Brody, L. C. and Izumo, S. (1998). Molecular cloning of the human HAND2 gene. *Biochim Biophys Acta* **1443**, 393-9.
Rutenberg, J. B., Fischer, A., Jia, H., Gessler, M., Zhong, T. P. and Mercola, M. (2006). Developmental patterning of the cardiac atrioventricular canal by Notch and Hairy-related transcription factors. *Development* **133**, 4381-90.
Sabherwal, N., Bangs, F., Roth, R., Weiss, B., Jantz, K., Tiecke, E., Hinkel, G. K., Spaich, C., Hauffa, B. P., van der Kamp, H. et al. (2007). Long-range conserved non-coding SHOX sequences regulate expression in developing chicken limb and are associated with short stature phenotypes in human patients. *Hum Mol Genet* **16**, 210-22.
Saga, Y., Miyagawa-Tomita, S., Takagi, A., Kitajima, S., Miyazaki, J. and Inoue, T. (1999). MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. *Development* **126**, 3437-47.
Sahgal, N., Canham, L. N., Canham, B. and Soares, M. J. (2006). Rcho-1 trophoblast stem cells: a model system for studying trophoblast cell differentiation. *Methods Mol Med* **121**, 159-78.
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning : a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.
Sandelin, A., Wasserman, W. W. and Lenhard, B. (2004). ConSite: web-based prediction of regulatory elements using cross-species comparison. *Nucleic Acids Res* **32**, W249-52.
Satou, Y., Imai, K. S., Levine, M., Kohara, Y., Rokhsar, D. and Satoh, N. (2003). A genomewide survey of developmentally relevant genes in Ciona intestinalis. I. Genes for bHLH transcription factors. *Dev Genes Evol* **213**, 213-21.
Satou, Y., Imai, K. S. and Satoh, N. (2004). The ascidian Mesp gene specifies heart precursor cells. *Development* **131**, 2533-41.
Schwartz, R. J. and Olson, E. N. (1999). Building the heart piece by piece: modularity of cis-elements regulating Nkx2-5 transcription. *Development* **126**, 4187-92.
Sedmera, D., Pexieder, T., Vuillemin, M., Thompson, R. P. and Anderson, R. H. (2000). Developmental patterning of the myocardium. *Anat Rec* **258**, 319-37.
Sedmera, D., Reckova, M., DeAlmeida, A., Coppen, S. R., Kubalak, S. W., Gourdie, R. G. and Thompson, R. P. (2003). Spatiotemporal pattern of commitment to slowed proliferation in the embryonic mouse heart indicates progressive differentiation of the cardiac conduction system. *Anat Rec* **274A**, 773-7.
Sellin, J., Albrecht, S., Kolsch, V. and Paululat, A. (2006). Dynamics of heart differentiation, visualized utilizing heart enhancer elements of the Drosophila melanogaster bHLH transcription factor Hand. *Gene Expr Patterns* **6**, 360-375.
Sharan, R., Ben-Hur, A., Loots, G. G. and Ovcharenko, I. (2004). CREME: Cis-Regulatory Module Explorer for the human genome. *Nucleic Acids Res* **32**, W253-6.
Shelton, E. L. and Yutzey, K. E. (2007). Tbx20 regulation of endocardial cushion cell proliferation and extracellular matrix gene expression. *Dev Biol* **302**, 376-88.
Shiratori, H. and Hamada, H. (2006). The left-right axis in the mouse: from origin to morphology. *Development* **133**, 2095-104.

Simmons, D. G., Fortier, A. L. and Cross, J. C. (2007). Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. *Developmental Biology* **doi: 10.1016/jydbio.2007.01.009**.

Singh, M. K., Christoffels, V. M., Dias, J. M., Trowe, M. O., Petry, M., Schuster-Gossler, K., Burger, A., Ericson, J. and Kispert, A. (2005). Tbx20 is essential for cardiac chamber differentiation and repression of Tbx2. *Development* **132**, 2697-707.

Slack, J. M. W. (1991). From egg to embryo : regional specification in early development. Cambridge, England: Cambridge University Press.

Sparrow, D. B., Kotecha, S., Towers, N. and Mohun, T. J. (1998). Xenopus eHAND: a marker for the developing cardiovascular system of the embryo that is regulated by bone morphogenetic proteins. *Mechanisms of Development* **71**, 151-63.

Srivastava, D., Cserjesi, P. and Olson, E. N. (1995). A subclass of bHLH proteins required for cardiac morphogenesis. *Science* **270**, 1995-9.

Srivastava, D. and Olson, E. N. (2000). A genetic blueprint for cardiac development. *Nature* **407**, 221-6.

Srivastava, D., Thomas, T., Lin, Q., Kirby, M. L., Brown, D. and Olson, E. N. (1997). Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. *Nat Genet* **16**, 154-60.

Stainier, D. Y., Lee, R. K. and Fishman, M. C. (1993). Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation. *Development* **119**, 31-40.

Stennard, F. A., Costa, M. W., Elliott, D. A., Rankin, S., Haast, S. J., Lai, D., McDonald, L. P., Niederreither, K., Dolle, P., Bruneau, B. G. et al. (2003). Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. *Dev Biol* **262**, 206-24.

Stennard, F. A., Costa, M. W., Lai, D., Biben, C., Furtado, M. B., Solloway, M. J., McCulley, D. J., Leimena, C., Preis, J. I., Dunwoodie, S. L. et al. (2005). Murine T-box transcription factor Tbx20 acts as a repressor during heart development, and is essential for adult heart integrity, function and adaptation. *Development*.

Sugi, Y., Yamamura, H., Okagawa, H. and Markwald, R. R. (2004). Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice. *Dev Biol* **269**, 505-18.

Takebayashi-Suzuki, K., Yanagisawa, M., Gourdie, R. G., Kanzawa, N. and Mikawa, T. (2000). In vivo induction of cardiac Purkinje fiber differentiation by coexpression of preproendothelin-1 and endothelin converting enzyme-1. *Development* **127**, 3523-32.

Takeuchi, J. K., Mileikovskaia, M., Koshiba-Takeuchi, K., Heidt, A. B., Mori, A. D., Arruda, E. P., Gertsenstein, M., Georges, R., Davidson, L., Mo, R. et al. (2005). Tbx20 dose-dependently regulates transcription factor networks required for mouse heart and motoneuron development. *Development*.

Takeuchi, J. K., Ohgi, M., Koshiba-Takeuchi, K., Shiratori, H., Sakaki, I., Ogura, K., Saijoh, Y. and Ogura, T. (2003). Tbx5 specifies the left/right ventricles and ventricular septum position during cardiogenesis. *Development* **130**, 5953-64.

Tallquist, M. D. and Soriano, P. (2000). Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extra-embryonic gene function. *Genesis* **26**, 113-5.

Tam, P. P. (1998). Postimplantation mouse development: whole embryo culture and micro-manipulation. *Int J Dev Biol* **42**, 895-902.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N. and Izumo, S. (1999). The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* **126**, 1269-1280.

Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A. and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. *Science* **282**, 2072-5.

Tevosian, S. G., Deconinck, A. E., Cantor, A. B., Rieff, H. I., Fujiwara, Y., Corfas, G. and Orkin, S. H. (1999). FOG-2: A novel GATA-family cofactor related to multitype zinc-finger proteins Friend of GATA-1 and U-shaped. *Proc Natl Acad Sci U S A* **96**, 950-5.

Tevosian, S. G., Deconinck, A. E., Tanaka, M., Schinke, M., Litovsky, S. H., Izumo, S., Fujiwara, Y. and Orkin, S. H. (2000). FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. *Cell* **101**, 729-39.

Thomas, P. S., Kasahara, H., Edmonson, A. M., Izumo, S., Yacoub, M. H., Barton, P. J. and Gourdie, R. G. (2001). Elevated expression of Nkx-2.5 in developing myocardial conduction cells. *Anat Rec* **263**, 307-13.

Thomas, T., Yamagishi, H., Overbeek, P. A., Olson, E. N. and Srivastava, D. (1998). The bHLH factors, dHAND and eHAND, specify pulmonary and systemic cardiac ventricles independent of left-right sidedness. *Developmental Biology* **196**, 228-36.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-80.

Togi, K., Kawamoto, T., Yamauchi, R., Yoshida, Y., Kita, T. and Tanaka, M. (2004). Role of Hand1/eHAND in the dorso-ventral patterning and interventricular septum formation in the embryonic heart. *Mol Cell Biol* **24**, 4627-35.

Togi, K., Yoshida, Y., Matsumae, H., Nakashima, Y., Kita, T. and Tanaka, M. (2006). Essential role of Hand2 in interventricular septum formation and trabeculation during cardiac development. *Biochem Biophys Res Commun* **343**, 144-51.

Uemura, O., Okada, Y., Ando, H., Guedj, M., Higashijima, S., Shimazaki, T., Chino, N., Okano, H. and Okamoto, H. (2005). Comparative functional genomics revealed conservation and diversification of three enhancers of the isl1 gene for motor and sensory neuron-specific expression. *Dev Biol* **278**, 587-606.

Venkatesh, B., Kirkness, E. F., Loh, Y. H., Halpern, A. L., Lee, A. P., Johnson, J., Dandona, N., Viswanathan, L. D., Tay, A., Venter, J. C. et al. (2006). Ancient noncoding elements conserved in the human genome. *Science* **314**, 1892.

Verzi, M. P., McCulley, D. J., De Val, S., Dodou, E. and Black, B. L. (2005). The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field. *Dev Biol*.

Victor, S., Nayak, V. M. and Rajasingh, R. (1999). Evolution of the ventricles. *Tex Heart Inst J* **26**, 168-75; discussion 175-6.

Vlieghe, D., Sandelin, A., De Bleser, P. J., Vleminckx, K., Wasserman, W. W., van Roy, F. and Lenhard, B. (2006). A new generation of JASPAR, the open-access repository for transcription factor binding site profiles. *Nucleic Acids Res* **34**, D95-7.

Vong, L., Bi, W., O'Connor-Halligan, K. E., Li, C., Cserjesi, P. and Schwarz, J. J. (2006). MEF2C is required for the normal allocation of cells between the ventricular and sinoatrial precursors of the primary heart field. *Dev Dyn*.

Voronov, D. A., Alford, P. W., Xu, G. and Taber, L. A. (2004). The role of mechanical forces in dextral rotation during cardiac looping in the chick embryo. *Dev Biol* **272**, 339-50.

Waldo, K. L., Kumiski, D. H., Wallis, K. T., Stadt, H. A., Hutson, M. R., Platt, D. H. and Kirby, M. L. (2001). Conotruncal myocardium arises from a secondary heart field. *Development* **128**, 3179-88.

Wang, G. F., Nikovits, W., Jr., Bao, Z. Z. and Stockdale, F. E. (2001). Irx4 forms an inhibitory complex with the vitamin D and retinoic X receptors to regulate cardiac chamber-specific slow MyHC3 expression. *J Biol Chem* **276**, 28835-41.

Watt, A. J., Battle, M. A., Li, J. and Duncan, S. A. (2004). GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *Proc Natl Acad Sci U S A* **101**, 12573-8.

Woolfe, A., Goodson, M., Goode, D. K., Snell, P., McEwen, G. K., Vavouri, T., Smith, S. F., North, P., Callaway, H., Kelly, K. et al. (2005). Highly conserved non-coding sequences are associated with vertebrate development. *PLoS Biol* **3**, e7.

Xin, M., Davis, C. A., Molkentin, J. D., Lien, C. L., Duncan, S. A., Richardson, J. A. and Olson, E. N. (2006). A threshold of GATA4 and GATA6 expression is required for cardiovascular development. *Proc Natl Acad Sci U S A* **103**, 11189-94.

Xu, H., Morishima, M., Wylie, J. N., Schwartz, R. J., Bruneau, B. G., Lindsay, E. A. and Baldini, A. (2004). Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract. *Development* **131**, 3217-27.

Yamada, M., Revelli, J. P., Eichele, G., Barron, M. and Schwartz, R. J. (2000). Expression of chick Tbx-2, Tbx-3, and Tbx-5 genes during early heart development: evidence for BMP2 induction of Tbx2. *Dev Biol* **228**, 95-105.

Yamagishi, H. and Srivastava, D. (2003). Unraveling the genetic and developmental mysteries of 22q11 deletion syndrome. *Trends Mol Med* **9**, 383-9.

Yamagishi, H., Yamagishi, C., Nakagawa, O., Harvey, R. P., Olson, E. N. and Srivastava, D. (2001). The combinatorial activities of Nkx2.5 and dHAND are essential for cardiac ventricle formation. *Developmental Biology* **239**, 190-203.

Yanagisawa, H., Clouthier, D. E., Richardson, J. A., Charite, J. and Olson, E. N. (2003). Targeted deletion of a branchial arch-specific enhancer reveals a role of dHAND in craniofacial development. *Development* **130**, 1069-78.

Yelon, D. (2001). Cardiac patterning and morphogenesis in zebrafish. *Dev Dyn* **222**, 552-63.

Yelon, D., Ticho, B., Halpern, M. E., Ruvinsky, I., Ho, R. K., Silver, L. M. and Stainier, D. Y. (2000). The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development. *Development* **127**, 2573-82.

Yutzey, K., Gannon, M. and Bader, D. (1995). Diversification of cardiomyogenic cell lineages in vitro. *Dev Biol* **170**, 531-41.

Zaffran, S. and Frasch, M. (2002). Early signals in cardiac development. *Circulation Research* **91**, 457-69.