The Role of Hand1 in the Development of the Lateral Plate Mesoderm in Xenopus

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Abstract

The transcription factor hand1 is expressed in the heart, lateral plate mesoderm (LPM) and neural crest cells during development. As Hand1-null mice die early in embryogenesis, identification of the precise role of Hand1 in development is difficult. In Xenopus, we observed that hand1 expression patterns correlates very closely with development of LPM derivatives, leading us to hypothesize that hand1 is required for normal LPM development. Using hand1 knockdown and overexpression models in Xenopus, development of LPM derivatives were assessed by whole mount in situ hybridization. I found that hand1 is required for proper heart morphogenesis. Furthermore, hand1 is required for the formation of a complex vasculature plexus within embryos, by maintaining early endothelial cell populations however Hand1 is not sufficient to induce endothelial cell differentiation. These findings confirm a conserved role of Hand1 in heart morphogenesis and suggest a new role for hand1 in development of the vasculature plexus.

Keywords: Hand1, Xenopus, lateral plate mesoderm, vascular development, heart development, CRISPR-Cas9
Co-Authorship Statement

I personally performed all of the experiments described within this thesis. Co-authorship belongs to my supervisor, Dr. Thomas Drysdale for his supervision, assistance in design and analysis of experiments and writing edits.
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List of Abbreviations

°C  Degrees Celsius
α  Aplha (Greek letter)
β  Beta (Greek letter)
µg  microgram
µL  microliter
αmi  Gene encoding adipsin
aplnr  Gene encoding apelin receptor
aVBI  anterior VBI
bHLH  basic helix-loop-helix
BMP-4  bone morphogenetic protein 4
bp  Base Pairs
Cas9  CRISPR associated protein 9
cDNA  complementary DNA
ChIP  Chromatin immunoprecipitation
CRISPR  Clustered regularly interspaced short palindromic repeats
DIG  Digoxygenin
DNA  deoxyribonucleic acid
DLP  Dorsal lateral plate
E-box  Enhancer box
EtBr  Ethidium Bromide
ETS  E-twenty-six
etv2  *Xenopus* gene encoding ETS variant 2
Etv2  Mouse gene encoding ETS variant 2
FGF  fibroblast growth factor
FGF-2  fibroblast growth factor 2
fli1  Gene encoding Fli-1 proto-oncogene
foxf1  Gene encoding forkhead box F1
Fwd  forward
Gata4  Gene encoding GATA binding protein 4
Gata6  Gene encoding GATA binding protein 6
hand1  \textit{Xenopus} gene encoding heart and neural derived factor 1
Hand1  Mouse gene encoding heart and neural derived factor 1
Hand1  Heart and neural derived factor 1 protein
HAND1  Human gene encoding heart and neural derived factor 1
HAND1  Heart and neural derived factor 1 protein (Human)
hand2  \textit{Xenopus} gene encoding heart and neural derived factor 2
Hand2  Mouse gene encoding heart and neural derived factor 2
Id  Inhibitor of DNA-binding protein
isl1  Gene encoding ISL LIM homeobox 1
IU  International units
kb  Kilobase
L  Liter
LB  Luria Broth
LPM  Lateral plate mesoderm
M  Molar
mA  Milliamps
MAB  Maleic Acid Buffer
Mash1  Achaete-scute family bHLH transcription factor 1
mL  Milliliter
mM  Millimolar
mRNA  Messenger RNA
MT  Myc-Tag
MyoD  Myogenic differentiation factor
ng  Nanogram
nKx2.5  \textit{Xenopus} gene encoding NK2 homeobox 5
Nkx2.5  Mouse gene encoding NK2 homeobox 5
Nkx2.5  NK2 homeobox 5 protein
NKX2.5  Human gene encoding NK2 homeobox 5
nL  Nanoliter
PAM  Protospacer-adjacent motif
PCR  Polymerase chain reaction
Pecam  Gene encoding platelet and endothelial cell adhesion molecule 1
pVBI  Posterior VBI
RA  Retinoic acid
Rev  Reverse
RNA  Ribonucleic acid
rpm  Revolutions per minute
RT  Room Temperature
rtTA  Reverse tetracycline-controlled transactivator
sall3  Gene encoding Spalt-like transcription factor 3
scl  Gene encoding T-cell acute lymphocytic leukemia 1
sgRNA  Single guide RNA
slc45a2  Gene encoding solute carrier family 45 member 2
SNP  Single nucleic polymorphism
sox17  Gene encoding SRY box-17
sox7  Gene encoding SRY box 7
spib  Gene encoding Spi-B transcription factor
Srf  Gene encoding serum response factor
Tβ4  thymosin beta 4
Tbx5  Gene encoding t-box 5
Tbx20  Gene encoding t-box 20
TdT  Terminal deoxynucleotidyl transferase
TE  Tris-EDTA Buffer
TIDE  Tracking of INDELS by Decomposition
tinman  Drosophila gene encoding NK2 homeobox transcription factor
tnii3  Gene encoding Cardiac troponin I
VBI  Ventral blood island
VEGF  Vascular endothelial growth factor
vwf  Gene encoding Von Willebrand factor
wnt  Wingless-type MMTV integration site family member 1
Chapter 1: Introduction

1.1 Early Embryogenesis

1.1.1 Development of the Germ Layers

Embryogenesis is defined as the formation and development of an embryo. Gastrulation is a critical event in that process and marks the first morphogenetic event following fertilization. During gastrulation, the three germ layers of the embryo— the endoderm, mesoderm and ectoderm— become clearly segregated. The formation of the three germ layers outlines the basic body plan where each germ layer will give rise to specific tissues and organs of the organism (Dale and Slack, 1987a).

Amphibians have been crucial in establishing key molecular events during germ layer induction (Kiecker et al., 2016). In particular, *Xenopus laevis* is a widely used model for studying early embryogenesis due to some key features: *in vitro* fertilization and development, which allows for direct observation and manipulation of early embryos; large number of eggs per fertilization (a female can produce around 2000 eggs/day); lack of extra-embryonic germ layers, allowing for direct gene analysis in the embryo proper and extensive knowledge of early development including established fate maps (Blitz et al., 2006; Cao, 2015). Furthermore, *Xenopus* do not require a functional cardiovascular system until late tadpole stages, making them an ideal model to study abnormal cardiovascular phenotypes, as these same studies would otherwise be lethal in mouse models (Blitz et al., 2006; Levine et al., 2003). Additionally, the external development of the embryo and the transparency of the tadpole make visualizing and imaging the heart very accessible (Blitz et al., 2006).

In *Xenopus*, the ectoderm, the outer germ layer, forms in the animal region of the pre-gastrulation embryo, the mesoderm forms from the middle region and the endoderm forms in the yolky, vegetal region (Cao, 2015). Although the morphogenetic movements of gastrulation occur differently in mammals because of the presence of extra-embryonic tissues and topological differences, the molecular factors that specify the germ layers are conserved (Kiecker et al., 2016). Nieuwkoop (1969) demonstrated that when explants from either the animal or vegetal pole of a blastula stage embryo were isolated and cultured, only ectodermal (animal) or endodermal (vegetal) tissues were derived. However, when the explants from the animal and vegetal pole were combined, mesodermal tissues were then formed from the
ectodermal explant, indicating that mesodermal tissues depend on an inductive event to form within the embryo (Dale and Slack, 1987a; Nieuwkoop, 1969). The induction of mesoderm from ectodermal tissues was further shown to require signals that emanate from the vegetal half of the embryo (Nieuwkoop, 1969; Sud, 1971).

1.1.2 Mesoderm specification

There have been many studies done to identify the signals required for mesodermal induction within the embryo. The signals that emanate from the vegetal half of the embryo to initially induce the mesoderm to form were determined to be from maternally derived mRNA, as mesoderm induction takes place very early, before new transcription occurs (Woodland and Jones, 1987). In addition to the animal-vegetal axis that helps drive endodermal and ectodermal fates, there is a dorsal-ventral axis that arises during gastrulation. It was discovered that when the most dorsal lip of the embryo was transplanted onto the ventral side of a host embryo, the transplanted tissue induced dorsal tissues to form in the surrounding ventral tissue (De Robertis, 2009; Harland and Gerhart, 1997).

Because the ability to induce and organize a second axis was discovered by Hans Spemann, leading to the 1935 Nobel Prize, the dorsal region of the mesoderm was termed the Spemann’s organizer (Harland and Gerhart, 1997). The remainder of the mesoderm is termed the ventral mesoderm (Harland and Gerhart, 1997). The organizer is formed prior to gastrulation, and acts during gastrulation to organize embryonic development, primarily by secreting signals to differentiate surrounding cells into certain tissue types (Harland and Gerhart, 1997). Although the Spemann organizer is located in the dorsal mesoderm, it is responsible for patterning over half of the embryo. Numerous signaling molecules involved in specifying the animal-vegetal axis and the Spemann organizer have been identified including activin, FGF, BMP-4, wnt and Noggin (Smith, 1995). Altering these signaling pathways and gradients during embryogenesis demonstrates the importance of these factors, as loss of their activities disrupts normal body axis and positioning (Smith, 1995).

Prior to gastrulation, fate map studies using *Xenopus* have shown that morphogenetic fields are determined quite early in development (Dale and Slack, 1987b; De Robertis, 2009). A fate map showing mesodermal fates of a pre-gastrulation embryo is shown in Figure 1.
Figure 1. Fate map of *Xenopus* embryo.

The lateral plate mesoderm (LPM) is induced to form in the middle of the embryo as seen in red. The mesoderm bordering the animal side of the embryo is fated to become the somites. The dorsal mesoderm is fated to become pharyngeal endoderm and head mesoderm, while the region ventral to that is fated to become the heart and blood islands. The remainder of the mesoderm bordering the vegetal side is fated to become to LPM.
The most dorsal lip of the mesoderm forms the notochord, head mesoderm and pharyngeal endoderm fields, the ventral mesoderm located closest to the animal side constitutes the somite field, and the remainder of the ventral mesoderm on the vegetal side constitutes the lateral plate mesoderm (LPM) field of the embryo, with the most dorsal area of that field comprising the heart field of the embryo. Although it should be noted that a fate map alone does not give information regarding the specification or commitment of a particular lineage, but rather the potential of each region to give rise to a particular organ or tissue within the embryo (Dale et al., 1985).

The Spemann organizer is also the area of the embryo where the movements of gastrulation begin. During gastrulation, the mesoderm as well as the endoderm invaginate through the blastopore and line the interior of the embryo. The ectoderm surrounds the most outer layer of the embryo, where the mesoderm forms the second layer of the embryo. The cells located at the uppermost portion of the mesoderm will give rise the somites and notochord, while the mesoderm surrounding the remainder of the embryo will form the LPM as seen in Figure 2.

1.2 Lateral Plate Mesoderm

Shortly after gastrulation, once the LPM has formed around the middle of the embryo, the LPM splits into two layers during neurulation: the splanchnic layer and the somatic layer, with the space between forming the coelom, as seen in Figure 2. The somatic layer of the LPM will eventually line the body cavity, whereas the splanchnic layer gives rise to the circulatory system, including the heart, blood and vasculature system.

1.2.1 Patterning within the LPM

Although the mechanisms surrounding initial mesoderm induction have been highly studied, the molecular basis for the regional patterning within the LPM, specifically in the ventrolateral mesoderm have not been as extensively studied (Deimling and Drysdale, 2009, 2011; Saint-Jeannet et al., 1992). However, studies have shown that the underlying endoderm requires signals from the mesoderm for proper regional specification and differentiation (Horb and Slack, 2001; Rawdon, 2001; Wells and Melton, 2000), suggesting that the LPM has an intrinsic pattern of its own.
Figure 2. Development of the LPM.

After gastrulation (stage 14), the LPM forms a layer surrounding the outer layer of the embryo. The LPM is then splits into two layers during neurulation (stage 20), the splanchnic mesoderm and the somatic mesoderm. The splanchnic mesoderm gives rise to the circulatory system while the somatic mesoderm gives rise to the body wall. The space between the layers is termed the coelom and forms the body cavity later in development.
Traditionally the LPM was considered quite uniform, outside of the two described domains of the heart field located at the anterior lateral mesoderm, and both the anterior and posterior blood islands located on the ventral side of the embryo (Deimling and Drysdale, 2009). However, our lab has previously identified four distinct domains based on expression of specific genes along the anterior-posterior axis of the LPM. The marker genes *nkx2.5, foxf1, hand1* and *sall3* are first expressed after neurulation demonstrating that patterning of the LPM occurs earlier than previously thought (Deimling and Drysdale, 2009). It is noted that these domains are not fully distinct from one another, as some overlap exists at the borders of the domains (Deimling and Drysdale, 2009, 2011).

The locations of each of the four expression domains are shown in Figure 3. The primary heart field, which is located in the ventral-anterior mesoderm (Sater and Jacobson, 1989) is marked by the expression of *nkx2.5*, a transcription factor known to be vital for the development of the heart (George et al., 2015; Jamali et al., 2001; Zhang et al., 2014). The expression of *nkx2.5* first appears broadly in the anterior-ventral region during neurulation, and later becomes restricted to the heart where it is maintained throughout development (Cleaver et al., 1996; Tonissen et al., 1994). Moving posteriorly from the *nkx2.5* domain is the *foxf1* domain, which is localized in the anterior region of the embryo. *Foxf1* expression appears first during neurulation, initially having a restricted expression domain in the ventral-anterior LPM, however throughout development the expression of *foxf1* expands posteriorly along the LPM (Deimling and Drysdale, 2009; El-Hodiri et al., 2001; Koster et al., 1999). *Hand1* is expressed posteriorly to that of *foxf1* and expression begins during neurulation, forming a saddle shape around the middle of the LPM (Deimling and Drysdale, 2009; Sparrow et al., 1998). *Hand1* expression domains become more defined and constricted throughout development, although expression in these regions remains high (Deimling and Drysdale, 2009; Sparrow et al., 1998). Finally, the most posterior domain of the LPM is marked by *sall3*, which is first expressed during neurulation (Hollemann et al., 1996). *Sall3* expression is located in the LPM posterior to that of *hand1*, however, expression is transient and rapidly declines shortly after neural tube closure (Deimling and Drysdale, 2009).
Figure 3. Distinct domains within the LPM.

An *in situ* hybridization of a stage 20 embryo probed for *hand1* expression, where it is located in the middle of the LPM. The remaining domains of the LPM are displayed. The primary heart field is marked by the expression of *nкс2.5* and is located at the anterior-ventral region of the embryo. The *foxl1* domain is located posterior of that the *nкс2.5* expression domain. Finally, the *sall3* domain is located at the posterior end of the embryo.
Using these four distinct domains as described, our lab was able to demonstrate that anterior-posterior patterning within the LPM is regulated by fibroblast growth factor (FGF) and retinoic acid (RA), because there are shifts in the four domains when either FGF or RA signaling pathways were disrupted (Deimling and Drysdale, 2009, 2011). Most importantly, when the domains were transiently manipulated using embryonic treatments during neurulation, the initial changes in the four expression domains persisted after the treatment was removed, and were associated with later developmental defects, such as disrupted heart morphogenesis when nkgx2.5 domain was lost, and a corresponding shift in the location of the developing vascular plexus within the ventral region of the embryo (Deimling and Drysdale, 2009, 2011). Thus, the patterning within the LPM occurs early in development, and is important for normal patterning and development of the LPM derivatives. As the focus of this thesis is on the development of the LPM, it is imperative to discuss the derivatives of the LPM, as these will be used to assess the regional patterning of the LPM.

1.2.2 LPM derivatives

As previously described (Fig. 2) the LPM will split into both the splanchnic layer and somatic layer during neurulation, each giving rise to a different subset of tissues. However, the focus of this thesis is specifically on the development of the splanchnic mesoderm, which forms the circulatory system. The circulatory system is comprised of the heart, vasculature (blood vessels) and hematopoietic lineages (blood and myeloid cells), each of which will be discussed in further detail.

1.2.2.1 Early cardiac development

Cardiac progenitors are first specified during gastrulation (Fig. 4) and these precursor cells are located as two bilaterally patches at the most dorsal region of the LPM (Sater and Jacobson, 1989), which has been determined through fate mapping studies and expression of early cardiac transcription factors (Gessert and Kühl, 2009). Although the progenitors of the heart are first specified in this region, during gastrulation the heart fields migrate as two bilateral regions and relocate in the anterior region of the LPM termed the cardiac crescent. This region is where the heart is going to form the functional organ system (Brand, 2003; Mohun et al., 2003). The heart tube then begins to form from primary heart field progenitor cells within the cardiac crescent (Hempel and Kühl, 2016; Waardenberg et al., 2014).
Figure 4. Development of the heart in *Xenopus*.

Prior to gastrulation, the cardiogenic fields are specified and located within two bilateral patches of the embryo, at stage 10. At stage 20, the cardiac cells are specified within the cardiac crescent. Both primary and secondary heart fields are intermingled together and located within the same region. By stage 24-28 the primary and secondary heart field have separated. The heart tube is thus induced to form in this region and by stage 35 the tube has looped to the right. The secondary heart field contributes to the heart during looping. Finally, by stage 46 the formation of the heart is complete, with chamber formations and atrium and ventricles separated. This image was arranged from (Hempel and Kühl, 2016) following Creative Commons Attribution (CC-BY) license (http://creativecommons.org/licenses/by/4.0/).
Once the primary heart tube is formed, the tube undergoes rightwards looping, which is accompanied by the elongation of the heart tube through addition of cells from the secondary heart field (Dyer and Kirby, 2009; McFadden and Olson, 2002; Mohun et al., 2000). The looping of the heart is a critical step during heart morphogenesis, as heart looping determines the position and location of the future cardiac chambers (Blitz et al., 2006; McFadden and Olson, 2002; Mohun et al., 2000).

Although there exist differences in heart morphology between Xenopus and mammals, as the Xenopus heart only has three chambers (2 atria, 1 ventricle), as opposed to the four chambered mammalian heart, the early events leading to heart development and molecular pathways are conserved (Mohun et al., 2000; Warkman and Krieg, 2007).

Nkx2.5 represents one of the first transcription factors that is activated during the specification of the heart field (Brand, 2003; Lints et al., 1993). Nkx2.5 is the murine homolog of tinman, which was first discovered in Drosophila, where tinman mutants failed to develop a heart, as well as the precursors to cardiomyocytes (Bodmer, 1993; Bodmer et al., 1990). Given the importance of tinman in fly heart development, the role of nkx2.5 in heart development in other species has been studied. Nkx2.5 in mouse and nkx2.5 in Xenopus were found to have the same early cardiac expression pattern as tinman, marking the earliest cardiogenic precursors (Lints et al., 1993; Newman and Krieg, 1998; Sparrow et al., 2000; Tonissen et al., 1994).

Unlike in Drosophila, knockout models of nkx2.5 in other species are still able to differentiate cardiomyocytes, due to presumed redundancy of other genes involved in cardiac specification, such as Gata4/6, Srf, Tbx5 and Tbx20, although the loss of individual genes still has severe consequences on heart morphogenesis (Brand, 2003; Haworth et al., 2008; Peterkin et al., 2005; Tandon et al., 2012; Waardenberg et al., 2014). Knockouts of Nkx2.5 in mice are lethal between E9.5-11.5 with defects in heart morphogenesis, failing to form a separation between atria and ventricles (Lyons et al., 1995; Yamagishi et al., 2001). In dominant negative nkx2.5 models in Xenopus, mutant phenotypes exhibited loss of morphologically recognizable cardiac tissue, and decreased cardiac marker expression on the injected side of the embryo (Fu et al., 1998; Grow and Krieg, 1998). Interestingly, over expression of nkx2.5 in Xenopus led to an increase in number of differentiating myocardial cells, and thus an increase in overall heart size, but function was not compromised (Cleaver et al., 1996). Lack of ectopic myocardial cells in Xenopus over expressing nkx2.5 implies that nkx2.5 is not a master regulator of
cardiomyocyte differentiation (Cleaver et al., 1996; Grow and Krieg, 1998). Importantly, heterozygous loss of function NNX2.5 mutations in people results in congenital heart defects (Kasahara et al., 2000).

In addition to the role nkv2.5 has during heart morphogenesis, nkv2.5 has recently been shown to also be involved in specification of the secondary heart field (George et al., 2015; Guner-Ataman et al., 2013; Zhang et al., 2014). The secondary heart field represents a group of cells that remain proliferative until after the differentiation of the primary heart field, and then undergo differentiation later on to contribute to elongation of the heart tube during looping morphogenesis (Dyer and Kirby, 2009; Waardenberg et al., 2014). Loss of the secondary heart field reduces the population of cardiac cells that contribute to the heart, and thus causes defects in heart morphogenesis (Dyer and Kirby, 2009). Isl1 has been shown to mark the cells representing the secondary heart field, and loss of isll in Xenopus led to phenotypes ranging from looping failure and pericardial edema, to a small beating nub where the heart should be and thus is required for normal heart development (Brade et al., 2007; Ma et al., 2008). In Xenopus, the expression patterns of isll and nkv2.5 are similar within the cardiac crescent, but represent two distinct populations of progenitors cells within the embryo that are vital for heart development (Brade et al., 2007; Dyer and Kirby, 2009; Ma et al., 2008).

The first terminal differentiation event of cardiomyocytes can be identified by the expression of cardiac troponin I (tnni3) in Xenopus (Drysdale et al., 1994). When expression of tnni3 first appears, it is located as two bilateral regions within the heart field at stage 28, and is maintained throughout development of the heart (Drysdale et al., 1994).

Although the complete molecular pathways involved in looping morphogenesis have not been eluded, knockouts of either nkv2.5, tbx5, hand1, hand2 and BMP-4 in mice result in looping defects suggesting they play a role in looping morphogenesis (Brand, 2003). Defects in looping is one of the major causes of congenital heart defects, and thus the molecular events underlying this process are of high importance (Blitz et al., 2006; Mohun et al., 2000).

1.2.2.2 Early vasculature development

Development of the vascular system begins with the differentiation of mesodermal progenitor cells to an endothelial lineage. After initial differentiation, these cells will coalesce to form a
primary vascular plexus, which then undergoes remodeling later in development to form the final functional vasculature system within the embryo (Marcelo et al., 2013). Vasculature development can be separated into two distinct phases: vasculogenesis and angiogenesis (Lawson and Weinstein, 2002; Mills et al., 1999; Wilting et al., 1995). Vasculogenesis is defined as the \textit{de novo} formation of endothelial cells from precursor cells (Wilting et al., 1995). Angiogenesis occurs when endothelial cells arise from pre-existing endothelial cells, and is typically accomplished through sprouting of vessels from a previously formed vessel (Wilting et al., 1995).

The identity of the precursor cell that will give rise to the vasculature is still unclear, although the hypothesized cell is typically referred to as a hemangioblast, holding potential to differentiate into either a endothelial cell or blood cell lineage (Iraha et al., 2002; Mills et al., 1999). This support for the idea of a hemangioblast precursors lies with the fact that blood and endothelial cells differentiate in such close proximity to each other early in embryo development (Iraha et al., 2002; Marcelo et al., 2013; Mills et al., 1999).

Many of the key signaling molecules required for differentiation toward an endothelial fate have been eluded, including FGF-2 and BMP-4 (Iraha et al., 2002; Marcelo et al., 2013).

The most studied signaling factor during vascular development is vascular endothelial growth factor (VEGF) which is required for the growth and migration of endothelial cells, however it is noted that VEGF is not sufficient to induce differentiation of precursor cells to an endothelial lineage (Liang et al., 2001). VEGF is upregulated in hypoxic areas of developing embryos, and serves as the signaling factor to promote vascular growth into those areas of the embryo (Wilting et al., 1995).

Additionally, the transcription factor \textit{etv2} is required for differentiation of precursors cells to an endothelial fate (Lammerts van Bueren and Black, 2012; Neuhaus et al., 2010; Salanga et al., 2010). The requirement of \textit{etv2} for vasculature formation has been demonstrated by several loss of function studies. Morpholino knockdown of \textit{etv2} in zebrafish and \textit{Xenopus} result in a complete loss of vasculature formation (Neuhaus et al., 2010; Sumanas and Lin, 2006). Similarly, knockout of \textit{Etv2} in mice result in early lethality of embryos exhibiting no evidence of blood vessel or blood formation (Lee et al., 2008). Overexpression of \textit{etv2} in \textit{Xenopus}, zebrafish and stem cells results in ectopic expression of endothelial markers, thus \textit{etv2} is not
only required but also sufficient for endothelial cell differentiation (Kataoka et al., 2011; Marcelo et al., 2013; Neuhaus et al., 2010; Salanga et al., 2010).

In *Xenopus*, *etv2* expression begins during neurulation, marking precursor endothelial cells (Neuhaus et al., 2010). High levels of *etv2* expression are found in developing vascular structures and expression is rapidly lost once endothelial cells begin to mature (Marcelo et al., 2013; Neuhaus et al., 2010).

Similarly, *aplnr*, a G-protein coupled receptor is also required for vascular development as demonstrated by loss of function models in *Xenopus* (Cox et al., 2006; Inui et al., 2006). Similarly, loss of *aplnr* in mice is lethal due to both vasculature and cardiac defects (Kang et al., 2013). In *Xenopus*, the expression of *aplnr* begins after gastrulation and is localized to the endothelium layer within endothelial cells, and is similarly downregulated later in development (Devic et al., 1996).

Endothelial cells arise from two distinct locations within the *Xenopus* embryo (Fig. 5A). One population arises at the ventral side of the embryo in close proximity to the ventral blood islands, which will form the vitelline vasculature (Cleaver et al., 1997). The second population of endothelial cells arise in dorsal region of the embryo where the cardinal veins will develop (Cleaver et al., 1997).

As development continues, the cardinal veins mature and extend to the tail of the embryo, with intersomitic vessels beginning to sprout from the cardinal vein. The intersomitic veins are considered one of the first forms of angiogenesis within the embryo. Sprouting of the intersomitic vessels occurs in a very regulated and controlled manner, such that small perturbations in angiogenesis can be observed in these vessels (Helbling et al., 2000; Levine et al., 2003). Meanwhile, the vitelline vessels have coalesced and attach to the cardinal veins and aortic arches to form a functional vasculature system (Cleaver et al., 1997; Levine et al., 2003). The locations of these vessels within the embryo are shown in Figure 5B. Blood circulation begins through the vasculature system at stage 33 as the heart begins to beat (Levine et al., 2003). The expression of *ami*, a serine protease, is found to correspond to the developing vasculature, and is used as a marker for later vasculature development in *Xenopus* (Inui and Asashima, 2006).
Figure 5. Vasculature formation in *Xenopus* embryos.

An embryo probed with *etv2* at stage 25 showcasing the two distinct regions where endothelial cells arise (A). The cells arising from the ventral region of the embryo will form the vitelline veins while the cells arising from the dorsal region of the embryo will form the posterior cardinal vein. Additionally, embryos were probed with the marker *ami*, to showcase the vasculature development at stage 37 (B). The vitelline veins form within the broad region of the embryo, with the precursors arising from the ventral region of the embryo and link up to the cardinal vein. The cardinal vein runs along the dorsal length of the embryo and arises from precursors in the dorsal region of the embryo. The intersomitic vessels arise from sprouting angiogenesis coming from the cardinal vein. Embryos are oriented with anterior side to the left, posterior side to the right.
1.2.2.3 Early hematopoietic development

There are two distinct blood islands within the *Xenopus* embryo, the posterior ventral blood island (pVBI) and the anterior ventral blood island (aVBI). They both originate from different regions of the gastrula, with the pVBI arising from the ventral gastrula mesoderm and the aVBI arising from the dorsal gastrula mesoderm. After the movements of gastrulation, they are juxtaposed beside each other, with the aVBI located next to the developing heart, and the pVBI posteriorly to that of the aVBI as seen in Figure 6 (Costa et al., 2008; Mace et al., 2012). Each blood island gives rise to different cell types, with the aVBI giving rise to the myeloid cells of the embryo, and the pVBI giving rise to the erythrocytes (red blood cells) of the embryo (Costa et al., 2008; Mace et al., 2012). Another distinction between the two blood islands is the timing of differentiation, with the aVBI differentiating into myeloid cells during neurulation (stage 18), while erythrocytes do not differentiate until later in development, around stage 28 (Costa et al., 2008; Mace et al., 2012). Thus, the signals involved in differentiation of each lineage appear distinct from one another.

Myeloid cells first appear in *Xenopus* during neurulation, and expression is initially restricted to a small population of cells located posterior to the heart on the ventral side of the embryo (Smith et al., 2002). These cells then rapidly proliferate and migrate throughout the embryo during early tailbud stages (Smith et al., 2002). These myeloid cells are capable of responding to wounds and infections in the embryo, thus the myeloid cells are both differentiated and functional a day prior to the differentiation of erythrocytes and formation of a functional vasculature system (Costa et al., 2008; Smith et al., 2002). Furthermore it has been shown that myeloid cells play a role in normal embryogenesis, through the clearance of apoptotic cells, and are also essential for normal heart morphogenesis in *Xenopus* (Agricola et al., 2016; Smith and Mohun, 2011).

The first gene that is expressed in myeloid precursors cells is spib, an ETS (E-twenty-six) transcription factor which is required for both specification and differentiation of myeloid cells in *Xenopus* (Agricola et al., 2016; Costa et al., 2008). Loss of spib in *Xenopus* results in a loss of other early and late myeloid cell markers, however this does not lead to an increase in erythrocyte specification in these embryos, thus the separate blood islands are not able to compensate for one another (Costa et al., 2008).
Figure 6. Locations of the blood islands in *Xenopus*.

A ventral view of a stage 21 embryo is shown, depicting the locations of the aVBI and pVBI within the embryo after gastrulation. The aVBI is located near the developing heart of the embryo, and will give rise to the myeloid cells, while the pVBI will give rise to erythrocytes.
The requirement of spib for myeloid cell development is conserved. In mice, when the DNA binding domain of the spib gene is removed, the mice lack the myeloid cell lineages of macrophages, neutrophils, and B and T lymphocytes. However the precursors cells for these lineages are not abolished (Mckercher et al., 1996; Scott et al., 1994).

Blood progenitors arise from the pVBI, and contribute to the embryonic blood (Ciau-Uitz et al., 2000; Walmsley, 2002). Additionally, adult blood arises from a separate region of the embryo, the dorsal lateral plate (DLP) (Ciau-Uitz et al., 2000; Walmsley, 2002). The blood cells that arise in the pVBI differentiate prior to the blood cells that arise from the DLP, and the pVBI derived blood cells have been shown in Xenopus to be the only blood cells present in circulation throughout early development of the embryo (Kau and Turpen, 1983). Lineage tracing of the two blood populations demonstrated that each blood compartment arises from different regions of the embryo during development, suggesting that each population relies on different signals to induce their differentiation (Ciau-Uitz et al., 2000). For example, BMP (bone morphogenetic protein) signaling is required for the specification and differentiation of embryonic blood lineages, but is dispensable for the differentiation of adult blood lineages (Ciau-Uitz et al., 2000; Walmsley, 2002). Loss of BMP-4 signaling in Xenopus lead to a decrease in expression of globin, which is a marker of differentiated blood cells, while ectopic BMP-4 signaling in dorsal explants of embryos lead to an induction of globin expression in the tissue (Iraha et al., 2002; Kumano et al., 1999).

BMP signaling has also been shown to induce scl (stem cell leukemia) expression (Mead, 1998). Scl is the first known marker of the blood lineages and expression of scl appears in the ventral side of Xenopus embryos just after gastrulation (Iraha et al., 2002; Mead, 1998; Nastos et al., 2002). Loss of scl in mice and Xenopus results in disruption of normal hematopoiesis, and is thus required in committing cell lineages to a blood cell fate (Mead, 1998; Nastos et al., 2002).

Differentiated blood cells are located in the most ventral region of the embryo forming a V-shape and first appear in late tailbud stages (Zon, 1995). Shortly after the heart begins beating, the anterior region of the blood islands begin to enter the heart through the forming primitive vasculature, thereby establishing circulation throughout the embryo (Zon, 1995).
1.3 Expression pattern of hand1 throughout Xenopus development

Although hand1 is not a pan lateral plate marker, it occupies majority of the LPM (as seen in Figure 3) and has been correlated with vasculature formation, thus the focus of this thesis is on the role of hand1 in LPM development. The expression of hand1 first appears in Xenopus at stage 14, once gastrulation is completed, with no maternal transcripts of hand1 identifiable in unfertilized eggs (Sparrow et al., 1998). The expression of hand1 is localized to the middle of the LPM, forming a saddle shape around the embryo (Deimling and Drysdale, 2009). Expression of hand1 increases throughout development, reaching peak levels at early tailbud stages (Sparrow et al., 1998). As development continues, hand1 expression is maintained in the broad region of the embryo, however expression does not extend to the very posterior end. The expression of hand1 also appears in the heart, beginning at stage 28, and is maintained during heart development, however expression of hand1 decreases to barely detectable levels once the embryos reach stage 42 and is undetectable in adult tissues (Sparrow et al., 1998).

1.4 Hand1

1.4.1 Hand1 structure and function

Hand1 (Heart And Neural Derived factor 1) is a Class B bHLH transcription factor, consisting of both a DNA binding domain, which is composed of a number of basic amino acids, and a second dimerization domain, which consists of an amphipathic α-helix, followed by a loop that varies in length and then another α-helix (Firulli et al., 2003). Hand1 was first discovered using a yeast two-hybrid system, screening for novel bHLH transcription factors that dimerize with the Class A E12 bHLH in mouse (Cserjesi et al., 1995). Originally named eHAND, hand1 encodes a protein that is 216 amino acids in length, and the genetic code of hand1 has been highly conserved throughout evolution (Cserjesi et al., 1995; Firulli, 2003). Hand1 mediates its actions by binding to DNA through an E-box sequence as either a homo- or heterodimer to regulate gene expression (Firulli, 2003; Firulli et al., 2003). The dimerization that occurs between the two bHLH proteins confers a unique combined DNA binding domain that recognizes a specific E-box sequence (Firulli, 2003).

Unlike most Class B bHLH that typically only form dimers with Class A proteins, Hand1 has been shown to dimerize with a multitude of different Class A and B bHLH factors (Firulli et
al., 2000). Using a mammalian two-hybrid system, it was shown that Hand1 is able to form homodimers and heterodimers with Hand2, Mash1, MyoD and the Hairy-related transcription factors (Firulli et al., 2000). Furthermore, Hand1 is able to dimerize with the class A bHLH proteins, E12 and E47 (Firulli et al., 2000).

Hand1 has been shown to act as both a transcriptional activator and repressor (Hill and Riley, 2004). Furthermore, studies have shown that Hand1 can negatively affect the function of other bHLH transcription factors through dimerization with them, similar to what is seen with Id (inhibitor of DNA) HLH proteins (Firulli et al., 2000; Scott et al., 2000). Hand1 has been shown to inhibit MyoD function by forming stable dimers with MyoD and E12 and thereby disrupting MyoD DNA binding (Firulli et al., 2000). Additionally, Hand1 can inhibit Mash1 function by decreasing the pool of binding partners (Scott et al., 2000).

Post-translational modifications have also been shown to affect the dimerization properties of Hand1 by altering the affinity of Hand1 to dimerization partners (Firulli et al., 2003). When phosphorylation of Hand1 in mice is disrupted, this led to defects in limb morphogenesis, however, complete loss of Hand1 has no effect on limb morphogenesis, suggesting that the control of Hand1 binding partners through phosphorylation events is critical for its function (Firulli et al., 2017). The unique ability of Hand1 to bind to a variety of partners, and regulation of the binding partners through post-translational modifications reveals the complex mechanisms in which Hand1 regulates transcription and allows for the various different functions of Hand1 within different tissues (Conway et al., 2010; Firulli et al., 2000).

Although the function of Hand1 has been highly studied, there are still limited insights into the direct transcriptional targets of Hand1. Thymosin β4 (Tβ4), an actin monomer binding protein, has been confirmed as a direct target of Hand1 (Smart et al., 2010). Differential gene expression comparisons have identified genes mis-regulated in Hand1 null as compared to Hand1 wildtype embryoid bodies, such as fibronectin, cardiac actin, cyclin D2, Wnt2 and cystatin C, however it is not known if these are direct targets (Smart et al., 2002). Recently, chromatin immunoprecipitation (ChIP) sequencing of Hand1 in primed multipotent cardiovascular mesoderm cells identified potential targets of Hand1, such as Pecam, Vwf, Sox17, Sox7 and Fli1, although it is unclear if Hand1 functions to activate or repress them (Org et al., 2015).
1.4.2  *Hand1* expression

*Hand1* has been highly conserved throughout evolution, and although the expression pattern of *hand1* between species is generally consistent there are some interspecies variations in tissue specific expression patterns (Firulli, 2003). The expression of *hand1* is highly embryonic in all species, and is found in low to undetectable levels in adult tissues, except in disease phenotypes (Cserjesi et al., 1995; Firulli, 2003). During development, *hand1* is highly expressed in the extra-embryonic tissues, lateral mesoderm, the heart and in neural crest derivatives (Cserjesi et al., 1995). In mice, although initially expressed throughout the developing heart, *Hand1* becomes restricted to the future left ventricle, however this asymmetrical expression pattern is not seen in either chick or *Xenopus* embryos (Angelo et al., 2000; Cserjesi et al., 1995; Srivastava et al., 1995).

1.4.3  *Hand* family

The most closely related transcription factor to *hand1* is *hand2*. Both are closely related to the *twist* family of transcription factors (Conway et al., 2010). Both *hand* factors are highly expressed during embryo development and are found at lower levels in adult tissues (Conway et al., 2010). The bHLH domain of *hand2* is 90% conserved compared to the bHLH of *hand1* suggesting that they have a similar function, although the remaining identity of *hand2* is less conserved (Conway et al., 2010; Srivastava et al., 1995).

Support for functional redundancy between the two *hand* factors comes from chick experiments, where loss of either *hand1* or *hand2* using antisense oligonucleotides were viable, but loss of both hand factors resulted in lethality due to cardiac defects (Liang et al., 2001). Interestingly, in zebrafish, there is only a single *hand* gene, which more closely resembles the sequence and expression of *hand2*, suggesting that *hand2* may be the ancestral member of the subclass of *hand* proteins (Angelo et al., 2000; Yelon et al., 2000). The differing expression patterns of *hand1* and *hand2* within the embryo suggests that they have a similar function within tissues, with *hand2* expressed in the decidium, heart, autonomic nervous system and neural crest derivatives (Srivastava et al., 1995). Furthermore, in mice, while both *Hand* factors initially overlap in expression within the heart, upon the beginning of morphogenesis, the expression pattern of each factor shifts; *Hand1* expression becomes restricted to the future left ventricle and *Hand2* expression becomes restricted to the future right ventricle (Firulli et al.,...
This differing expression pattern forms a gradient of Hand factors throughout the developing heart, where the Hand factors can form heterodimers with each other in the tissues where they are co-expressed (Firulli et al., 2000).

Although initially thought to have redundant functions, knock-in of Hand2 to the Hand1 locus in mice was lethal, providing evidence that both Hand factors have a unique and non-redundant roles in development (Firulli et al., 2010). Furthermore, Hand2 null mice are lethal beyond E10.5 due to defects in heart morphogenesis and disorganized vasculature formation (Srivastava et al., 1997; Yamagishi et al., 2000). Thus, both hand genes play important roles in development and are unable to fully compensate for one another.

1.4.4 Hand1 loss of function experiments

The first loss of function experiments of hand1 were done in chick embryos using antisense oligonucleotides targeting hand1 mRNA (Srivastava et al., 1995). The loss of hand1 did not have any effect on cardiovascular development. However, when antisense oligonucleotides were used targeting both the hand1 and hand2 gene in chick, heart development arrested at the looping stage (Srivastava et al., 1995). The embryos died due to hemodynamic insufficiency resulting from the failure of the heart valves to form accompanied by heart distention and pericardial edema (Srivastava et al., 1995). In contrast, knockout of Hand1 in mice led to early lethality of the embryo at E8.5, a timepoint before the heart is required for viability of the embryo, suggesting the lethality was due to defects in extra-embryonic tissues (Firulli et al., 1998; Riley et al., 1998). A tetraploid-aggregation model was then used to rescue the extra-embryonic defects, however embryos were still not viable beyond E10.5, exhibiting defects in heart looping and pericardial edema (Riley et al., 1998). The heart phenotype of the tetraploid-aggregation model of Hand1-null mice was very similar to the phenotype seen in Nkx2.5-null mice, suggesting that Nkx2.5 might function by inducing Hand1, as Hand1 expression is lost in Nkx2.5-null mice (Biben and Harvey, 1997; Riley et al., 1998; Thomas et al., 1998).

Other conditional knockout models of Hand1 have been employed to determine the cause of early lethality in Hand1-null mice. Hand1 has been shown to be important in the differentiation of the trophoblast cells, suggesting the early lethality of embryos may be due to a defect in this lineage, however rescue experiments did not rescue the extra-embryonic or embryonic defects observed (Cross et al., 1995; Morikawa and Cserjesi, 2004; Scott et al., 2000). Using a gene
replacement strategy, \textit{Hand1} was deleted in the yolk sac to examine the role of \textit{Hand1} in the vasculature development of the extra-embryonic mesoderm (Morikawa and Cserjesi, 2004). Loss of \textit{Hand1} in the yolk sac lead to vascular defects as well as abnormal folding of the endoderm (Morikawa and Cserjesi, 2004). In order to determine the precise cause of early lethality of \textit{Hand1}-null mice, Maska et al. (2010) created a conditional \textit{Hand1}-null mouse, deleting \textit{Hand1} in the lateral mesoderm and the extraembryonic mesoderm except the trophectoderm. Deletion in both of these lineages still led to early lethality, therefore another conditional \textit{Hand1}-null mouse was created to only delete \textit{Hand1} in the lateral mesoderm (Maska et al., 2010). Of the conditional lateral mesoderm \textit{Hand1}-null mice, only 2 of 8 survived until birth; the phenotypes of surviving embryos ranged in severity, with the most severe phenotype exhibiting defects in ventral closure leading to gut hernias, showing a disorganization and thinning of the endothelial smooth muscle layer (Maska et al., 2010). Although this model confirms that the early lethality of \textit{Hand1}-null mice is due to defects in the extra-embryonic mesoderm, defects were still observed in conditional lateral mesoderm \textit{Hand1}-null mice suggesting \textit{Hand1} plays an important role within the lateral mesoderm during development as well (Maska et al., 2010).

To further understand the role \textit{Hand1} plays during heart development, Riley et al. (2000) created a chimeric knockout of \textit{Hand1} in mice and found that when less than 50\% of cells lost \textit{Hand1} there was no observable phenotype of the \textit{Hand1} mutants. However, when 60\% of cells were \textit{Hand1}-null, the hearts of these chimera embryos resembled \textit{Hand1} mutant mice, demonstrating that the remaining wild type cells were not sufficient to rescue the defects (Riley et al., 2000). Additionally, a heart-specific conditional \textit{Hand1} knockout, where cre-recombinase was driven by an \textit{Nkx2.5} enhancer, was created to specifically delete \textit{Hand1} expression in the heart cells (McFadden et al., 2005). These cardiac specific conditional \textit{Hand1}-null mice survived until birth, but only survived for two days postnatally (McFadden et al., 2005). Hearts of these mice were observed to have congenital heart defects, including septal defects, a double outlet right ventricle and hyperplastic atrioventricular valves (McFadden et al., 2005). The limitations of the timing and mosaicism of Cre-driver \textit{Nkx2.5} used have been implied for the difference in phenotype severity compared to the tetraploid rescued \textit{Hand1}-null mice (McFadden et al., 2005), thus the precise role of \textit{Hand1} in heart development remains incompletely understood.
The congenital heart defects observed in conditional Hand1-null mice suggests that defective HAND1 could play a role in development of congenital heart defects in humans (McFadden et al., 2005). A study looked at genetic mutations in patients with isolated congenital heart defects and found a patient with a novel mutation in the HAND1 gene, a heterozygous SNP in the bHLH domain (Wang et al., 2017). The SNP resulted in an amino acid substitution at position 118 from an arginine to a cysteine, altering an evolutionary conserved amino acid (Wang et al., 2017). This SNP resulted in a decrease the transcriptional activity of HAND1 and was correlated with Tetralogy of Fallot in the patient (Wang et al., 2017). Septum deviations in human hearts have also been correlated to a decrease or loss of function of HAND1 in patients, caused by a frameshift mutation in the bHLH domain (Reamon-Buettner et al., 2009). Interestingly, two other novel amino acid substitutions of Hand1 were found in patients with septal defects, however these substitutions result in an increased ability of HAND1 to form homodimers (Cheng et al., 2012). Patients with hypoplastic hearts have also been found to have frame shift mutations in the HAND1 bHLH domain which result in a truncated protein and ultimately a decrease in the mutant protein levels (Reamon-Buettner et al., 2008). The wide range of congenital heart defects resulting from different HAND1 mutations suggest HAND1 has a complex role during development of the heart but is nevertheless crucial for its proper development.

1.4.5 Hand1 gain of function experiments

Due to the lethality of Hand1-null mice, a Hand1 over expression mouse model was created as to bypass the lethality to study Hand1 function in heart development (Risebro et al., 2006). Hand1 was over expressed using the Hand1 promoter, in order to limit over expression to tissues that typically express Hand1 (Risebro et al., 2006). Hearts of Hand1 over expressing embryos had disrupted heart morphogenesis, with abnormal looping and elongation of the outflow tract as well as defects in left ventricle development (Risebro et al., 2006). To further examine the role of Hand1, embryonic stem cells were induced to over express Hand1 which resulted in an increase in proliferation of precursor cells and a lack of differentiation. In contrast, embryonic stem cells lacking Hand1 had an increase in differentiation towards cardiomyocytes (Risebro et al., 2006). Both the in vivo and in vitro results suggest that the role of Hand1 in the developing heart is to regulate proliferation and differentiation of precursor
cells to ensure proper balance of myocardial precursors required for normal development (Risebro et al., 2006).

Hand1 appears to have a similar regulatory function in trophoblast cells. Over expression of Hand1 in trophoblast cells led to an increase in giant cell differentiation, whereas a loss of Hand1 in trophoblast cells resulted in a failure of giant cell differentiation and an increase in proliferation of precursor cells (Scott et al., 2000). Hand1 appears to have a contradicting role in different tissues, as over expression of Hand1 led to an increase in proliferation of cardiac precursors, but an increase in differentiation of trophoblast cells to giant cells. However, the ability of Hand1 to regulate a balance between proliferation and differentiation in these different lineages is consistent.

Interestingly, Hand1 expression in very low in adult human hearts, however, expression is found to increase in patients with cardiac hypertrophy (Breckenridge et al., 2009; Wang et al., 2017). This increase in Hand1 expression in diseased hearts raises the question whether Hand1 is a biproduct of this hypertrophy or a cause of it. Breckenridge et al. (2009) created a doxycycline-inducible Hand1 cardiac over expression mouse model by generating a transgenic mouse where Hand1 expression is regulated by a rtTA-responsive promoter. Doxycycline was then used induce Hand1 over expression in hearts of adult mice. This over expression caused an increase in cardiac hypertrophy compared to controls and was accompanied by a significant increase in mortality of these mice due to a predisposition to ventricular arrhythmias (Breckenridge et al., 2009). Furthermore, when the doxycycline treatment was stopped, it led to a reversal of cardiac hypertrophy in these mice (Breckenridge et al., 2009). Thus regulation of Hand1 is important in terms of both developmental of the heart and in cardiac disease phenotypes (Breckenridge et al., 2009; Wang et al., 2017).

1.5 Rationale

Hand1 expression begins shortly after gastrulation, and marks the anterior and middle of the LPM of Xenopus, however, hand1 is not expressed in the most posterior tail region of the embryo, an area which is an area devoid of a vascular plexus (Deimling and Drysdale, 2009). Our lab has previously noted a correlation between the size of the developing vascular plexus and the hand1 expression domain in Xenopus. Embryonic treatments used to increase or decrease the size of the vascularized region of the embryo resulted in a corresponding increase
or decrease in the size of the hand1 expression domain, suggesting that hand1 has a role in defining the tissue (Deimling and Drysdale, 2011). Furthermore, loss of hand1 expression in previously expressed areas of the embryo occurs in a very controlled manner. The most robust example of this occurs around stage 32 in Xenopus, when we observe loss of hand1 expression along the ventral side of the embryo, in the same location where the ventral blood islands begin to form, suggesting that hand1 is playing a role in defining the tissues within the LPM. Given the importance of hand1 during development and the strong correlation between hand1 and the derivatives of the LPM, we aim to determine the function of hand1 within the LPM of Xenopus.

1.6 Hypothesis and Objectives

We hypothesize that hand1 is required for normal development of the LPM derivatives in Xenopus.

Objectives:

1. Create and characterize the phenotype of a hand1 mutant

2. Create and characterize the phenotype of hand1 over expression model
Chapter 2: Materials and Methods

2.1 *Xenopus laevis* embryo harvesting

Female *Xenopus laevis* were injected with 600-700 IU of human chorionic gonadotrophin (Intervet Canada Corporation), depending on the size of the female, to induce ovulation. Eggs were collected the following morning and fertilized *in vitro* in 80% Steinburg’s Solution with minced testis harvested from male *Xenopus laevis*. Embryos were flooded with 20% Steinburg’s solution following fertilization, de-jellied in 2.5% cysteine solution pH 8, and cultured in 20% Steinburg’s solution.

Working concentrations of Steinburg’s solution were made by mixing 2000% Steinburg’s Stock A (1.16M NaCl, 13mM KCl, 16mM MgSO₄•7H₂O, 7mM Ca(NO₃)₂) and 2000% Steinburg’s Stock B (92mM Tris-HCl pH 7.4) in a 1:1 ratio. Stock solutions were diluted with dH₂O into 200% Steinburg’s for storing male testis, 80% Steinburg’s for fertilization, and 20% Steinburg’s for embryo culture.

Embryonic stages were determined using the standard *Xenopus* staging table (Nieuwkoop and Faber, 1994). For fixation, embryos were fixed in MEMPFA solution (4% paraformaldehyde, 1mM MgSO₄, 2mM EDTA (ethyleneglycol-bis-(β-aminoethyl ether) N’,N’,N’,N’-tetra-acetic acid) pH 8.0, 0.1 M MOPS pH 7.4), rocking either at room temperature (RT) for two hours, or at 4°C overnight and stored in 100% methanol at -20°C.

2.2 Single guide RNA generation and synthesis

Single guide RNAs (sgRNA) targeting the *hand1* gene were generated. Cas9 target sites were identified in the *hand1* gene from the Xenbase *Xenopus laevis* genome database v9.1, identifying 18-20 base pair sequences upstream of a protospacer-adjacent motif (PAM) site (3’-NGG-5’) by input into CrisprScan Software (http://www.crisprscan.org/). Due to *Xenopus laevis* being tetraploid, sites conserved between both copies of *hand1* (Chromosome 1L and 1S) were considered, with preference for sequences prior to the bHLH domain. Potential off-target sites were evaluated by probing the *Xenopus laevis* genome for similar sequences using the website GGGGenome, and sequences with one or more base pair mismatches to off-target sites in the genome were not considered. Four target sites were obtained, one targeting both
chromosomes (sgT1), one targeting specifically the long chromosome (sgT2) and two targeting specifically the short chromosome (sgT3 and SgT4; Fig. 7).

To obtain the functional sgRNA for injection, a two-step PCR and \textit{in vitro} transcription reaction was performed. The PCR reaction was carried out following the PCR conditions listed in Table 1. Sequences for each unique forward target oligonucleotide and the universal reverse primer are listed in Table 2. Each forward primer contained a T7 RNA polymerase binding site, followed by the unique target sequence and a universal reverse sequence. The universal reverse primer contained a universal reverse sequence complementary to the forward primer and a Cas9 association site as outlined in Figure 8. Following the PCR reaction, 5xPB Buffer (QIAGen) and 10µL 3M Na Acetate pH 5, were added to the PCR reaction. The mixture was then applied to QIAGen Quickspin PCR Columns, and slow spun (6000rpm) for 30 seconds, followed by a fast spin (13,000rpm) for 10 seconds. The flow-through was discarded and column fast spun again for 1 minute. To wash the column, 750µL PE buffer was added and fast spun for 10 seconds. The flow-through was discarded and the column was fast spun for 5 minutes. PCR product in the column was incubated with 40µL of EB Buffer warmed to 50°C 30 seconds before elution by a slow spin for 30 seconds, followed by a 2 minute fast spin. The quantity and quality of the PCR product was determined using a Nanodrop and 1% agarose gel containing EtBr.

Using the purified PCR product, an \textit{in vitro} transcription using T7 RNA polymerase (Ambion Megascript Kit) was performed in a 20µL reaction containing 300ng DNA, 2µL of each ATP, CTP, GTP and UTP, 2µL 10X reaction buffer and 2µL T7 RNA polymerase, incubating at 37°C overnight. DNA was removed by incubating reaction with 1µL Turbo DNase (Invitrogen) at 37°C for 15 minutes. The sgRNA was then purified using GE Illustra Sephadex G-50 NICK columns. Prior to use, columns were equilibrated by washing with 3mL TE Buffer pH 8 (10mM Tris pH 8, 1mM EDTA pH 8). The sgRNA reaction mixture was applied to the column and allowed to enter gel column completely before the column was washed with 400µL TE buffer. When dripping ceased, the sgRNA was eluted by applying 400µL TE Buffer to the column and collecting the elute in an Eppendorf tube containing 1mL 100% EtOH and 10µL NH₄ Acetate (Megascript Kit).
Figure 7. **Hand1 sgRNA target sites within Xenopus.**

Locations of *Hand1* guide sequences on both the 1S chromosome (A) and 1L chromosome (B).
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Table 1. PCR cycling conditions for sgRNA template generation.
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<td>sgT4</td>
<td>CAGCTAAATACGACTCACTATAGCTCCCAATCAAGTTCAATTTTATTCGAGCCAGGGAAGTTGTCAGCCACGAAAA</td>
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**Table 2. Primer sequences for the generation of sgRNA for injection.**

The first nucleotides correspond to the T7 promoter site, which is located at the beginning of each sgRNA. Underlined are the unique target sequences corresponding to a region of the *hand1* gene. Bolded is the universal primer sequences which anneals both the target primers and the Cas9 association site located on the universal reverse primer together. The remainder of the universal reverse primer sequence is the Cas9 association site.
Figure 8. Schematic showcasing the two oligonucleotides used to create the final sgRNA.

The target oligonucleotide contains a T7 RNA Polymerase promoter, target sequence and universal primer sequence. The universal reverse primer contains the complementary universal primer sequence, which allows the two strands to anneal during PCR, and the Cas9 association site, to form the final functional sgRNA.
After inverting the tube several times, the sgRNA was incubated at -20°C for 45 minutes, and then -80°C for 15 minutes before centrifugation at maximum rpm for 20 minutes at 4°C. The pellet was washed with 70% EtOH, dried on a 55°C heat block and resuspended in 20µL dH₂O. Quality and quantity of the sgRNA was determined using a Nanodrop and 2% agarose and TAE gel containing EtBr. sgRNA was diluted to 1500ng/µL and aliquots were stored at -20°C until use.

Cas9 protein was obtained from PNA Bio Inc. (Cat# CP01-200; Newbury Park, CA). Cas9 protein was resuspended in 50µL nuclease free H₂O according to manufactures instructions and aliquots were stored at -20°C until use.

2.3 Embryo Microinjection

Embryos to be microinjected were transferred into a 3% Ficoll in 1xMMR solution (1mM mgSO₄, 2mM CaCl₂, 5mM Hepes pH 7.8, 0.1mM NaCl, 0.1mM EDTA pH 8 and 2mM KCl) following the de-jelling procedure to ensure stability during the injection process by removal of liquid between the fertilization envelope and embryo. After incubation in 3% Ficoll in 1xMMR at 14°C for 15 minutes embryos were microinjected at the one-cell stage using the Microinjuctor Nanoject 3 (Drummond Scientific Company; Broomall, Pa). Seven nanoliters of injection stock were injected into each embryo. For hand1 loss of function, 1.5 ng of Cas9 protein and 750 pg of corresponding sgRNA were injected per embryo. Injection stock solutions were diluted in dH₂O. sgRNA was heated to 70°C for 2 minutes prior to addition to the stock solution. For hand1 gain of function experiments increasing concentrations of synthetic hand1 mRNA, either 100pg, 500pg or 1000pg were microinjected per embryo. Control embryos were injected with water to control for the injection process itself. After the injection process, the embryos were cultured in 3% Ficoll in 1xMMR solution at 14°C for 4 hours before being transferred to 20% Steinburg’s, where successful injected embryos were allowed to develop until the desired stage prior to fixation in MEMPFA.

2.3.1 Determining Cas9 efficiency

Before injecting embryos with the hand1 sgRNAs, we sought to assess the function of the Cas9 protein in Xenopus, as cells quickly divide during embryogenesis. We therefore first designed sgRNA targeting the slc45a2 gene (solute carrier family 45 member 2). Slc45a2 is a carrier
protein that mediates melanin synthesis (Dooley et al., 2013), therefore knockdown of slc45a2 would result in embryos lacking melanin providing a direct visual assay to assess Cas9 function. Slc45a2 was chosen over other melanin production enzymes as slc45a2 is only located on the long chromosome, therefore only one pair of chromosomes would need to be targeted. The sequence of the forward target oligonucleotide was as follows: CAGCTAATACGACTCATTAGGAGCCTCCCAGAAGATCTAGTTTTAGAGCTAG AAATAG. The underlined portion corresponds to the target region of the slc45a2 gene and the bolded sequence corresponds to the universal primer sequence. Functional sgRNA was generated as described previously, and embryos were injected with 7nL of injection stock, containing 750pg sgRNA and 1.5ng Cas9 protein. Embryos were then allowed to develop until stage 40, when pigmentation is evident, and fixed in MEMPFA before imaging.

2.4 T7 Endonuclease Assay

2.4.1 Xenopus DNA extraction

DNA was extracted from 10 individual embryos at stage 30 every time injections occurred to ensure the injections were successful for that day. Embryos were homogenized overnight at 55°C in homogenization buffer (1% SDS (sodium disulfate), 10mM EDTA pH 8, 20mM Tris pH 7.5, 100mM NaCl) containing 0.1mg protease K. Following homogenization, 0.1 volumes 5M NH₄ Acetate was added to the homogenate and DNA was extracted with 1:1 phenol chloroform, followed by 1:1 chloroform extraction. DNA was precipitated with 0.1 volumes 5M NH₄ Acetate pH 5 and 0.6 volumes isopropanol, pelleted by centrifugation at high speed for 10 minutes. The pellet was washed with 70% EtOH and resuspended DNA was treated with 0.5µg RNase A for 30 minutes at RT, followed by precipitation in 1 volume isopropanol before centrifugation at high speed for 10 minutes. Pellet was dried, and DNA was resuspended in 20µL dH₂O.

2.4.2 T7 Endonuclease Digestion

To determine the effectiveness of the sgRNA being used, a T7 Endonuclease Assay was then performed on the extracted DNA. PCR reactions were performed on extracted DNA, using primers that amplified the area of hand1 gene surrounding the sgRNA target sites, one set for each the 1S and 1L chromosome. Primers for the T7 Endonuclease Assay were chosen so that
the product amplified was 700-800 base pairs, with the sgRNA target site located offset from the center of the amplicon so that resolution on a gel would result in two distinct product sizes. Primers were as follows: \textit{hand1.L} Fwd: TGCAGTGTAGACCTTGCTTGGG; \textit{hand1.L} rev: CCTATATTCATACTAGGCCTTGGGA; \textit{hand1.L} Fwd: GTCTATATTCATACTAGGCCTTGGGA; \textit{hand1.L} Rev: CCAATTTTGAGCGATTTCTACTC. PCR cycling conditions were as follows: denaturing at 95°C for 1 minute, annealing at 55°C (\textit{hand1.S}) or 59°C (\textit{hand1.L}) for 30 seconds, and amplification at 72°C for 30 seconds, repeating the cycle 40 times. Before continuing, a sample of the PCR product was run on a 1% agarose gel and TAE containing EtBr to ensure a single product was amplified. The remaining PCR product was then denatured at 95°C for 1 minute and reannealed by decreasing the temperature 1°C/minute until 4°C was reached. After reannealing, 0.3 U of T7 endonuclease enzyme (NEB) and NEB Buffer 2 was added to the remaining PCR product and incubated at 37°C for 1 hour. Fifteen microliters of the digest were resolved on a 2% agarose gel and TAE containing EtBr at 100V for 50 minutes. Mismatched DNA would expect to resolve 3 products on the gel, which would indicate that Cas9 cut the DNA at the predicted guide target site.

2.5 Sequencing of \textit{hand1} target region and TIDE Analysis

PCR products for \textit{hand1} gene of extracted single embryo DNA were also sent for sequencing. Primers were used as listed above, following the same conditions and the product was purified using the QIAgen PCR purification kit following manufactures instructions. PCR product was sequenced using both primer sets as in amplification. Electrogram results from sequencing were input into the program TIDE (Tracking of Indels by Decomposition; https://www.deskgen.com/landing/tide.html) comparing non-injected embryos to embryos injected with sg1 alone; sg2 and sg3; or sg2 and sg4. TIDE output was then able to determine estimations for the number of mutations, the number of wildtype sequences remaining and the number of in-frame mutations for each embryo analyzed.

2.6 mRNA probe synthesis for whole mount \textit{in situ} hybridization

2.6.1 Bacterial competent cell culture and plasmid transformation

In order to generate competent cells for transformations Escherichia coli (DH5-\textalpha) cells were grown in 4 mL liquid Luria Broth (LB; for 1L: 10g bacto-tryptone, 5g bacto-yeast extract, 10g
NaCl, pH 7) overnight agitating at 37°C. An aliquot of cells was removed and 100-200µL was used to inoculate 50mL culture of LB and agitated at 37°C for 4 hours, or until the culture reached log phase growth. The cultures were chilled on ice for 20 minutes and pelleted for 10 minutes at 4°C at 3000 rpm. Cells were re-suspended in 30mL ice cold Solution A (0.1M CaCl₂, 0.1M MgCl₂, 1mM Tris pH 7.5) and left on ice for 20 minutes. Cells were then centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was removed, and cells were resuspended in 2.5mL ice cold Solution B (0.1M CaCl₂, 0.1M MgCl₂, 1mM Tris pH 7.5, 0.1% glycerol) and aliquots were stored at -80°C until use.

When transforming plasmid into bacteria, 10ng of plasmid was added to 60mL of ice cold competent cells. The competent cell/DNA solution was kept on ice for 30 minutes and then heat-shocked at 42°C for 90 seconds. Following heat shock, cells were incubated on ice for 2 minutes before being transferred to 1mL liquid LB and incubated at 37°C for 45 minutes, rocking at 225rpm. Two hundred microliters of cells were then plated on LB agar plates (for 500mL: 5g bacto-tryptone, 2.5g bacto-yeast extract, 5g NaCl, 7.5g bacto-agar) containing the antibiotic ampicillin (50mg/mL) and cultured overnight at 37°C. Colonies were then picked using a sterile pipette tip and grown at 37°C overnight in 4mL liquid LB, rocking at 225rpm. DNA was then isolated from bacterial cells using QIAgen Miniprep Spin Kit, following manufactures instructions.

2.6.2 Restriction Digest of Plasmid

Plasmids were cut with the appropriate enzymes to yield the template for generating antisense mRNA probe. In a reaction volume of 50µL, 15µL plasmid DNA, 28µL dH₂O, 5µL of the appropriate restriction buffer and 2µL of the appropriate restriction enzyme were incubated at 37°C for 2 hours. Refer to Table 3 for the specific buffer and enzyme for each cDNA plasmid used. Digested DNA was purified by phenol: chloroform extraction. The aqueous layer was extracted and precipitated with 0.1 volumes 5M NH₄ Acetate and 95% EtOH. DNA was pelleted at 14000rpm for 10 minutes and washed with 70% EtOH. DNA was resuspended in 10µL dH₂O and the product from the digestion was visualized by running 1µL of digestion on a 1% agarose and TAE gel containing EtBr.
2.6.3 *In Vitro* Transcription of Probe

Transcription of RNA was carried out with either T7, T3 or SP6 RNA polymerase, depending on the probe being synthesized. To a new, clean 1.5mL Eppendorf tube, a 20µL reaction containing 1ng linearized plasmid, 1.5µL dH2O, 4µL DIG-NTP mix (2.5 mM ATP, 2.5mM GTP, 2.5mM CTP, 1.625mM UTP, 0.875mM DIG-11-UTP (Roche)), 4µL 10mM DTT (Dithiothreitol), 0.5µL RNase inhibitor (RNase Out (Invitrogen)), 4µL transcription buffer and 2µL of the appropriate RNA polymerase as seen in Table 3 were added and incubated at 37°C for 2 hours. One microliter of DNase I (Invitrogen) was added to the reaction and incubated at 37°C for 15 minutes. To check the quality of the resultant RNA probe, 1µL was run on a 1% agarose and TAE gel containing EtBr. To the remainder reaction, 80µL 1% SDS in TE Buffer, 15µL 5M NH₄ Acetate and 100µL EtOH was added and precipitated on ice until results from gel were known. If the quality of the RNA was good, as determined by one clean band on the gel, the RNA was then centrifuged at 14000rpm for 15 minutes at 4°C. The supernatant was removed by glass pipette and RNA pellet was briefly dried before resuspending in 1mL RNA hybridization buffer (50% formamide, 5xSSC (diluted from a 20xSSC stock: 3M NaCl, 0.3M sodium citrate, pH 7), 5mM ETDA pH 5, 1mg/mL Yeast RNA extract (Boehringer), 1M Denhart’s solution (2% bovine serum albumin, 2% polyvinylpyrrolidone (PVP-40), 2% Ficoll 40) and 0.1% Tween-20) and warmed to 37°C to help RNA move into suspension. The RNA probe was then diluted in a 15mL conical tube with RNA hybridization buffer to 8-15mL depending on the level of RNA present as determined by the previous agarose gel. Prepared RNA probe was then stored at -20°C.

2.7 Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed on fixed embryos following the protocol by Deimling *et al.* (2015). The procedure was carried out in small glass vials 1.5 cm in diameter on LabQuake shakers. All steps were carried out at RT unless specified. Embryos stored in methanol were rehydrated in a methanol series (5 minute washes in 75%, 50%, 25%) followed by 3 washes for 10 minutes in TTW buffer (tris buffered saline: 50mM Tris pH 7.4, 200mM NaCl, 0.1% Tween-20). Embryos were then fixed with MEMPFA for 20 minutes, followed by three washes of five minutes each in TTW buffer.
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Table 3. Restriction enzyme and appropriate buffer for linearizing each plasmid for transcription with appropriate RNA polymerase.
To prepare for RNA probe hybridization, embryos were washed for 10 minutes in RNA hybridization buffer. RNA hybridization buffer was then replaced with warmed buffer and incubated at 65°C for 1 hour. Pre-warmed RNA probe was added to the vials (1.5mL/vial) and incubated overnight at 65°C.

The following day, probe solutions were removed (reused if stored at -20°C) and replaced with warmed RNA hybridization buffer for 10 minutes at 65°C. Embryos were then washed twice for 20 minutes in 2xSSC at 37°C followed by 3 washes in 0.2xSSC for 1 hour at 65°C. Embryos were blocked for 30 minutes in blocking solution (MAB pH 7.5 (100mM maleic acid, 150mM NaCl) containing 2% Blocking reagent (Roche; Indianapolis, IN) and 20% sheep serum treated for 2 hours at 65°C) and then overnight in blocking solution containing DIG-labelled antibody (Cat#11093274910, Anti-Digoxigenin-AP, Fab Fragments; Roche) diluted at 1:5000. The following day, embryos were washed 12 times in MAB for 30 minutes. Embryos were then stained using BM purple (Roche) as the alkaline phosphatase substrate overnight. The colour reaction was fixed in MEMPFA for 30 min followed by three 5 min washes with 25%. Embryos were bleached in bleaching solution (5% formamide, 0.5xSSC, 1% hydrogen peroxide) for 2-3 hours, until the endogenous pigmentation was removed. Once the bleaching solution was removed, embryos were dehydrated in a methanol series and stored in 100% methanol at -20°C until imaging.

2.7.1 Embryo Imaging

Embryos were rehydrated through a methanol series, transferred in 1xPBS solution and imaged on a 1% agarose plate using a Leica M205 FA microscope. Images were captured and processed using Leica Application Suite.

2.8 Whole mount TUNEL staining

Fixed embryos were rehydrated in a methanol series and then washed twice in 1xSSC. Embryos were then bleached in bleaching solution, under direct light for 1-2 hours. Embryos were washed twice with PBS/0.1% Tween-20 for 15 mins before incubation with terminal deoxynucleotidyl transferase (TdT) Buffer (Thermo Fisher Scientific) for 1 hour. Embryos were incubated with end-labeling solution (TdT Buffer, 150U/ml TdT enzyme (Thermo Fisher Scientific), 1µM DIG-11-dUTP (Roche)) overnight to label cells undergoing apoptosis.
Embryos were washed twice with 1mM EDTA pH 8 in PBS for 1 hour at 65°C, followed by 4 washes in PBS at RT. Embryos were incubated with blocking solution for 1 hour followed by overnight incubation with blocking solution containing anti-DIG antibody coupled to alkaline phosphatase diluted to 1:3000 overnight at 4°C. The following day, embryos were washed 5 times for 1 hour with MAB. Embryos were then washed with AP buffer (100mM Tris pH 9.5, 50mM MgCl2, 100mM NaCl, 0.1% Tween, 5mM levamisole (Sigma)). Staining was developed using the phosphate substrate NBT/BCIP (4.5µL nitro blue tetrazolium (75mg/mL 70% dimethylformamide) and 3.5µL 5-bromo-4-chloro-3-indolyl (50mg/mL 100% dimethylformamide) in 1mL AP buffer). Colour reaction was stopped with a MAB wash, and embryos were dehydrated in methanol and cleared in benzyl benzoate/benzyl alcohol (2:1 ratio) for imaging.

2.9 Synthetic hand1 mRNA synthesis

2.9.1 Xenopus RNA isolation

A total of 8 embryos were pooled and used for cDNA library preparation. Embryos at stage 35 were homogenized in a homogenization buffer consisting of 4M guanidine thiocynate, 25mM sodium citrate, 0.5% N-lauroyl sarcosine and 0.1M β-mercaptoethanol. After homogenization, 0.1 volume of 2M Na Acetate pH 4 was added followed by phenol: chloroform extraction. The aqueous layer was removed and 2.5x volume of 70% ethanol was added. This solution was then transferred to QIAgen RNeasy spin columns, and RNA was purified according to manufactures instructions. RNA concentration and quality were measured using a spectrophotometer and 1% agarose in TAE gel containing EtBr.

2.9.2 Cloning of hand1 into pCS2 vector

Xenopus cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) with Oligo-dT primers (Invitrogen) and RNase Out (Invitrogen). One microgram of RNA was used for the reaction to generate cDNA. Primers were designed flanking the coding region of the hand1 mRNA transcript and restriction sites were added that corresponded to sites in the polylinker of the pCS2+ expression vector. The enzyme site for BamHI was added to the 5’ end of the forward primer, and the enzyme site for ClaI was added to the 3’ end of the reverse primer. Primers were as follows: fwd: GCGAGGATCCGGAGGTGCCAGAAGTTGTTTTT,
Rvs: CTCGCATCGATATCAGGGGTTTAGTTCCAGGG. PCR reaction using 2µl cDNA was carried out using Q5 High Fidelity Polymerase (NEB). The following conditions were used, DNA was denatured at 95°C for 1 minute, annealed at 57°C for 30 seconds, and amplified for 1 minute at 72°C, repeating the cycle 35 times. PCR product was purified using QIAgen PCR Purification Kit, following manufactures instructions. The PCR product was verified by running 2µl on a 1% agarose in TAE gel containing EtBr. After attaining both the insert and the plasmid, restriction digests with the enzymes BamHI and ClaI were performed. After digestion, the vector and insert were gel extracted, and purified using QIAgen gel extraction kit, following manufactures instructions. Quantity of vector and insert were determined using a spectrophotometer. Insert and vector were then ligated overnight using T4 Ligase (Thermo Fisher Scientific) and transformed into DH5-α cells as described previously. Colonies that formed were picked and grown overnight in LB broth. Plasmids were isolated using QIAgen Miniprep Kit, following manufactures instructions and sent for sequencing to ensure there was no mutations in the hand1 mRNA prior to mRNA synthesis.

Xenopus hand1 cDNA was also cloned into a pCS2+MT (myc tag) vector. Insert was generated through a PCR reaction using template cDNA and PCR conditions as described above, with the following primers Fwd: GCGAGGATCCGGAGGTGCCAGAAGTTGTTTTT, rvs: TATACATCGATTGGGGTTTAGTTCCAGGGCC. The forward primer contained a BamHI enzyme site on the 3’ end and reverse primer contained a ClaI enzyme site on the 5’ end. The pCS2+MT vector and insert were digested with BamHI and ClaI, gel purified, and ligated overnight following the protocol above followed by transformation and isolation of DNA from positive colonies. Plasmids containing pCS2+MT hand1 sequence was verified by sequencing before mRNA synthesis for injection.

2.9.3 mRNA synthesis and purification

The pCS2+ hand1 vector and pCS2+MT hand1 vector were digested using NotI for 2 hours at 37°C to linearize the plasmid after the polyA tail segment. Digest was confirmed by running a 1% agarose and TAE gel containing EtBr. One microgram of digested plasmid was transcribed using SP6 mMessage mMACHINE (Ambion), following manufactures instructions. After 2 hours incubation with SP6 enzyme mix at 37°C, synthetic mRNA was extracted with 1:1 phenol: chloroform, followed by extraction with 1:1 chloroform. The aqueous phase was
transferred to a clean Eppendorf tube and 1:1 isopropanol was added and incubated at -20°C for 15 mins, followed by centrifugation at 4°C for 15 minutes at 14000rpm. The supernatant was removed and once dried, the synthetic mRNA was resuspended in 20µl nuclease-free H₂O. Quality and quantity of mRNA was verified using a 1% agarose and TAE gel containing EtBr and Nanodrop, respectively. mRNA was diluted to desired concentrations for injections and aliquots were stored at -20°C until use.

2.10 Western Blot

Embryos were pooled into groups of 10, and protein was extracted from Hand1-MT injected embryos and control embryos at stage 20 and stage 30 of development. Embryos were lysed in RIPA buffer (150mM NaCl, 1% Triton-X-100, 1% deoxycholic acid, 0.1% SDS, 1mM EDTA, 10mM Tris-HCl, pH 7.5) containing a cocktail of protease inhibitors (1mM PMSF, 10mM NaF, 10mM NaNO₃, 0.1M DTT, 20µM Leupeptin, 20µM Pepstatin and 1µM Aprotinin). A syringe with a 25-gauge needle was then used to draw up and push out the embryos until a cell lysate was formed. This lysate was then centrifuged at 1000g at 4°C. The syringe and needle step were repeated 4 times to the supernatant to completely remove the yolk layer. Protein was quantified by Bradford Assay.

Twenty micrograms of each protein sample were heated at 95°C for 5 min prior to loading on a 12% SDS-PAGE gel. A PVDF (polyvinylidene difluoride) membrane was pre-activated with 100% methanol and washed with transfer buffer (25mM Tris, 192mM glycine) prior to transfer of proteins from the gel. Wet-transfer was carried out for 90 min at 200mA. Following transfer, the membrane was rinsed with TBST (10mM Tris pH8,150mM NaCl, 0.1% Tween-20) before blocking in 5% non-fat dried milk (NFDM) diluted in TBST for one hour at RT. Rabbit anti-c-Myc primary antibody (Cat# - C3956, Sigma, St. Louie, MO) was diluted 1:500 in 5% NFDM and incubated overnight at 4°C. Membrane was washed with TBST before incubation with secondary antibody (Goat Anti-Rabbit IgG, horseradish peroxidase conjugated, Cat#- 65-6120, Thermo Fisher Scientific) for one hour at RT. Membrane was washed with TBST and colour reaction using ECL (enhanced chemiluminescence) Western blot Substrate (Thermo Fisher Scientific) was carried out prior to protein visualization using a Versa-doc gel system.
2.11 Image Analysis

Due to the mosaicism seen within the embryos, each side of the embryo was used as a separate n-value for the measurements, and classification.

2.11.1 Vasculature Classification

In order to characterize the formation of the vasculature, embryos were classified into one of four categories: normal, mild, moderate and severe, at stage 35 and 37. Vasculature formation was based both on size of the vascularized region as well as complexity of the region, with severe phenotype determined by less than 5% marker expression in the ventral region of the embryo. Moderate vasculature was determined if there was less than 5 branch points within the developing vasculature plexus. Mild vasculature was characterized by a combination of both 50% of the area of vascular plexus, and/or less than 10 branch points within the developing vasculature plexus. Normal vasculature was characterized based on the average vasculature marker expression within wildtype control embryos.

2.11.2 Vasculature Complexity

To measure the complexity within the vasculature plexus, a line was drawn from the top of the cement gland to the point where the proctodeum meets the somites to account for the size and position of the embryos. A parallel line was then matched intersecting the bottom of the heart, where ImageJ Line Analysis Tool was used to determine the number of times the vasculature plexus crossed this line.

2.11.3 Size of Vasculature Free Zone

Furthermore, the size of the vasculature free zone of the embryo was measured at Stage 35 and 37, with the probes *aplnr* and *ami*, respectively. To measure the size of the region, a ratio between two lines, x and y was used. To obtain the x value, a line of best fit was drawn along the end of the vasculature in the ventral region of the embryo, and the length between the region where proctodeum meets the somites, to the center of the previous line was the x value.

The y value was obtained by measuring the length of a line from the bottom of the cement gland to the point where the proctodeum meets the somites, to account for the size of each of the embryos.
2.12 Statistical Analysis

A one-way ANOVA was used comparing the complexity of the vasculature between *hand1* mutants and control embryos. Similarly, a one-way ANOVA was used to compare the size of the vasculature free zone between *hand1* mutants and control embryos. A one-way ANOVA was also used to compare the size of the blood and myeloid cells between *hand1* overexpressing embryos and control embryos. For all statistical analysis, a p-value < 0.05 was considered significant. Statistical analysis was done using GraphPad Prism program.
Chapter 3: Results

3.1 Expression of LPM derivatives throughout development

As a correlation between the regions where there is loss of *hand1* expression in the embryo and the differentiation of specific LPM derivatives in those same regions has been only partially demonstrated (Deimling and Drysdale, 2011), I decided to more completely document the spatial distribution of markers of specific LPM derivatives in parallel with that of *hand1* expression throughout development. Control embryos were fixed at various stages from stage 20-37 and probed with dig-labelled antisense mRNA probes to visualize expression patterns of *hand1*, *etv2* (endothelial precursors), *globin* (blood), *spib* (myeloid cells), and *ami* (endothelial cells).

At stage 20 (Fig. 9) we saw expression of *hand1*, *etv2* and *spib*. The expression domain of *etv2* at stage 20 overlapped with the expression domain of *hand1* along the ventral region of the embryo, although *hand1* was additionally expressed broadly along the side of the embryo. *Spib* was expressed at stage 20 in a tight cluster of cells marking the aVBI.

At stage 23 (Fig. 9), *spib* expressing cells began to migrate away from their initial site of differentiation and continued to migrate throughout development until they are located throughout the embryo. At stage 23, *hand1* expression was located in the middle of the LPM and wrapped around the ventral region of the embryo. Similar to that seen at stage 20, *etv2* was expressed in overlapping regions to that of *hand1* expression. At stage 25 (Fig. 9) *etv2* expressing cells appeared to migrate dorsally with loss of both *hand1* and *etv2* expression along the ventral side of the embryo where the pVBI will arise. The expression domain of *hand1* at stage 25 was still located within the middle of the LPM and the border of *hand1* expression appeared to overlap with the expression of *etv2*.

As development continued into stage 29 (Fig. 10), the expression domain of *etv2* continued to overlap with the *hand1* expression domain, which was seen throughout stages 20-25. At stage 29 the cells expressing *etv2* originating from the ventral side of the embryo migrated into the region where the vitelline veins will form, which was also *hand1* positive. Additionally, expression of *hand1* at stage 29 could be seen in the forming heart field, along the anterior side of the embryo.
Figure 9. Expression pattern of *hand1*, *etv2*, *spib*, *globin* and *ami* of WT embryos from stages 20-25.

Control embryos were fixed at stage 20, 23 and 25 and probed with dig-labelled antisense mRNA probes against *hand1*, *etv2*, *spib*, *globin* or *ami*. Both a side view and ventral view of each embryo is shown above. Embryos are oriented with anterior side to the left and posterior to the right. At stage 25, expression of *hand1* is lost along the ventral side of the embryo, following a similar expression of *etv2*. *Spib* expressing cells initially are in a restricted domain, and then migrate throughout the embryo. Both expression of *globin* and *ami* are not yet present.
At stage 29 globin expression appeared, marking the pVBI in the classic V-shape along the ventral side of the embryo. Of note, expression of globin appears in a region devoid of hand1 expression.

At stage 32 (Fig. 10) the vascular plexus began to form and could be visualized by the expression of ami, as etv2 expression becomes down regulated once the vascular plexus begins formation. Expression of hand1 was still maintained along the broad region of the embryo where the vasculature plexus was forming, and within the heart at stage 32 and 35. Expression of hand1 did not extend to the most posterior region of the embryo, a region that has been termed the vascular free zone as it also does not express either vasculature marker, etv2 or ami. At stage 37, the vasculature became more complex, and appeared to be functional as globin expression could be seen to migrate throughout vascularized regions of the embryo, and hand1 expression was still maintained. As the formation of the vitelline veins continued, hand1 expression appeared speckled in the broad region of the embryo, potentially being down regulated in terminally differentiated endothelial cells (Fig. 10).

3.2 slc45a2 knockdown

Before beginning to target hand1 in Xenopus using CRISPR technology, we sought to determine the efficiency of Cas9 in Xenopus. In order to have a direct visual assay of Cas9 function, we first targeted the slc45a2 gene, which is required for melanin production. Successful mutations in slc45a2 would likely produce albino embryos. When compared to wild type controls, slc45a2 mutants exhibited a reduction in melanin when assayed at stage 40 of development (Fig. 11A,B). The phenotype of pigmentation loss was classified into four groups: none, mild, moderate and severe. The phenotype was considered mild if the embryo had >50% pigmentation, moderate if the embryo had <50% pigmentation and severe if the embryo had no pigmentation. Compared to non-injected embryos that exhibited 100% normal phenotype, slc45a2 mutant embryos had an average of 25% severe, 47% moderate, 27% mild and 7% normal pigmentation (Fig. 11C). This variation in phenotype severity was expected due the rapid division of Xenopus embryos that could result in different indels created in different cells within each embryo. Particularly, non-edited genes, or mutations resulting in a functional protein within the embryo would lead to mosaicism of the phenotype.
Control embryos were fixed at stage 29, 32, 35 and 37 and probed with dig-labelled antisense mRNA probes against *hand1, etv2, spib, globin* or *ami*. Both a side view and ventral view of each embryo are shown above, with anterior side to the left and posterior to the left. Expression of *hand1* is maintained throughout development and begins to appear again in the ventral region of the embryo around stage 35. *etv2* expression begins to disappear as the vasculature plexus becomes more defined, and expression of *ami* first appears, marking the forming vasculature plexus. *Spib* expression is maintained, with cells expressing *spib* now located throughout the embryo. *Globin* expression begins to appear, and cells remain in the ventral blood islands.

**Figure 10.** Expression pattern of *hand1, etv2, spib, globin* and *ami* of WT embryos from stage 29-37.
Figure 11. Cas9 efficiency determined by knockdown of slc45a2.

Slc45a2 mutants generated using CRISPR-Cas9 demonstrate the efficiency of the Cas9 protein as seen by the lack of pigment (B) compared to full melanin production in the wild type embryos (A) at stage 40. Classification of phenotype was determined for each group: mild phenotype was characterized by >50% loss of pigment, moderate phenotype was characterized based on <50% loss of pigment, and severe phenotype was characterized when there was no pigmentation evident on the embryos. Percentage of embryos with each phenotype are displayed for both control and slc45a2 injected embryos (C). As approximately 30% of the embryos exhibited complete loss of pigmentation, we can conclude that Cas9 is able to efficiently knockdown genes of interest in Xenopus.
3.3 Generation of hand1 mutants

After establishing the efficiency of Cas9 in *Xenopus* based on the phenotype of the *slc45a2* mutants, I then proceeded to target the *hand1* gene. To ensure the sgRNAs used were targeting the correct area of the genome, a T7 Endonuclease Assay was performed. Compared to the control embryo (first column on agarose gel; Fig. 12A,B) that had intact DNA, embryos injected with Cas9 protein and *hand1* sg1 had three bands (either groups of 5 embryos or single embryos) indicating that there were mutations in the DNA around the target site caused by Cas9 (Fig. 12A,B). Successful cuts were made in 94% of the embryos that were tested from various injection days (n=421).

To further validate the efficiency of the sgRNA used, DNA surrounding the target site of the gene was sequenced. The chromatogram of a wild type embryo shows the correct DNA sequence around the sg1 target site (labeled with a black line; Fig. 12C). However, when the DNA of *hand1* mutants was sequenced, the chromatogram becomes unreadable after the PAM site (labeled with a red line), indicating that there is a mixture of sequences after the PAM site as a result of multiple different mutated copies of DNA (Fig. 12D). Furthermore, when the reverse sequence was amplified (displayed is the reverse compliment sequence; Fig. 12E) it was evident that the gene was only altered in the region surrounding the PAM site, as the remaining sequence of the *hand1* gene is intact after that region.

The chromatograms for each guide were compared using TIDE analysis and TIDE indicated that each of the guides used modified the DNA in a mosaic fashion. The average number of indels estimated by TIDE for each guide sequence, from an average of 3 single embryos, was 7 unique sequences for each embryo. Efficiency predictions for each guide was based on the percent of the DNA that was mutated within each embryo. Sg1 was predicted to have 85% efficiency for both chromosome 1S and 1L, creating an average of 65% out-of-frame mutations in chromosome 1L and 54% out-of-frame mutations in chromosome 1S (Fig. 12F,G). Sg2 was predicted to have 92% efficiency, with an average of 86% out-of-frame mutations chromosome 1L (Fig. 12H). Sg3 was predicted to have 93% efficiency, with 66% out-of-frame mutations in chromosome 1S (Fig. 12I). Lastly, sg4 was predicted to have 28% efficiency, with 7% out-of-frame mutations in chromosome 1S (Fig. 12J).
Figure 12. sgRNA targeting hand1 are effective at mutating Xenopus DNA.

DNA was extracted from hand1 knockout embryos and a T7 assay was performed on either pools of five or single embryos. Compared to controls, embryos injected with sg1 resolve 3 bands on the gel indicating the DNA has been mutated, compared to the one band in WT controls (A,B). Chromatogram from Sanger sequencing results, first showing the hand1S sequence surrounding the sgRNA target site from a control embryo, the sgRNA site is underlined in black, while the associated PAM site is underlined in red (C). Both the forward (D) and reverse complementary sequence (E) of one hand1 mutant embryo is shown, where it is evident that the DNA has undergone mutations near the PAM site. The reverse complementary sequence shows that correct sequence is re-aligned shortly after the PAM site, indicating only indels surrounding the sgRNA target site were introduced in the DNA. When chromatographs were compared using the program TIDE, estimations of the percent of in-frame mutations, out-of-frame mutations and DNA with no-editing are reported for each hand1 sgRNA target (F-J).
To assay the expression domain of hand1 in hand1 mutants, embryos were probed with dig-labelled antisense mRNA against hand1 at stage 20. There was no observable difference in the expression domains of hand1 compared to control embryos (Fig. 13). Staining for hand1 appeared to be less intense in hand1 mutants, although this is not a strictly quantifiable measure of hand1 expression levels within the embryo.

3.4 hand1 mutant phenotype

3.4.1 hand1 loss disrupts heart morphogenesis

After establishing a knockdown of hand1, I first examined heart development in the hand1 mutants. Embryos were fixed at stage 35, when looping morphogenesis has normally been completed and probed with dig-labelled antisense mRNA against tnni3, a myocardial-specific transcript. A schematic showcasing normal rightwards heart looping is shown in Fig. 8A. Representative images for each experimental group are displayed (Fig. 14 B-I). Heart looping was classified into three groups based on phenotype severity: normal, defective looping and linear. Hearts were considered to have looped normally if looping occurred to the right, hearts were classified as having defective looping if the linear heart tube had begun looping but the loop was not as tightly coiled as it is in normal looping, and hearts were classified as linear if there was no obvious looping. Percentages for each phenotype are displayed for each experimental group. Non-injected embryos show 100% normal looping and water injected embryos show 92% normal looping. However, sg1 injected and sg1,2,3 injected embryos show an increase in both defective looping and linear phenotypes, with 33% of sg1 hearts having defective looping and 25% linear heart tubes, and 30% of sg 1,2,3 injected hearts with defective looping and 46% linear heart tubes (Fig. 14J).

As nkx2-5 and isl1 are factors known to be involved in heart looping morphogenesis, I next examined the expression of both nkx2-5 and isl1 in the hand1 mutants. Changes in the size of these expression domains could lead to defects in heart morphogenesis. Compared to controls, there was no discernible difference in the expression patterns of nkx2-5 or isl1 in sg1,2,3 injected embryos when assessed at stage 18 (Fig. 15).
Figure 13. *Hand1* mutants show similar *hand1* expression patterns.

Embryos were fixed at stage 20 and probed with dig-labelled antisense mRNA probes for *hand1*. Both a side (A, C, E, G) and ventral view (B, D, F, H) of each embryo is shown. There appears to be no change in *hand1* expression domain between control embryos (A-D) and *hand1* mutants (E-H).
Phenotype: Normal | Defective looping | Linear

Non-Inj

H20 Inj
n=24

sg1 inj
n=21

sg1,2,3 inj
n=16

% of embryos with Phenotype
Figure 14. Loss of *hand1* disrupts heart morphogenesis.

Schematic showcasing heart looping of a stage 35 embryo, where the linear heart tube has looped to the right (A). Embryos were fixed at stage 35 probed with dig-labelled antisense mRNA probes against *tnni3* transcripts. Shown is a ventral view of the embryos. Control embryos display a similar rightwards heart looping (B-E) while *hand1* mutants display disturbed heart looping, with an increase in cardiac edema (F-I). Phenotypes of the heart were classified into 3 groups; normal looping, when the heart was observed to loop to the right, moderate, which was characterized based on the extent of reduced looping, H being an example of moderate looping, where the heart has incomplete heart looping, and lastly, linear, where the heart had no indication of looping morphogenesis occurring (J).
Figure 15. Loss of *hand1* does not impact differentiation or size of heart fields.

Embryos were fixed at stage 18 and probed with dig-labelled antisense mRNA probes against *nkx2-5* and *isl1* transcripts. No differences in the primary heart field marker *nkx2-5* was found between control (A,B) and *hand1* mutant embryos (C). No differences in the secondary heart field marker, *isl1*, were found between control (D,E) and *hand1* mutant embryos (F).
3.4.2  *hand1* loss decreases vasculature plexus size and complexity

To examine the formation of the vasculature plexus embryos were probed at stage 35 and 37, with dig-labelled antisense mRNA against *aplnr* and *ami* transcripts, respectively. To quantify the phenotype of the *hand1* mutants, vasculature formation was grouped into four different classes: normal, mild, moderate, and severe. Representative images for each classification are shown in Figure 10A. The frequency of each phenotype is displayed for each of the experimental groups at both stage 35 (Fig. 16B) and 37 (Fig. 16C). Embryos injected with sg1 or sg1,2,3 had an increase in the percentage of embryos exhibiting mild, moderate and severe phenotypes as compared to control embryos (Fig. 16B,C). Interestingly, there was a further increase in the severe phenotype for sg1,2,3 injected embryos as compared to sg1 injected embryos (Fig. 16C).

The size of the non-vascularized region at the posterior end of the embryo was then compared in order to quantify the overall size of the plexus being formed in the embryos. To standardize and quantify the region, a ratio of x (length from where the proctodeum meets the somites to the middle of the line of best fit along the end of the vasculature) / y (length from the cement gland to where the proctodeum meets the somites) was compared between the embryos (Fig. 17A,B). There was a significant increase in the size of the non-vascularized region in *hand1* mutant embryos both stage 35 (Fig. 17C) and stage 37 (Fig. 17D). Furthermore, the size of the non-vascularized region in sg1,2,3 injected embryos was significantly greater when compared to sg1 injected embryos and controls at stage 37 (Fig. 17C).

To quantify the complexity of vasculature plexus, a line analysis was used to determine the number of intersection points between an artificial line through a standardized position on the embryo and the vasculature plexus (Fig. 18A,B). When quantified, there was a significant decrease in the number of intersection points in *hand1* mutants as compared to control embryos (Fig. 18C). Therefore, loss of *hand1* results in a decreased size and complexity of the vascular plexus within *Xenopus* embryos.
Embryos in both control and hand1 mutant groups were classified into one of four groups based on the complexity of the vascular plexus: normal, mild, moderate and severe, at stage 35 and 37 with either the probe aplnr or ami, with a representative image of each classification shown (A). Percent of embryos in each classification are shown for stage 35 (B) and stage 37 (C). At both stages, there was an increased frequency of mild, moderate and severe phenotypes in hand1 mutants as compared to control embryos. Embryos are oriented with anterior to the right, posterior to the left.

Figure 16. Loss of hand1 disrupts vasculature formation.
Embryos were fixed at stage 35 and 37 and probed with dig-labelled antisense mRNA probes against *aplnr* and *ami*, respectively. Size of the non-vascularized region was measured as a ratio of $x/y$ for all embryos (A-B). Embryos are oriented with anterior to the right, posterior to the left. In each case, in the *hand1* mutants, the area of the non-vascularized region was increased at both stage 35 (C; student’s t-test, $p>0.01$) and stage 37 (D; one-way ANOVA, $p>0.01$), with different lowercase letters representing statistically significant differences.

**Figure 17. Loss of *hand1* increases the size of the non-vascularized region.**
Figure 18. *hand1* mutants show a decrease in complexity of the vasculature plexus.

Embryos were fixed at stage 37 and probed with dig-labelled antisense mRNA probes against *ami*. Complexity was measured based on number of times the vasculature intersected along a line across the middle of the embryo (A-B). Embryos are oriented with anterior to the right, posterior to the left. *Hand1* mutants showed a significant decrease in number of line intersections as compared to the non-injected and water injected controls (C; one-way ANOVA, p>0.001), with different lowercase letters representing statistically significant differences.
3.4.3 *hand1* loss decreases the number of differentiated endothelial cells

Next, to discern potential causes for the reduced vasculature plexus formation, I examined earlier time points in development to determine if the vasculature was reduced at earlier stages. First, I looked at stage 28 of development using a dig-labelled antisense mRNA probe against *etv2* transcripts as they are a marker of early endothelial cells. Control embryos at this stage showed *etv2* expression in the cardinal vein and vitelline vein region (Fig. 19A,B). When examining *etv2* expression from the ventral side of the embryo, the endothelial cells created a defined "border" surrounding the blood islands (Fig. 19C). The expression of *etv2* within the cardinal vein appeared unchanged in both sg1 and sg1,2,3 injected embryos (Fig. 19D,G). However, the vasculature within the broad region of the *hand1* mutant embryos appeared to have a reduced number of endothelial cells (Fig. 19E,H), and similarly had a less dense “border” surrounding the ventral blood islands (Fig. 19F,I).

Given the decrease in endothelial cells at stage 28, I then examined expression of *etv2* even earlier in development. *In situ* hybridization demonstrating *etv2* expression at both stage 23 and 25 shows the earliest endothelial cells populations. In control embryos, ventral *etv2* expression at stage 23 formed a wide V-shape just posterior of the cement gland (Fig 20A, B, E, F). Although *etv2* expression was evident in *hand1* mutants the wide V-shape was less dense and appeared smaller in size compared to controls (Fig 20 I, J, M, N). At stage 25, *etv2* expression in controls was more dense in the vitelline vein region of the embryo, with minimal expression on the most ventral side of the embryo (Fig 20C, D, G, H). In *hand1* mutants at stage 25, *etv2* expression appeared more sparse within the broad region of the embryo, however there was no change in the location of *etv2* expression within these embryos (Fig 20K, L, O, P). Thus, the loss of *hand1* affects the early formation of vasculature cells within the embryos.

To determine if the decrease in endothelial cells was due to increased apoptosis of endothelial cells in the embryos, a whole-mount TUNEL assay was performed on stage 28/30 embryos. *Slc45a2* sgRNA and Cas9 injected embryos were used as an additional control for Cas9 function as it has recently been shown that double stranded breaks created by Cas9 may activate the p53 pathway triggering cell death (Haapaniemi et al., 2018; Ihry et al., 2018).
Figure 19. Loss of hand1 decreases number of endothelial cells in the LPM.

Control embryos and hand1 mutants were fixed at stage 30 and probed with dig-labelled antisense mRNA probes against etv2. Both side view (A, D, G), enlarged side view (B, E, H) and ventral view (C, F, I) of each embryo is shown above, with anterior to the right, posterior to the left. Control embryos show a large number of endothelial cells within the LPM (A,B), forming a fine boarder along the ventral side of the embryo (C). Hand1 mutants show both a decrease in number of endothelial cells (D-E, G-H), and loss of populated boarder surrounding the ventral blood islands (F,I).
**Figure 20. Loss of hand1 reduces the population of differentiated endothelial cells.**

Control embryos and *hand1* mutants were fixed at stage 23 and 25 and probed with dig-labelled antisense mRNA probes against *etv2* to observe endothelial cells prior to vascular plexus formation. Both side view (A, C, E, G, I, K, M, O) and ventral view (B, D, F, H, J, L, N, P) of each embryo are shown, oriented anterior to the right, posterior to the left. Control embryos at both stages show a large population of differentiation endothelial cells within the ventral region of the embryo (A-H). Loss of *hand1* does not disturb the localization of the endothelial cells within the embryo, but reduces the population of cells within these areas (A-P).
Apoptosis patterns observed in all embryos are similar to that as reported in the literature, with apoptotic cells located in the neuronal, midbrain, and optic nerve regions of the embryo (Hensey and Gautier, 1998). However, there appeared to be no apoptosis occurring in the broad region where hand1 is normally expressed in either hand1 mutants or control embryos (Fig. 21), thus the decrease in endothelial cells observed in hand1 mutants is likely not due to increases in cell death.

3.4.4 hand1 loss does not affect hematopoietic development

As I have shown that Hand1 has a vital role in both heart and vasculature formation in Xenopus, the role of Hand1 in the development of other LPM derivatives was examined.

To observe the differentiation of myeloid cells, embryos were fixed at both stage 20 and stage 34 and probed with dig-labelled antisense mRNA against spib. At stage 20, spib was expressed in a compact region at the anterior ventral side of the embryo (Fig. 22A). There was no difference in the expression domain of spib between control and hand1 mutant embryos (Fig. 22A, C, E). Similarly, no differences in expression of spib were observed at stage 34 between control and hand1 mutant embryos (Fig. 22B, D, F), therefore loss of hand1 does not appear to affect the differentiation or migration of the myeloid cells marked by spib.

To assess the differentiation of blood cells, dig-labelled antisense mRNA probes for scl and globin were used against stage 34 embryos. There was no observable difference in expression of either scl or globin (Fig. 23). Thus, Hand1 does not appear to affect the differentiation of the blood cell lineages.
Figure 21. Loss of handl does not increase apoptosis within the LPM.
Control embryos and handl mutants were fixed at stage 28/30 of development, and TUNEL assay was performed to determine the number of apoptotic cells. Embryos are oriented with anterior to the right, posterior to the left. Both control (A-D) and handl mutant (F-I) embryos show a normal pattern of apoptosis for their developmental stages. Although CRISPR injected embryos appear to have an increase number of positive nuclei as compared to controls at stage 28, there is no positive nuclei within the handl positive region of the LPM.
Figure 22. Loss of *hand1* does not affect the differentiation of myeloid cells as marked by expression of *spib*.

Control embryos and *hand1* mutant embryos were fixed at both stage 18 and 35 and probed with dig-labelled antisense mRNA probes against *spib*. Stage 34 embryos are oriented with anterior to the left, posterior to the right. No differences in differentiation of lineages expressing *spib* were observed between the control embryos (A-D) and *hand1* mutants (E-F).
Figure 23. Loss of hand1 does not affect the differentiation of blood lineages as marked by scl and globin.

Control embryos and hand1 mutant embryos were fixed at stage 35 and probed with dig-labelled antisense mRNA probes against globin and scl. Both a side view (A, C, E, G, I, K, M, O) and ventral view (B, D, F, H, J, L, N, P) of each embryo is shown above. Embryos are oriented with anterior to the left, posterior to the right. No differences in differentiation of lineages were observed between the control embryos (A-H) and hand1 mutants (I-P).
3.5 Consequences of *hand1* over expression

To further explore the role of Hand1 in development of the lateral plate in *Xenopus*, a *hand1* over expression model was used. This was accomplished by injecting synthetic *hand1* mRNA into embryos and assessing the differentiation of LPM derivatives. To confirm that synthetic *hand1* mRNA was present in embryos, a whole mount *in situ* hybridization was performed on injected embryos at stage 20, probing for *hand1*. Compared to control embryos who exhibit normal *hand1* expression patterns (Fig. 24A-C), there was an increase in *hand1* expression in all embryos injected with synthetic *hand1* mRNA (Fig. 24D-F, K-M). This was based on both staining intensity and distribution. Embryos injected with synthetic *hand1* mRNA had expression of *hand1* throughout the entire embryo indicating that the injections were successful. Furthermore, a portion of the embryos injected with *hand1* were observed to have an increase in *hand1* expression in only one half of the embryo (Fig. 24G-J). Increased expression in one half of the embryo suggests that the mRNA injected did not diffuse throughout the cell prior to the first cell division, thus rendering only half of the embryo containing synthetic *hand1* mRNA.

To confirm that the *hand1* mRNA was being translated into protein, a western blot was performed probing for Hand1-MT protein at stage 20 and stage 30. Hand1-MT protein, as seen by the band at 40kD was detected at both stage 20 and stage 30 in embryos injected with synthetic *hand1*-MT mRNA and was not detected in control embryos (Fig. 24N).

3.5.1. *hand1* overexpression disrupts heart morphogenesis

To assess the effects of *hand1* over expression on heart morphogenesis, a whole mount *in situ* hybridization was performed on stage 35 embryos using dig-labelled antisense mRNA probe against *tnn3*. Embryos over expressing *hand1* had defects in heart morphogenesis accompanied by an increase in cardiac edema (Fig. 25A-J). Defects in heart morphogenesis were classified into three groups: normal rightward looping, defective looping (determined if there was incomplete looping or leftward looping), and linear heart tubes. Control embryos exhibited normal heart looping in 90% of the embryos, compared to *hand1* over expressing embryos which exhibited 10% linear, 40% defective looping and 50% normal phenotype when injected with 100pg or 500pg of synthetic *hand1* mRNA, and 35% linear, 35% defective looping and 30% normal phenotypes when injected with 1ng of synthetic *hand1* mRNA.
Non-inj

500pg Hand1 mRNA

1ng Hand1 mRNA

Stage 20

Stage 30

Non-inj

OE

Non-inj

OE

40kD
Figure 24. Embryos over expressing *hand1* display an increase in *hand1* mRNA throughout the embryo and the mRNA is being translated into protein.

Control embryos and *hand1* over expressing embryos were fixed at stage 20 and probed with dig-labelled antisense mRNA probes against *hand1* transcripts. A side view (A, D, G, K, J) ventral view (B, E, H, L) and dorsal view (C, F, I, M) are shown of each embryo. Control embryos show normal *hand1* expression that appears as a saddle shape along the middle of the LPM (A-C). Embryos over expressing *hand1* lose this expression pattern, and instead have *hand1* expressed throughout the embryo (D-F, K-M). Some embryos were also shown to have only increased expression in one half of the embryo, with one side of the embryo displaying the normal *hand1* expression pattern (G), while the opposing side had a ubiquitous expression pattern (J), which is most evident in the ventral (H) and dorsal (I) photos. The one-sidedness suggests that *hand1* mRNA was introduced later during the 1-cell stage, and only was present one of the two cells after the first division in those embryos, leading to only half of the embryo showing increased *hand1* transcripts later on in development. Myc-tagged-Hand1 protein is correctly translated to yield the Hand1 protein as seen by the 40kB band in OE embryos at stage 20 and stage 30, which is not detected in the control embryos (N).
Phenotype: Normal  Defective looping  Linear

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>% Embryos</th>
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<tbody>
<tr>
<td>Non-Inj</td>
<td>30</td>
<td>100%</td>
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<tr>
<td>H2O Inj</td>
<td>20</td>
<td>100%</td>
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<tr>
<td>100 pg hand1 mRNA</td>
<td>22</td>
<td>100%</td>
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<tr>
<td>500 pg hand1 mRNA</td>
<td>12</td>
<td>100%</td>
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<tr>
<td>1 ng hand1 mRNA</td>
<td>30</td>
<td>100%</td>
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</table>
Figure 25. Over expression of hand1 causes defects in heart morphogenesis.

Control embryos and embryos injecting with varying amounts of hand1 mRNA (100pg, 500pg, 1ng) were fixed at stage 35 and probed with dig-labelled antisense mRNA probes against tnni3 to visualize heart shape. Ventral view of the embryos are shown. Compared to the control embryos which showcase looping of the heart towards the right (A-D), embryos over expressing hand1 show a range of defects, from loss of looping to leftward looping, along with an increase in cardiac edema (E-J). Phenotypes of the heart were classified into 3 groups; normal looping, when the heart was observed to loop to the right, moderate, which was characterized based on the extent of looping, where the heart appears to begun to loop, but not complete, and lastly, linear, where the heart had no indication of looping morphogenesis occurring. Percentage of each phenotype is displayed for each experimental group (K).
To examine if hand1 over expression resulted in changes in altered expression of other genes involved in heart looping, embryos were probed with dig-labelled antisense mRNA against nkx2-5 at stage 20. There was no observable difference in expression of nkx2-5 in embryos over expressing hand1 as compared to control embryos (Fig. 26).

3.5.2 hand1 over expression does not affect vascular plexus formation

In order to assess the effect of hand1 overexpression on vasculature development, in situ hybridization to assess expression of ami was performed at stage 37. Over expression of hand1 had no apparent effect on vasculature formation within the embryo (Fig. 27). When looking at earlier vasculature plexus formation at stage 28 using the probe etv2, there was no observable difference in etv2 expression in hand1 over expressing embryos as compared to controls (Fig. 28). At both stages, ectopic hand1 expression did not result in ectopic expression of vasculature markers, thus hand1 does not appear to be sufficient to drive endothelial cell differentiation.

3.5.3 hand1 over expression decreases differentiation of hematopoietic cells

In order to assess the differentiation of blood in hand1 over expressing embryos, embryos were probed with scl at stage 28. There did not appear to be any change in expression domain of scl in embryos over expressing hand1 as compared to controls (Fig. 29). In contrast, embryos over expressing hand1 showed a reduction in the size of the globin expression domain as compared to non-injected controls (Fig. 30A-P). The area of globin expression was measured as a ratio between the area of the globin expression (a’) compared to the area of the embryo (a) as illustrated in Figure 30C. There was a significant decrease in the ratio between the area of globin expression as compared to the area of the embryo (Fig. 30Q).

To assess the differentiation of myeloid cells in hand1 over expressing embryos, embryos were probed by in situ hybridization for spib transcripts at stage 20, when the myeloid cells are still compact within the embryo. Embryos over expressing hand1 appeared to have a decrease in the size of the expression domain of myeloid cells as compared to non-injected controls (Fig. 31A-F). When the size of spib expression was measured as a ratio between the area of spib expression (a’) compared to the size of the embryo (a) as seen in Figure 31A. A significant decrease in the size of the spib expression domain was confirmed in embryos injected with 500pg of synthetic hand1 mRNA as compared to controls (Fig. 31G).
Figure 26. Over expression of hand1 does not affect differentiation of the heart field as marked by nkm2-5 expression.

Control embryos and embryos over expressing hand1 were fixed at stage 20 and probed with dig-labelled antisense mRNA probes against nkm2.5. There appears to be no difference in the expression of nkm2.5 between control embryos (A-C) and hand1 over expressing embryos (D-F).
Figure 27. Overexpression of hand1 has no apparent effect on vascular plexus formation.

Embryos were fixed at stage 37 and probed with dig-labelled antisense mRNA probes against ami transcripts. Over expression of hand1 (C-F) does not appear to affect vasculature formation as compared to control embryos (A-B). Embryos are oriented with anterior to the left, posterior to the right.
Figure 28. Over expression of *hand1* has no observable effect on endothelial cell differentiation.

Control embryos and embryo overexpressing *hand1* were fixed at stage 28 and probed with dig-labelled antisense mRNA probes against *etv2* transcripts. Both side view (A-C, G-I) and ventral view (D-F, J-L) of each embryo are shown. No apparent differences in endothelial cell differentiation were observed between control embryos (A-F) and *hand1* over expressing embryos (G-L). Embryos are oriented with anterior to the right, posterior to the left.
**Figure 29. Hand1 over expression does not impact blood cell progenitors.**

Control embryos and embryo over expressing *hand1* were fixed at stage 28 and probed with dig-labelled antisense mRNA probes against *scl* transcripts. Both side view (A, C, E, G) and ventral view (B, D, F, H) of each embryo are shown above. No apparent differences in blood cell progenitor cells were observed between control embryos (A-D) and *hand1* over expressing embryos (E-H). Embryos are oriented with anterior to the right, posterior to the left.
Figure 30. Over expression of hand1 does not impact blood cell differentiation but alters the size of the globin expression domain.

Control embryos and embryo over expressing hand1 were fixed at stage 28 and probed with dig-labelled antisense mRNA probes against globin. Both a side view (A, B, E, F, I, J, M, N) and ventral view (C, D, G, H, K, L, O, P) of each embryo are shown above. There appeared to be a decrease in globin expression in hand1 over expressing embryos (I-P) as compared to control embryos (A-H). Area of globin expression (a’) was measured as a ratio compared to the area of the whole embryo (a), as illustrated in (C). There was a significant decrease in the ratio between a’/a for both embryos injected with either 100pg or 500pg hand1 mRNA as compared to controls (J; one-way ANOVA, p>0.001). Embryo are oriented with anterior side to the right, posterior to the left.
Non-Inj  OE 100pg  OE 500pg

Ratio between a'/a

G

Ratio between a/a

Non-Inj  OE 100pg  OE 500pg

**
**Figure 31. Hand1 does not affect differentiation of myeloid cells.**

Control embryos and embryo over expressing *hand1* were fixed at stage 20 and probed with dig-labelled antisense mRNA probes against *spib* transcripts. *Spib* expressing cells appear to be more compact when *hand1* is over expressed (D-F) as compared to control embryos (A-C), however over expression of *hand1* does not appear to ablate *spib* expression. Area of *spib* expression (a’) was measured as a ratio compared to the area of the whole embryo (a), as illustrated in (A). There was a significant decrease in the ratio between a’/a for both embryos injected with 500pg *hand1* mRNA as compared to controls (G; one-way ANOVA, p>0.001).
Chapter 4: Discussion

This study investigated the role of Hand1 in development of the LPM in *Xenopus* by analyzing the differentiation of LPM derivatives in *hand1* loss of function and over expression models. Our results show that the role of Hand1 in heart morphogenesis is conserved. Hand1 does not appear to function by specifying cardiac cell fate but is required for proper morphogenesis. Additionally, Hand1 is required for formation of a complex vasculature plexus by maintaining a large endothelial cell population early in development. Finally, we showed that downregulation of Hand1 is critical for proper development of the hematopoietic lineages in *Xenopus*. The molecular mechanisms behind Hand1 function remain elusive and more studies are warranted for further identification of targets and functions of Hand1, but this study provides guidance as to which lineages may contain important Hand1 targets.

4.1 Confirming CRISPR applicability in *Xenopus*

Confirmation of CRISPR-Cas9 efficiency was demonstrated by knockdown of *slc45a2* which provided a direct assay for Cas9 function. Approximately 25% of embryos displayed complete loss of pigmentaion, while the remaining embryos exhibited mosaic phenotypes suggesting that a successfully mutated gene is not typically achieved at the one cell stage, but instead at the two or four cell stage. This is in line with other studies, where knockdown of genes required for pigmentation, either *tyrosinase* or *slc45a2*, in *Xenopus tropicalis* and *Xenopus laevis*, show similar levels of mosaicism (Delay et al., 2018; Guo et al., 2014; Shigeta et al., 2016). Mosaicism can be attributed to either untargeted alleles or mutations that do not alter protein function (Delay et al., 2018; Guo et al., 2014; Shigeta et al., 2016).

TIDE analysis on *hand1* mutant embryos indicated that there was an average of seven different sequences within each embryo, providing more evidence that Cas9 was introducing different indels in the DNA of separate cells. Deletions were the most common mutation, while insertions and base pair changes were more rare, which is similar to what is noted in other studies (Delay et al., 2018; Shigeta et al., 2016). The frequency of mosaicism can be attributed to the rapid division of *Xenopus* embryos, although a study has shown that all embryos had mutations in their genome two hours post Cas9-sgRNA injection, thus formation of the
majority of indels is occurring early in development rather than at later stages (Bhattacharya et al., 2015).

The overall effectiveness of each sgRNA as estimated by TIDE showed that sg1, sg2 and sg3 were highly effective at causing indels in the target region of the genome, with sg2 producing the greatest frequency of out-of-frame mutations compared to the other guides. Furthermore, sg4 was shown to only have an 28% efficiency, with majority of the sequence unedited. Differences in sgRNA efficiency have been noted in other studies, and have been attributed the GC content of the guide, the binding affinity of the sgRNA to Cas9 and the location of the target site on the chromosome (Bassett et al., 2013; Wang et al., 2014). The location of target site is predicted to be more efficient in non-transcribed regions compared to transcribed regions of the gene and also predicted to be less efficient the closer the target site is to the last exon (Wang et al., 2014). Therefore, these could be potential reasons as to why sg4 had a very low efficiency as compared to the other three sgRNAs tested. TIDE analysis therefore holds a great importance in determining the effectiveness of sgRNA in *Xenopus*, in addition to the T7 endonuclease assay.

Notable differences exist in the utilization of the CRISPR/Cas9 system for generation of mutant embryos between mice and *Xenopus*. In mice models, Cas9 and sgRNA can similarly be injected into a zygote, transplanted back into a surrogate mother to produce chimera offspring (Wang et al., 2013; Yang et al., 2014, 2013). However, these mice need to undergo further breeding and genetic transmission to produce a homozygous mutant embryo (Wang et al., 2013), and this mutant embryo is thus studied further. In contrast, the CRISPR/Cas9 system when utilized in *Xenopus* can easily produce a large number of mutant embryos within a very short time (about one week), thus providing an inexpensive tool to knockdown genes of interest. However, the one disadvantage of this method is that the pooled population of mutant embryos generated will be mosaic, thus instead of studying the effects of one particular mutation as one would in mice mutant embryos, the pooled population of *Xenopus* mutant embryos result in a broad range of phenotypes. However, because of the large number of mutant embryos that can be generated, the frequency of phenotypes can be classified based on severity and thus still provide valuable information on the function of the gene of interest.

There is currently no antibody that recognizes Hand1 in *Xenopus*, thus we were unable to assess the effectiveness of the knockdown by protein detection. Therefore, we sought to determine if
there was a change in the mRNA levels of hand1. However, no observable differences were found hand1 mRNA expression patterns within hand1 mutant embryos as compared to controls. Embryos injected with sg1,2,3 appeared to have a speckled expression pattern of hand1 suggesting that hand1 mRNA levels may be decreased, however this was not quantifiable. Non-sense mediated decay, could account for a potential decrease in hand1 mRNA levels. Non-sense mediated decay is mediated by targeting mRNA with a premature stop codon for degradation (Popp and Maquat, 2016). However, the premature stop codon must be located more than 50-55 nucleotides upstream of an exon-exon junction to be recognized (Nagy and Maquat, 1998; Popp and Maquat, 2016). As hand1 is only comprised of two exons, and the target sites are approximately 450-500 nucleotides upstream of the exon-exon junction, non-sense mediated decay could be playing a role in the stability of the hand1 transcript, and thus leading to the speckled appearance in the sg1,2,3 hand1 mutants. Furthermore, the probability of inserting a stop codon is small, which could also account for why there is not a drastic loss of hand1 mRNA in the hand1 mutants. Despite the lack of an antibody to definitively test the loss of hand1, the data from TIDE provides confidence that a large proportion of Hand1 was non-functional, as majority of the mutations caused by the guides are out-of-frame deletions.

Furthermore, embryos injected with sg1,2,3 exhibited a more severe phenotype for each assay than sg1 alone, presumably due to increased chance of producing a non-functional protein because of two targets sites within the same gene. Studies have shown that injection of two sgRNAs targeting the same gene in both Caenorhabditis elegans and mouse models are able to effectively delete regions up to 25kb long between the two target sites (Chen et al., 2014; Han et al., 2014). For each hand1 chromosome the two sgRNA sites are only 50-60bp apart from each other, therefore it is plausible that there is a larger deletion could occur in the embryos injected with sg1,2,3, which could account for the increased phenotype severity seen in sg1,2,3 injected embryos compared to sg1 injected embryos.

4.2 Hand1 is required for proper heart morphogenesis

Hand1 is required for heart development in mice (Firulli et al., 1998; Risebro et al., 2006; Srivastava et al., 1995), therefore we first sought to assess the role of Hand1 in Xenopus heart development. Loss of hand1 in Xenopus resulted in defective looping morphogenesis, with
phenotypes ranging from disrupted looping to complete loss of looping of the heart. The linear heart phenotype we observed is quite similar to the phenotype of tetraploid-rescue hand1 null mice (Riley et al., 1998). The similarities in heart phenotype provides evidence that hand1 has a conserved role in heart morphogenesis in Xenopus, and also provides confidence that the Hand1 protein is non-functional. Similarly, chimera Hand1-null mice with greater than 70% mutant Hand1 displayed hearts that did not loop, and those with 60-70% mutant Hand1 had a turned heart tube, however they were much smaller than controls and were still located in the midline (Riley et al., 2000). The Hand1 chimera mice with a mild looping defect is similar to the disrupted looping phenotype we observed in hand1 mutant Xenopus, thus these embryos would be expected to have a mosaic cardiac cell population in regard to functional Hand1. Furthermore, Hand1 chimera mice with less than 50% mutant Hand1 had morphologically similar hearts to those of controls (Riley et al., 2000). These results from Hand1-null chimera mice aligns with variance in phenotype severity in hand1 mutants due to mosaicism, as sufficient functional Hand1 within cardiac cells could rescue the phenotype. Furthermore, sg1,2,3 injected embryos showed an increase in heart phenotype severity compared to sg1 injected embryos which aligns with the idea that more cells have non-functional Hand1 in sg1,2,3 injected embryos. The smaller size of the turned heart in hand1 mutant embryos supports the hypothesized role of Hand1 in regulating the balance of cell proliferation and differentiation within cardiac cells (Risebro et al., 2006).

Interestingly, over expression of hand1 in Xenopus also led to disruptions in heart morphogenesis, with embryos displaying both linear phenotypes and disrupted looping phenotypes. However, the linear phenotype was less common in hand1 over expressing embryos compared to the frequency of linear hearts in hand1 mutants. Other studies showed that over expression of Hand1 in mice under the Hand1 reporter have abnormal looped hearts with an elongated outflow tract presumably due to increased cardiac cell proliferation (Risebro et al., 2006). Although there was not an overt increase in heart size of hand1 over expressing embryos, the heart was nonetheless larger in the defective looping phenotype of hand1 over expression compared to hand1 mutants. The lack of a stable overexpression of Hand1 throughout development could have attributed to the difference in phenotypes between mice and Xenopus. As shown previously, doxycycline-induced over expression of hand1 in adult mice hearts resulted in hypertrophy of the heart, however when doxycycline was removed, the hearts were able to revert back to their normal state (Breckenridge et al., 2009). As Hand1
levels were not maintained at consistent high levels throughout development, a decrease from over expressing Hand1 levels at the time of looping may have rescued or prevented defects due to Hand1 over expression.

I have further shown that the defects in heart morphogenesis are not due to alterations of cardiac cell fate. Expression patterns of isl1 and nkh2-5 were unaltered in both handl mutant and overexpression embryos, however quantification analysis would be required to further validate this. Although nkh2-5 has been shown to regulate handl expression (Fu et al., 1998), there does not appear to be any negative feedback of handl on nkh2-5 expression patterns in our Hand1 overexpression model. Interestingly, a study has shown that overexpression of VEGF in Xenopus resulted in defective cardiac morphogenesis, specifically 63% of embryos had a linear heart tube and similarly showed decreased handl expression in the heart (Nagao et al., 2007). Although this is only correlational, the loss of handl showcasing a similar phenotype supports the idea that nkh2-5 and VEGF may function to induce handl expression and loss of handl is ultimately what led to defects in heart morphogenesis.

Overall, handl does not appear to specify cardiac cell fate, but could function to regulate the balance between differentiation and proliferation as shown in in vitro cardiac cell models (Risebro et al., 2006). Although the role of Hand1 in heart development is still not fully understood, our data suggests that the function of Hand1 is conserved and precise control and regulation of Hand1 is critical for proper development of the heart.

4.3 Hand1 is required for formation of a complex vitelline network in Xenopus

As I have shown, handl expression is tightly correlated with the location of precursor endothelial cells expressing etv2 throughout development, and previous studies in our lab have shown a correlation between handl expression and the developing vascular plexus (Deimling and Drysdale, 2011). Thus, we predicted that handl plays a role in the development of this lineage. Loss of handl in Xenopus resulted in a decrease in both complexity and size of the vitelline veins. I further showed that the decrease in vascular complexity is likely caused by a decrease in the initial endothelial cell population early in development. The similar expression pattern of handl and etv2 throughout development provides further evidence of the relationship between handl and etv2. As apoptosis was absent in the region of vascular plexus development, the reduction of endothelial cells must then be due to disruptions in either cell
proliferation or differentiation. Additionally, we saw no difference in the development of the cardinal vein in *Xenopus*, which we expected as *hand1* is not expressed in the dorsal region of the embryo where the cardinal vein forms, and thus loss of *hand1* had no effect on cardinal vein development.

Based on *in vitro* studies using trophoblasts and cardiac cells, *hand1* plays an important role in regulating the balance between cell proliferation and differentiation of cell lineages (Risebro et al., 2006; Scott et al., 2000). Although *hand1* has contrasting roles in these cell models we speculate that *hand1* plays a similar role in the balance of proliferation and differentiation within the endothelial cells. Loss of Hand1 in cardiac cells led to a corresponding decrease in cyclin D2 (Risebro et al., 2006), therefore, it is plausible that Hand1 functions by regulating proliferation through the same mechanism within the endothelial cells. Cyclin D2 is involved in controlling cell proliferation such that decreases in cyclin D2 levels results in the halting of cell proliferation allowing cells to differentiate (Ruijtenberg and Van Den Heuvel, 2016). If Hand1 functions to regulate the levels of cyclin D2, then the loss of *hand1* and therefore cyclin D2 in cells would result in the early differentiation of cells and thus a smaller precursor population, as seen in *hand1* mutants.

Although in cardiac cells, over expression of Hand1 was associated with an increase in cyclin D2 and thus an increase in cell proliferation, over expression of Hand1 in *Xenopus* did not increase the population of endothelial cells or influence vasculature formation. It is plausible that different cell types could have different thresholds for the regulation of *hand1* and thus the levels of Hand1 within endothelial cells might not have been high enough to induce further proliferation.

Furthermore, over expression of *hand1* does not lead to mis-expression of vasculature cells, thus Hand1 does not appear to be sufficient to drive endothelial differentiation. Similarly, loss of Hand1 in mice did not result in a loss of endothelial cell markers in the yolk sac, supporting that Hand1 does not specify the endothelial lineage (Morikawa and Cserjesi, 2004). Tβ4 has been identified as a target of Hand1, however supplementation of Tβ4 in *Hand1*-null mice only appeared to rescue vasculogenesis of the yolk sac while angiogenesis of vessels still failed to occur (Smart et al., 2010). This study offers insights into the role of Hand1, suggesting that Hand1 can both regulate vasculogenesis and angiogenesis of blood vessels, however it appears to do this through separate mechanisms. This could explain why a complex vasculature plexus
in hand1 mutants was unable to form despite still expressing endothelial precursors, although they were ultimately still reduced in number. Therefore, Hand1 appears to be playing a more complex role in vasculature development in addition to regulating the balance between cell proliferation and differentiation. Examining proliferation markers such as Cyclin D2 within the vasculature plexus of hand1 mutant embryos may provide insight into the levels of proliferation within the endothelial cells, to confirm the role of Hand1 in regulating proliferation of endothelial cells during vasculature development.

4.4 Hand1 is dispensable for hematopoietic development in Xenopus

As Hand1 is vital for both heart and vasculature development, we next determined if Hand1 played a role within the development of the blood and myeloid cells. However, loss of hand1 had no effect on the differentiation of either myeloid or blood cell lineages. Similarly, loss of Hand1 in the yolk sac of mice resulted in normal blood formation despite defective vasculature development (Morikawa and Cserjesi, 2004). This result is not surprising based on hand1 expression patterns in Xenopus. Hand1 expression is not present in the ventral blood island region of the embryo at the time of blood differentiation, therefore this lineage does not appear to be dependent on hand1 expression, although the downregulation of Hand1 in this lineage appears critical for proper development. It is likely that myeloid cells also differentiate in areas devoid of hand1 expression. We predict that the small hole devoid of hand1 expression at the anterior-ventral side of the embryo at stage 20 corresponds to the region of the embryo where spib expressing cells originated, and thus could explain why loss of hand1 had no effect on their development.

Interestingly, when hand1 is overexpressed in Xenopus there is a significant decrease in myeloid and blood cells, however there is no noticeable difference in the expression of the progenitor blood cell marker scl. This unexpected loss of these two lineages could be attributed to hand1 acting as a regulator of proliferation and differentiation within these lineages, as seen in other cell models. Overexpression of hand1 could be preventing cell differentiation, which could explain why we see normal expression of blood progenitors despite a decrease in the differentiated blood cells. Alternatively, the ectopic expression of hand1 in hematopoietic cell lineages that normally exhibit a downregulation of hand1 during lineage differentiation could
result in inappropriate binding partner formation and thus inappropriate gene regulation within these lineages, thus preventing proper differentiation.

Over expression of hand1 in ectopic cells could deplete the pool of partner transcription factors thereby preventing transcription factors from activating genes required for lineage differentiation. Scl and hand1 have been shown using embryonic stem cell models to bind enhancer sequences of similar genes, however scl was found to bind similar enhancers with a greater affinity than hand1 (Org et al., 2015). They further suggest a model where differentiation of lineages requires binding of specific transcription factors to both silence genes of other lineages while promoting genes necessary for differentiation of the lineage of interest (Org et al., 2015). As there is no change in the expression of scl in hand1 over expressing Xenopus, the increased level of Hand1 could outcompete Scl for binding of genes required for erythrocyte differentiation resulting in the reduction of globin expression that was seen in Xenopus Hand1 mutants.

This study showed that Hand1 does not normally play a role in the development of myeloid or blood cells, however interpretation of the result of over expression of Hand1 on these lineages remains unclear, although abnormal binding and gene regulation of Hand1 could be implicated in the reason for the decrease of hematopoietic lineages.

4.5 Limitations and future directions

This study has shown that hand1 is required for proper heart morphogenesis and this requirement is conserved throughout species. Furthermore, Hand1 is required for the formation of a complex vasculature plexus in the embryo through maintaining proliferation of early endothelial cells. Finally, we show that Hand1 is not required for the development of the hematopoietic lineages, but downregulation of Hand1 is important for proper differentiation of these lineages. Overall, Hand1 appears to have tissue dependent roles, which most likely is due to the different binding partners available within each lineage, as well as post-translational modifications that alter the affinity of Hand1 dimerization partners. Future studies are still needed to elucidate the targets and binding partners of Hand1 that facilitate its downstream effects.
As mentioned previously, one of the disadvantages of utilizing the CRISPR/Cas9 system in *Xenopus* is the variation in mutations within the pooled population of mutant embryos. As Hand1 is a small protein, it would be interesting to use two guides to completely remove the gene from the genome or removing the start site of the protein could reduce variations in phenotype by eliminating potential indels resulting in a functional protein. Although TIDE provides good evidence that majority of the Hand1 protein should be non-functional, reducing the mosaicism may reduce phenotype variability to allow for greater clarification the role of Hand1.

Additionally, to further assess the development of the heart, either reconstruction of serial sections or confocal imaging could provide more insight into the precise defects that are occurring when Hand1 is altered in the embryo. Furthermore, as proper looping is necessary for proper heart chamber formation, studies looking at later development of the heart, and potential functional studies would provide better analysis as to consequences of hand1 loss in development of the heart. Additionally, staining for markers of proliferation within both the heart and the vasculature would allow for a better understanding of the role of Hand1 in regulating proliferation within these tissues.

Although over expression of Hand1 provided some insight into the role of Hand1 during development, over expression models can cause inappropriate actions in other tissues where it is typically not expressed. Unwanted binding of other proteins and abnormal activation or repression of nontarget genes make interpretation of over expression difficult. Over expressing Hand1 in tissue specific regions by injecting synthetic mRNA into specific cells at later stages could reduce abnormal binding partner formation and gene regulation by Hand1 in other lineages where Hand1 is not normally expressed.

Determining the targets of Hand1 will provide the insight into the mechanisms by Hand1 exerts its actions. Performing ChIP sequencing using a tagged Hand1 in *Xenopus*, will allow for the identification of Hand1 targets. By combining the results of ChIP sequencing targets with RNA sequencing of hand1 mutant embryos, we can determine which target genes are mis-regulated when hand1 is lost, and thus provide us with a more concise list of potential targets of hand1. Preliminary studies where I have over expressed Hand1-MT in *Xenopus* show a similar phenotype to that of untagged Hand1 over expressing embryos, thus showing the addition of a
Myc tag does not appear to effect Hand1 function and thus ChIP sequencing analysis using Hand1-MT would provide a robust list of potential Hand1 targets.

Although we are still far from a complete understanding of the molecular mechanisms by which Hand1 regulates development, this study confirmed the role of Hand1 within the heart is conserved and provided insight into a new function of Hand1 within the embryonic vasculature. Based on the decrease of endothelial cells in hand1 mutants, we speculate that Hand1 is required to keep cells in a proliferative state throughout development, ensuring proper development of lineages. Elucidating the targets and binding partners of Hand1 will provide better insight into how Hand1 functions throughout development and in disease states. As dysregulation of Hand1 function is correlated with development of congenital heart defects, understanding the molecular basis of Hand1 will be vital for beginning to prevent and treat these conditions.
Chapter 5: References


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