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# Regional Specification of the Xenopus Lateral Plate Mesoderm

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

# Regional Specification of the *Xenopus* Lateral Plate Mesoderm

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by

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## Abstract

Successful patterning of the embryo, from establishing the three primary axes to the regional specification of tissue progenitors is essential to generating a viable embryo. The three germ layers in the early embryo undergo patterning through slightly different mechanisms. The tissue of interest to this study is the lateral plate mesoderm (LPM), which will give rise to the lineages of the cardiovascular system and is essential for regional specification of adjacent germ layers. However, little is known about how the LPM itself undergoes regional specification and attains its intitial patterning after gastrulation. Here, I will demonstrate that a complex pattern of gene expression exists across the entire LPM shortly after gastrulation, much earlier than previously recognized. Furthermore, I will use molecular techniques to elucidate the signalling factors involved in the early patterning and regional specification the LPM. I hypothesize that both the retinoic acid (RA) and Fibroblast Growth Factor (FGF) signalling pathways are involved in the LPM regional specification in the neurula stage embryo. Through the use of exogenous modulators of the RA pathway, I will show that RA signalling is essential for patterning the anterior-dorsal and middle LPM domains. Secondly, by addition of a synthetic FGF receptor inhibitor I will demonstrate that FGF signalling is essential for establishing the anterior-ventral and posterior domains of the LPM and functions antagonistically to the RA pathway. I will also show that altering the activity of either of these two signalling pathways affects the specification of the early cardiovascular progenitors, particularly the cardiac and endothelial lineages. Finally I will provide preliminary evidence that one of the early LPM marker genes, *hand1*, is necessary for

normal cardiovascular development and thus provide a link between the early LPM pattern and later organogenesis. A thorough understanding of the mechanisms behind specifying embryonic lineages is of vital importance for basic biological knowledge, as well as for providing a basis for the emerging field of regenerative medicine, whereby researchers are attempting to generate organ progenitors *in vivo* to be used for cell therapies.

**Keywords:** *Xenopus laevis*, lateral plate mesoderm, cardiovascular system, retinoic acid, fibroblast growth factor, *Hand1*, patterning, specification

## Co-authorship

The initial observations that *hand1* was retinoic acid responsive contributed to a manuscript, published in 2006, primarily authored by Andrew Collop and Dr. Thomas Drysdale. Some excerpts appearing in this thesis are from a manuscript published in Mechanisms of Development, entitled "Retinoic acid regulates anterior-posterior patterning within the lateral plate mesoderm of *Xenopus*" co-authored by my supervisor, Dr. Thomas Drysdale. Other excerpts are from a manuscript, also co-authored by Dr. Thomas Drysdale, currently being revised for publication. I have personally performed all of the writing and experiments contained within this thesis. Dr. Drysdale's co-authorship has been attributed to his supervision and assistance in designing experiments, analysis of results and the generation of scientific manuscripts of publishable quality.

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## Table of Contents

## List of Figures

## List of Tables

## List of Abbreviations, Symbols and Nomenclature

| | |
|---|---|
| °C | degrees Celcius |
| α | alpha (Greek letter) |
| β | beta (Greek letter) |
| γ | gamma (Greek letter) |
| μg | Microgram |
| μl | Microliter |
| nl | Nanoliter |
| ADH | Alcohol Dehydrogenase |
| AGN 193109 | Retinoic acid receptor antagonist |
| AGN 194301 | Retinoic acid receptor alpha specific antagonist |
| cDNA | complementary deoxyribonucleic acid |
| *cTnI* | *Cardiac troponin I* gene |
| DEAB | Diethylaminobenzaldehyde: RALDH2 inhibitor |
| $dH_2O$ | distilled, autoclaved water |
| DIG | Digoxygenin |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| *et al*. | and others (Latin) |
| IU | International units |
| LPM | Lateral plate mesoderm |
| M | Molar |

| | |
|---|---|
| MAB | Maleic acid buffer |
| mM | Millimolar |
| mRNA | Messenger Ribonucleic acid |
| NTP | Nucleoside triphosphate |
| PCV | Posterior cardinal vein |
| RA | *all trans* retinoic acid |
| *Raldh2* | *Retinaldehyde dehydrogenase 2* |
| RAR | Retinoic acid receptor |
| RARE | Retinoic acid response element |
| RLS | Rostral Lymph Sac |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |
| RXR | Retinoid X receptor |
| st. | Embryonic stage |
| SU5402 | Fibroblast growth factor receptor inhibitor |
| TBS | Tris-buffered saline |
| TGF-β | Transforming growth factor beta |
| VAD | Vitamin A deficient |

# Chapter 1 Introduction

The complexity of patterning within the early LPM of *Xenopus*, immediately after gastrulation, has thus far been under appreciated. While some pattern has been described with respect to the early cardiac progenitors, the remainder of the LPM is viewed as a mostly homogenous tissue layer with little appreciable pattern. Here, I describe a novel patterning event occurring shortly after gastrulation that leads to a complex pattern of gene expression in both the anterior-posterior and dorsal-ventral axes of the early frog LPM. This suggests that regional specification of the LPM may be occurring much earlier than previously thought. Elucidating the mechanism of this early patterning event, and its relevance to later development is of interest both to basic biology, as well as to the *in vitro* derivation of cardiovascular stem cells for modern cell therapies.

## 1.1 The Early Frog Embryo.

*Xenopus laevis* has become one of the most popular models for use in early embryogenesis. However, a highly related amphibian species, *Xenopus tropicalis* has been gaining popularity in recent years due to its diploid nature and shorter generation time, making *Xenopus tropicalis* much better suited to genetic studies that *Xenopus laevis*. The *Xenopus laevis* egg is relatively large with a 1.3 mm diameter (1 μL volume), although those of some other anuran species, such as *Eleutherodactylus coqui* (diameter of 3.5 mm, volume of 20 μL) or those of amniotes, such as reptiles or birds can be significantly larger. However, unlike amniote embryos, once fertilized the entire volume of the *Xenopus* embryo will contribute to the embryo proper, making the early embryo itself comparatively large.

One of the biggest advantages to studying the *Xenopus* embryo is that once the eggs are laid, embryonic development occurs completely external to the body allowing direct access to the embryo during all stages of development. Amniote embryos that either develop in hard shells (birds and reptiles) or develop internally (mammals) are much more difficult to observe and manipulate. The easy access to embryos and comparatively large size are advantageous for many reasons, including easy use of specific inhibitors of signalling pathways, physical manipulations and live imaging.

Visually, the frog egg has two distinct poles: a darkly pigmented animal half, which upon fertilization will face upward and a lighter pigmented vegetal half (Figure 1A). The lighter pigmented vegetal pole contains more of the embryonic yolk that will provide nourishment to the embryo until it reaches the feeding tadpole stage approximately four to five days post fertilization. In addition to the yolk, the egg also contains enough maternal mRNA to sustain development until embryonic transcription begins at the mid-blastula transition.

The frog embryo is, like all higher organisms (from flat worms to mammals), a triploblastic embryo. Triploblastic embryos are composed of three germ layers: the ectoderm (the outer most germ layer), the endoderm (the inner most germ layer) and the mesoderm (the middle germ layer). Cells arising from the animal and vegetal poles of the one cell embryo become either the ectoderm (animal pole) or endoderm (vegetal pole) in isolation (Nieuwkoop, 1969). The mesoderm is induced later as a result of complex interactions between the cells of the vegetal and animal poles.

## 1.2 Establishing the Early Axes

Patterning of the three-primary axes (dorsal-ventral, rostral-caudal, and left-right) occurs early in the *Xenopus* embryo. In the *Xenopus* embryo the dorsal-ventral axis is the back to belly axis, while the rostral-caudal axis refers to the head-tail axis. The dorsal-ventral and anterior-posterior axes are somewhat linked and are established prior to the first cleavage division. However, establishing the left-right axis does not occur until later during gastrulation (approximately 10 hours post fertilization). Since this thesis discusses axial patterning across a tissue layer, a discussion on the topic of primary axis determination in the embryo is relevant.

### 1.2.1 The Dorsal-Ventral Axis

The dorsal-ventral axis is specified very early in development by events that are set in motion by the sperm entering the egg. Following fertilization of the egg, the outer cortex rotates by approximately 30° in relation to the inner core (Vincent et al., 1986). This process is termed cortical rotation and is dependent upon proper formation of a cortically located microtubule network (Charron et al., 1999; Elinson and Rowning, 1988). Cortical rotation leads to the localization of maternal *wnt11* mRNA (Tao et al., 2005), enrichment of *dishevelled* (Miller et al., 1999) and stabilization of β-catenin on the future dorsal side of the embryo (Figure 1A). How *wnt11* is localized to the dorsal side remains an unanswered question. It is currently thought that, upon fertilization, the microtubule network assembles and particles located at the vegetal pole of the embryo, namely GSK-binding protein, *dishevelled* and *wnt11*, are transported by a combination of cortical rotation (Scharf and Gerhart, 1980) and active transport along the microtubule

network (Miller et al., 1999; Weaver et al., 2003) away from the sperm entry point and toward the equator. The enrichment of these factors then leads to the stabilization of β-catenin and activation of the canonical *Wnt* signalling pathway on the future dorsal side of the embryo (reviewed in (Weaver and Kimelman, 2004; White and Heasman, 2008)).

At the initiation of gastrulation the dorsal lip becomes an essential signalling centre for specifying the dorsal pole of the embryo. When the dorsal lip is transplanted into a recipient embryo, it has the ability to ectopically induce a secondary axis (Spemann and Mangold, 1924). The Spemann-Mangold organizer secretes inhibitors of the bone morphogenic protein (BMP) pathway: Noggin (Zimmerman et al., 1996), Follistatin (Fainsod et al., 1997) and Chordin (Piccolo et al., 1996). It also secretes antagonists of the *Wnt* pathway: Dickkopf 1 (Dkk-1) (Glinka et al., 1998) and Frizzled-related protein (Frzb-1) (Leyns et al., 1997) and Nodal antagonist: Lefty (Sakuma et al., 2002) among others. These signalling molecules are also essential to anterior-posterior patterning (as discussed in 1.2.2), and therefore links dorsal-ventral and anterior-posterior axis specification.

### 1.2.2 The Anterior-Posterior Axis

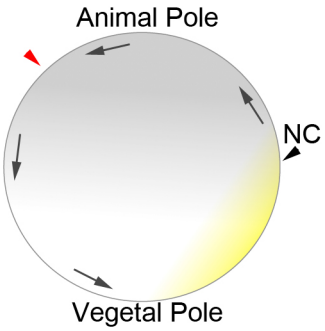
The anterior-posterior axis is tightly linked to the dorsal-ventral axis. The embryonic mesoderm that arises opposite to the sperm entry point, and where β-catenin becomes stabilized following cortical rotation, is not only fated to become dorsal, but also the anterior-most mesoderm cells of the embryo. That is, these future head mesoderm cells will travel through the dorsal lip of the blastopore first, and will travel the furthest towards the anterior end of the embryo.

The first active patterning process of the anterior-posterior axis occurs during gastrulation, as a result of signals originating from the dorsal lip. However, the organizer is not a static signalling center, and its ability to induce tissue changes with time, first hinted at by Hans Spemann who described the dorsal organizer (Spemann, 1938). The same inhibitors involved in specifying dorsal are also involved in specifying the anterior-posterior axis, although different combinations are required throughout the axis, leading to the suggestion that there may be three distinct organizers of the anterior-posterior axis (for a discussion see; (Niehrs, 2004)). To specify the head, antagonists to all of BMP, Wnt and Nodal pathways are required (Khokha et al., 2005; Piccolo et al., 1999), while the trunk requires both Nodal and canonical Wnt signalling (Agius et al., 2000; Hoppler et al., 1996). Finally, the tail, recently described in zebrafish, requires high levels of active BMP, Wnt and Nodal signalling (Agathon et al., 2003).

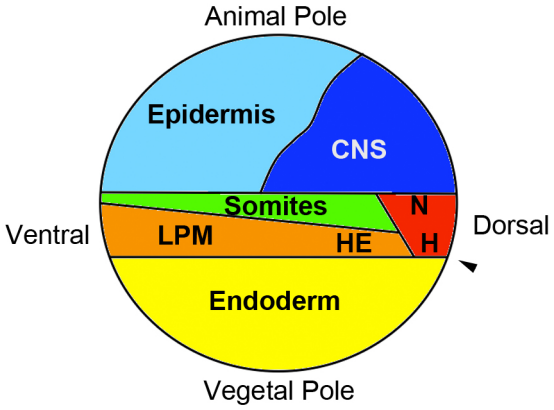
### 1.2.3 The Left-Right Axis

The intial symmetry breaking process of the left-right axis is generally considered to be generation of a leftward fluid flow via the actions of beating cilia in the node (gastrocoel roof) of *Xenopus* (Essner et al., 2002; Schweickert et al., 2007). This cilia-based symmetry-breaking event appears to be conserved in all vertebrates tested (Essner et al., 2002; Neugebauer et al., 2009; Nonaka et al., 1998; Okada et al., 1999). The leftward fluid flow in the node leads to a cascade of Nodal signalling (a member of the transforming growth factor β (TGFβ) super family) in the left side of the embryo that culminates in *pitx2* expression (Lohr et al., 1997; Lustig et al., 1996a; Ryan et al., 1998).

**Figure 1.** Early development of *Xenopus laevis*. A) One cell stage embryo (Stage 1). The sperm penetrates the egg on the vegetal pole, which leads to cortical rotation (grey arrows) away from the sperm entry point. This process culminates in the stabilization of β-catenin (yellow shading) on the side opposite the sperm entry point, and active Wnt signalling on the future dorsal side of the embryo. NC: Nieuwkoop Center. B) Blastula stage embryo (Stage 8). Fate map of the early gastrula embryo, showing the major components of the mesoderm in the marginal zone: the head mesoderm and notochord in red, somites in green, and lateral plate mesoderm in orange, with heart field marked. Position of the future dorsal lip is shown (black arrow). CNS: central nervous system, LPM: lateral plate mesoderm, H: head mesoderm, HE: heart, N: notochord. C) Early tail bud stage embryo (Stage 20). Previously described LPM patterning of cardiovascular progenitors. HE: heart, HEM: hemangioblasts, M: myeloid cells. D) Location of the three lineages of the cardiovascular system of interest to this thesis in the tail bud stage embryo. HE: heart, PCV: posterior cardinal vein, VP: vascular plexus, BI: blood islands (erythrocyte lineage).


Animal Pole
NC
Vegetal Pole

A


Animal Pole
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CNS
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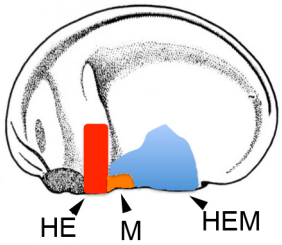
LPM
HE
H
Dorsal
Endoderm
Vegetal Pole

B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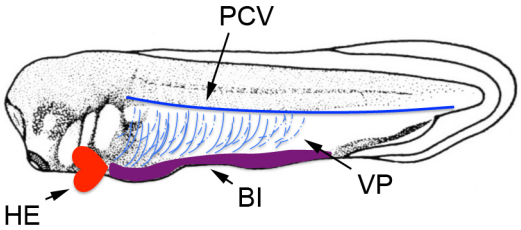

PCV
HE
BI
VP

D

However, recent reports have also implicated ion channels in the very early embryo to be necessary for generating left-right asymmetry (Adams et al., 2006; Aw et al., 2008; Aw et al., 2010; Levin et al., 2002). The mechanisms connecting early ion channel expression/function to leftward fluid flow in the node is currently unknown.

### 1.3 The Mesoderm

The mesoderm is the middle of the three germ layers. Immediately following gastrulation the mesoderm will form, from dorsal to ventral, the axial mesoderm (notocord and somites), the intermediate mesoderm (although this is not a distinct tissue in *Xenopus*), and the lateral plate mesoderm (LPM). Since the focus of this thesis is on the LPM, a discussion of the upstream events leading to LPM specification is pertinent.

The LPM is a subdivision of the embryonic mesoderm, that is present on the lateral and ventral sides of the embryo after gastrulation. By the end of neurulation the LPM will separate into two distinct layers, the outer somatic, and the inner splanchnic mesoderm with the body cavity (coelom) forming between them. The somatic layer forms the lining of the body cavity while the splanchnic layer forms the circulatory system and the mesenchyme surrounding the gut. The lateral plate mesoderm is also essential for proper regional specification of the underlying endoderm during the early tailbud stage of *Xenopus* development (Horb and Slack, 2001), which strongly suggests that the LPM itself must have anterior-posterior polarity before the mid tail bud stage. Therefore proper patterning of the LPM is crucial to both the mesodermal lineage, but likely also the underlying endodermal lineage. However, The mechanism and timing of LPM patterning is currently not well understood.

### 1.3.1 Mesoderm Induction

Roughly four decades ago, Pieter Nieuwkoop demonstrated that neither the animal pole, nor vegetal pole, could form mesoderm tissues in isolation (Nieuwkoop, 1969; Sudarwati and Nieuwkoop, 1971). More specifically, Nieuwkoop demonstrated that the mesoderm formed from the animal cap ectoderm as a result of an inductive signal from the endoderm of the vegetal pole. The inductive signal was later shown to be a maternal determinant, present in the ventral pole of the embryo long before the onset of zygotic transcription (Jones and Woodland, 1987).

A handful of genes have been implicated in mesoderm induction. Two genes that have been thoroughly studied, and are both present in the maternal pool of RNA are *growth differentiation factor 1* (*gdf1*; previously *Vg1*) and *vegt*. *Gdf1*, a Tgf β family member, has potent mesoderm inducing activity *in vivo* (Birsoy et al., 2006) in *Xenopus* as well as a number of other model organisms (Shah et al., 1997; Skromne and Stern, 2001). Furthermore, *gdf1* depleted embryos also show significantly reduced mesodermal gene expression during the late blastula stages suggesting that *gdf1* may also be necessary in mesodermal specification (Birsoy et al., 2006).

*Vegt* encodes a T-box transcription factor that is also a maternally supplied mRNAs and is vegetally localized in both the mature oocyte and early embryo (Lustig et al., 1996b; Stennard et al., 1996; Zhang and King, 1996). Loss of Vegt leads to a loss of endodermal marker expression and reduced ability for isolated *vegt* $^{-/-}$ vegetal pole explants to induce wild type animal pole caps to form mesoderm, and in severe cases, a complete loss of gastrulation (Kofron et al., 1999; Zhang et al., 1998). Interestingly,

depleting *vegt* mRNA disrupts the vegetal localization of other mRNAs important for early patterning including both *wnt11* and *gdf1* (Heasman et al., 2001).

After the mid-blastula transition, *vegt* initiates mesoderm induction through the regulation of specific TGFβ family members, such as the *Xenopus* Nodal genes (*nodal1, nodal2* and *nodal4*) and *derriere* (Clements et al., 1999; Kofron et al., 1999). Interestingly, *vegt*, *nodal1* and *nodal2* are responsive to canonical Wnt signalling through β-catenin, leading to these genes being more highly expressed in the presumptive dorsal side of the embryo (Agius et al., 2000). The area of overlap between Vegt and β-catenin activity is commonly called the Nieuwkoop center, or the area that will initiate the formation of the Spemann Organizer. Mesodermal genes, such as the T-Box genes *Brachyury* (*T*; *bra*) and *Eomesodermin* (*Eomes*) are activated downstream of a *vegt* and *nodal* signalling cascade leading to mesoderm specification (Kofron et al., 1999; Ryan et al., 1996; Smith et al., 1991).

### 1.3.2 Early Mesoderm Patterning and Fate Map

A great deal of research has been completed to create a fate map and locate cells destined to become each of the various mesodermal lineages as far back in development as the early blastula. Correlations have been made as to the location of cells with respect to the organizer, thus placing developmental 'fields' along a dorsal-ventral axis. In the late blastula stages only two definitive domains are present in the marginal zone: the organizer, characterized by expression of genes such as *noggin* (Smith and Harland, 1992) and *nodal3* (Smith et al., 1995), and the remaining marginal zone characterized by expression of *bmp4* (Fainsod et al., 1994) and *wnt8* (Christian et al., 1991). However, by

the early gastrula the marginal zone has begun to be subdivided along the dorsal-ventral axis as demonstrated by the differential expression of *vent1* (Gawantka et al., 1995), *myf5* (Dosch et al., 1997) and *sizzled* (Salic et al., 1997).

The current fate map of the *Xenopus* marginal zone has refined where specific mesodermal lineages arise from the early blastula embryo, by employing a variety of techniques including fate mapping with fluorescent dyes and explant strategies. The head and notochord mesoderm lay adjacent to the dorsal lip, while the somitic and lateral plate mesoderm lineages are arranged adjacent to one another in the animal to vegetal axis (Figure 1B) (Keller, 1991; Lane and Sheets, 2000; Lane and Smith, 1999). However, it should be noted that this fate map does not suggest that the cells inhabiting any of the presumptive mesodermal fields of the late blastula are patterned into any specific lineage. In fact, recent data suggests that these cells are not subdivided into the lineages of mesodermal derivatives until much later (discussed in more detail in section 1.4).

### 1.4 The Cardiovascular System and the Lateral Plate Mesoderm

The cardiovascular system is one of the first major organ systems to undergo specification and organogenesis during development. The cardiovascular system is also derived entirely from the lateral plate mesoderm. Recently there has been considerable interest in describing the signalling events that lead to the subdivision of the cardiovascular lineages from the mesodermal progenitors. For the purpose of this thesis, the cardiovascular system will generally be subdivided into three broad lineages: cardiac (or heart), endothelial (or vasculature) and hematopoietic (or blood). Although, note that in each case these lineages could be further subdivided. Recent data has suggested that

the myocardial, endothelial, hematopoietic, and smooth muscle cells (another vasculature lineage that differentiates after the endothelial lineage) are specified from a common progenitor pool in the lateral plate mesoderm of mouse (Kattman et al., 2006; Moretti et al., 2006), although this has yet to be demonstrated in *Xenopus*. Interestingly, both the heart (Tonissen et al., 1994) and the hemangioblast lineage (cells which will give rise to vascular and embryonic blood) (Mead et al., 1998) are detectable shortly after gastrulation (Figure 1C). However, while distinct populations of cells are identifiable by lineage specific gene expression, these progenitor cells remain somewhat plastic and are not determined until the early to mid tailbud stage of development, roughly six to eight hours later (Figure 1D).

Defining the cellular events that lead to specification of the three cardiovascular lineages is of direct clinical relevance. Recently, there have been a number of groups which have reported identifying cardiac stem cells in mammalian organisms, however clinical trials utilizing these stem cells in the treatment of cardiovascular disease has thus far not been encouraging (reviewed in (Tran et al., 2010)). Therefore, defining the combinatorial signals required to specify each lineage has become of great interest as it may allow researchers to derive a homogenous source of cardiovascular stem cells *in vitro* that may allow for better clinical outcomes when transplanted into patients.

The frog model is a convenient model to study early cardiovascular development. Mutations in amniote embryos that disturb early cardiovascular development tend to be early embryonic lethal, making a detailed analysis difficult. However, the *Xenopus* embryo is an incredibly resilient model able to survive until the swimming tadpole stages with major perturbations of the cardiovascular system, or even a complete ablation of the

heart (Copenhaver, 1926) . This enables researchers to alter one of the three lineages of the cardiovascular system, and subsequently assay for effects on specification of the other two lineages at later time points, a concept that is difficult to accomplish in some other model systems.

### 1.4.1 Early Cardiac Development

The heart develops from two patches of anterior-dorsal lateral plate mesoderm cells at the early neurula stage *Xenopus* embryo. In the approximately four hours after gastrulation these cells migrate ventrally to fuse at the ventral midline and form the heart field (Sater and Jacobson, 1990). In mammals and birds, the stage at which fusion occurs is called the cardiac crescent stage. At the crescent stage there are two distinct populations of cardiac cells, the primary heart field which will form the primary heart tube, and the secondary heart field (just anterior to the primary heart field) which migrates into the heart tube and contributes to the right ventricle and outflow tract (Cai et al., 2003). This secondary heart field has also recently been described in *Xenopus* (Gessert and Kuhl, 2009). The sheet of cells that represents the first heart field will then form the myocardial trough during mid tailbud stage (stage 29/30), and fuse dorsally to form the heart tube shortly thereafter (stage 32) (Mohun et al., 2000). Once the tube is formed it will quickly begin to loop in a leftward fashion, the earliest morphological consequence of the embryonic left-right axis. After the heart has looped, the walls of heart will undergo hypertrophy and thicken considerably. The atria and ventricles then undergo septation (although in frog there is a single ventricle) and the spiral valve and atrioventricular valves form (Mohun et al., 2000).

The signalling mechanisms required for heart specification and determination are complex, and in most cases are still not well understood. It has been well established that an interaction between the pre-cardiac mesoderm and adjacent axial mesoderm and endoderm is essential for inducing cardiac cell fate in *Xenopus* (Nascone and Mercola, 1995) as well as in chick (Schultheiss et al., 1995; Yatskievych et al., 1997) and mouse (Arai et al., 1997). While the relative contribution of specific signalling molecules is still uncertain, three signalling pathways have been implicated: the FGF, Wnt and BMP signalling pathways. While there is support for BMP signalling being involved in cardiac induction in both zebrafish (Reiter et al., 2001) and chick (Schlange et al., 2000; Schultheiss and Lassar, 1997), in *Xenopus* BMP signalling does not appear to be involved in heart field induction but rather maintenance of cardiac identity (Mandel et al., 2010; Walters et al., 2001). Non-canonical Wnt signalling has also been implicated in early heart specification, as a loss of *wnt11* in frog leads to a loss of early heart field markers, while *wnt11* is able to induce cardiogenesis in animal cap explants (Afouda et al., 2008; Pandur et al., 2002). Although Wnt signalling has also been shown to be detrimental after initial specification (Samuel and Latinkic, 2009). Lastly, FGF signalling has recently been implicated in heart field specification in frog (Keren-Politansky et al., 2009; Samuel and Latinkic, 2009), as well as zebrafish (Marques et al., 2008; Reifers et al., 2000) and chick (Alsan and Schultheiss, 2002).

The first marker of cardiac mesoderm in frog is *nkx2-5* (Tonissen et al., 1994), which is expressed throughout the presumptive myocardium and pharyngeal mesoderm. The *Drosophila* homologue *Tinman* was the first gene shown to be absolutely necessary for heart specification (Bodmer, 1993; Bodmer et al., 1990). While flies mutant for

*tinman* completely lack cardiac mesoderm, mice lacking *nkx2-5* specified cardiac tissue and a heart tube was still formed, though the tube does not loop and the embryos die of cardiac insufficiency (Lyons et al., 1995). This may be due to some functional redundancy between *Nkx* family members, such as *nkx2-3* which is also expressed in the early heart field in both frog (Cleaver et al., 1996) and chick (Buchberger et al., 1996). Studies in *Xenopus* where both *nkx2-5* and *nkx2-3* function was inhibited demonstrated a synergistic phenotype of the double mutant (Fu et al., 1998), suggesting at least some degree of functional redundancy. Furthermore, over expression of either *nkx2-3* or *nkx2-5* in *Xenopus* leads to an increased number of myocardial cells (Cleaver et al., 1996). This functional redundancy between vertebrate *Nkx* family members may explain the phenotypic anomaly between flies and vertebrates upon loss of *nkx2-5*. However, *Nkx2-3* is not expressed in the early mouse heart (Pabst et al., 1997), suggesting another family member may be responsible, if functional redundancy exists between *Nkx* family members in the mouse heart.

In *Xenopus*, expression of early cardiac markers is detectable shortly after gastrulation but cardiac differentiation does not occur until roughly 24 hours later with the onset of *cardiac troponin I* (*cTnI*) (Drysdale et al., 1994) and *myosin heavy chain alpha* (*MHCα*) (Logan and Mohun, 1993) expression. While the transcriptional control networks connecting early cardiac specification to later differentiation remain incomplete, one key family of genes is the *GATA* family. *Gata4*, *5*, and *6* have overlapping expression patterns during early heart and endodermal development in *Xenopus* (Jiang and Evans, 1996). *Gata4* has also been implicated in the control of *nkx2-5* gene expression (Jiang et al., 1999; Lien et al., 1999) and evidence suggests that *GATA* members can act as

cofactors with *nkx2-5* (Durocher et al., 1997; Sepulveda et al., 1998). Like the *Nkx* gene family, loss of function experiments have shown a high degree of functional redundancy between *GATA* family members. In *Xenopus*, all of the *gata4, 5,* and *6* genes function in the maturation and differentiation of the myocardium, with differing levels of redundancy (Peterkin et al., 2003; Peterkin et al., 2007). However, *gata5* is the only single morphant to have an affect on early heart progenitors in *Xenopus* (Haworth et al., 2008), whereas loss of *gata4* and *6* does not affect early expression of the cardiac specification markers *nkx2-5* and *nkx2-3* (Peterkin et al., 2007). It is worth noting that the role of *GATA* factors in cardiac differentiation and the high degree of functional redundancy is also highly conserved in both zebrafish (Holtzinger and Evans, 2007; Peterkin et al., 2007) and mice (Watt et al., 2004; Zhao et al., 2008; Zhao et al., 2005). Several other transcription factors have been implicated in regulating early cardiogenesis but a full discussion of these is beyond the scope of this thesis. These transcription factors include members of the *T-box* Family (Horb and Thomsen, 1999; Stennard et al., 2003; Yamada et al., 2000), *myocyte enhancer factor 2* (*mef2*) (Edmondson et al., 1994; Ghosh et al., 2009; Vincentz et al., 2008) and *Iroquois 4* (*Irx4*) (Bao et al., 1999; Bruneau et al., 2000).

Until now the focus of this discussion has been on the *nkx2-5* expressing cells of the cardiac crescent that form the first or primary heart field. However there is another population of mesodermal cells that down regulate the expression of *nkx2-5*, yet still contribute to the mature heart. This population of cells was termed the secondary heart field, because these cells do not migrate into the heart until later in development, after the presumptive myocardium has formed a tube (de la Cruz et al., 1977; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). This concept of a second, or later heart field

has been further refined with the characterization of the gene *Islet 1* (*isl1*). Mice lacking *isl1* are characterized by a loss of the outflow tract, right ventricle, and a large part of the atria, while the remainder of the heart is still intact (Cai et al., 2003). When *isl1* was inhibited in *Xenopus* with an antisense morpholino, the loss of function phenotype was much more severe than that reported for mouse and included defects in tissues of the primary heart field and vasculogenesis, leading the authors to suggest a much larger role for *isl1* than simply regulating the second heart field (Brade et al., 2007). A subsequent analysis of heart field markers in *Xenopus*, as well as lineage tracing the anterior-and posterior heart field has since demonstrated a clear divide between markers of the first and second heart field during the tail bud stage of development (Gessert and Kuhl, 2009), although no functional studies have been accomplished with these markers with the intent of understanding their role in the secondary heart field.

### 1.4.2 Early Endothelial Development

Endothelial progenitors have traditionally been thought to arise from a separate pool of mesodermal progenitors, termed the hemangioblast. The hemangioblast lineage, that will give rise to both endothelial and erythroid lineages, is first detectable shortly after gastrulation by the expression of *T-cell acute lymphatic leukemia 1* (*tal1*) (Mead et al., 1998) and *Ets variant* 2 (*etv2*) (Salanga et al., 2010) on the ventral side of the embryo shortly after the heart field is first detectable. However, it has recently been realized that the heart and vascular lineages might be much more closely linked than first appreciated. Evidence that the cardiovascular progenitors are initially generated from a common precursor arises from recent studies in mice examining *VEGFR-2*, an early marker of the

vascular lineage. Recently, it has been shown through use of a VEGFR2 reporter transgene that VEGFR2 is widely expressed in the early mesoderm during gastrulation as well as in cells of the cardiac crescent (Ema et al., 2006). This suggests the presence of a common mesodermal precursor between the cardiac and vascular populations.

The fully developed vascular system is formed by two distinct processes: vasculogenesis, the *de novo* differentiation of endothelial cells from a mesodermal progenitor, and angiogenesis, the sprouting of new blood vessels from pre-existing vessels. Many genes have been implicated in being important for endothelial development or for angiogenesis, but few have been shown to be necessary for initial vascular specification. However, there is evidence that a small number of transcription factors play an important role in vascular specification, including the Ets factors *fli1* and *etv2*. *Etv2*$^{-/-}$ mice die at midgestation with a loss of endothelial progenitors and without any detectable embryonic vessels suggesting that *Etv2* is necessary for initial endothelial specification (Lee et al., 2008). However, *y11* mutant zebrafish, harboring a mutant *etsrp* gene (the zebrafish homologue of *Etv2*), display a less severe phenotype, as they do specify endothelial cells, although the vascular cells fail to undergo tubular morphogenesis (Pham et al., 2007; Sumanas et al., 2008). The discrepancy between the mouse and zebrafish phenotypes could be, at least in part, due to redundancy between several Ets related factors (Pham et al., 2007).

### 1.4.3 Primitive Hematopoietic Development

The primitive blood islands form on the ventral side of the embryo and can be classified into two separate populations in early *Xenopus* development, the anterior blood islands (primitive myeloid cells), and the posterior blood islands (primitive erythroid

cells). The anterior blood islands are first detectable in the mid neurula stage embryo through expression of *tal1* (Mead et al., 1998). By the end of neurulation the anterior-ventral blood islands begin to down regulate expression of *tal1* and express markers of the myeloid lineage such as *mpo* (formerly *Xenopus peroxidase 2* (*Xpox2*)) (Smith et al., 2002) and *spib* (Costa et al., 2008) as they differentiate. Shortly after, the myeloid cells will migrate away from the anterior-ventral region and become diffuse throughout the entire embryo (Smith et al., 2002; Tashiro et al., 2006).

The posterior-ventral blood islands are present after the end of neurulation and are visualized as the remaining pool *tal1* expressing hemangioblast cells. The expression pattern of *tal1* now extends much further posterior on the ventral side. Primitive erythroid differentiation in the posterior-ventral blood islands does not occur until roughly a day later with the onset of *α-globin* expression, which begins at the anterior end of the hemangioblast domain, and proceeds in a wave of differentiation toward the posterior end (Mills et al., 1999).

The signalling pathways that regulate primitive erythroid development have been studied thoroughly both in frog and other model organisms, but questions do remain as to how hematopoietic development is specified in the LPM. Proper induction of primitive erythroid development requires signals from both the endoderm and ectoderm in *Xenopus* (Kikkawa et al., 2001). The BMP (Maeno et al., 1996; Schmerer and Evans, 2003), and FGF (Isaacs et al., 2007; Walmsley et al., 2008) signalling pathways have both been implicated in erythroid specification. As well a number of genes essential to early hematopoietic development have been identified in vertebrates, including *VEGRF2* (Shalaby et al., 1997; Shalaby et al., 1995), *tal1* (D'Souza et al., 2005; Endoh et al., 2002;

Liao et al., 1998; Mead et al., 1998; Porcher et al., 1996) and *etv2* (Liao et al., 1997; Stainier et al., 1995; Thompson et al., 1998). It is interesting that all of these genes have also been implicated in vascular development (discussed above), which is supporting evidence for the existence of a hemangioblast-like lineage in vertebrates. Furthermore, a number of GATA family members have been implicated in hematopoiesis, in addition to the heart and vasculature (see above) demonstrating a similar transcriptional regime in all three of the cardiovascular lineages. However, in hematopoiesis, *Gata1-3* have been implicated in development instead of *Gata4-6* genes important for the heart and vasculature (Bertwistle et al., 1996; Dalgin et al., 2007; Kelley et al., 1994). A signalling hierarchy is beginning to form in hematopoiesis, as BMP signalling during gastrulation has been shown to be upstream of *Fli1* (Liu et al., 2008) an Ets related transcription factor, that in turn regulates expression of both *tal1* (Liu et al., 2008; Mead et al., 1998) and *gata2* (Dalgin et al., 2007; Liu et al., 2008) in the hematopoietic lineage.

### 1.5 The Retinoic Acid Signalling System

*All trans* retinoic acid (RA, Figure 2A) is the biologically active metabolite of vitamin A (Mic et al., 2003). RA is synthesized *in vivo* from a precursor, retinal, by the actions of alcohol dehydrogenase (ADH) that catalyzes the conversion of retinol to retinal, and then by retinaldehyde dehydrogenase 2 (RALDH2), that converts retinal into RA and is the predominant RA synthesizing enzyme *in vivo* (Figure 2C) (Haselbeck et al., 1999; Mic et al., 2003). Once synthesized, RA will bind to the retinoic acid receptors (RAR α, β, or γ) that will dimerize with the retinoid X receptors (RXR α, β, or γ) and act as ligand activated transcription factors. The RARs

are nuclear hormone receptors with three important domains, a DNA binding domain, a ligand binding domain, and the transactivating domain that interacts with the RXRs (See figure 2B). The RAR/RXR heterodimer will bind retinoic acid response elements (RAREs: direct repeats of TGACCT) in the promoter regions of retinoic acid responsive genes. After the RAR/RXR heterodimer has bound its ligand (*all-trans* RA) it recruits the histone acetyl transferase (HAT) complex (along with other transcriptional co-factors) allowing chromatin to open and transcription to commence (Chambon, 1996; Petkovich et al., 1987).

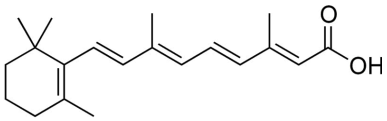
In addition to being an activator of gene expression, the non-ligand bound RAR/RXR heterodimer is required in a number of tissues not normally exposed to RA to act as a represser of transcription. The un-liganded form of the RAR/RXR heterodimer recruits the histone deacetylase (HDAC) complex and causes the regionalized compaction of DNA (Heinzel et al., 1997; Nagy et al., 1997). The retinoic acid signalling pathway is highly conserved throughout evolution and is essential for normal development (for a discussion of the evolutionary conservation see: (Albalat, 2009; Campo-Paysaa et al., 2008)).

### 1.5.1 Vitamin A Deficiency

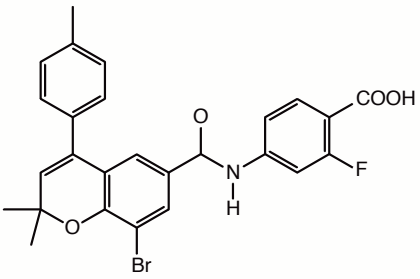
Retinoic acid was initially implicated as being essential for normal development by studying offspring of vitamin A deficient (VAD) pigs (Hale, 1935) and rats (Warkany and Schraffenberger, 1946). In these studies, female rats were kept on a diet free of vitamin A prior to and throughout pregnancy such that the

**Figure 2.** The retinoic acid signalling pathway. A) The chemical structure of the synthetic molecules used in this thesis to alter retinoic acid signalling. *All trans* retinoic acid is an agonist that will increase retinoic acid signalling. AGN 194301 is an RARα specific antagonist, while AGN 193109 is a pan-RAR antagonist. B) Generalized structure of the RAR receptors, with the DNA binding domain (DBD) and ligand binding domain (LBD) illustrated. C) Schematic of the retinoic acid signalling pathway, the predominant enzymes responsible for each step in the pathway are shown underlined above the arrows.

**A**

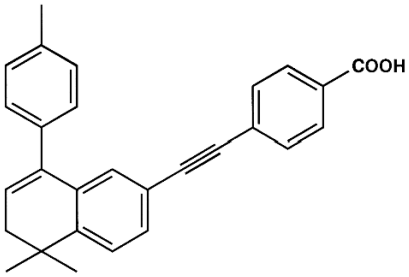



*All trans* Retinoic Acid




AGN 194301

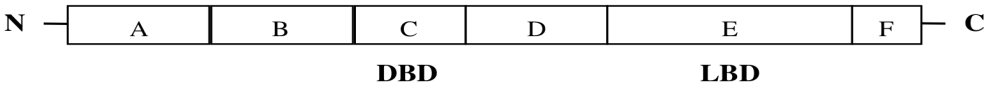
RARα antagonist



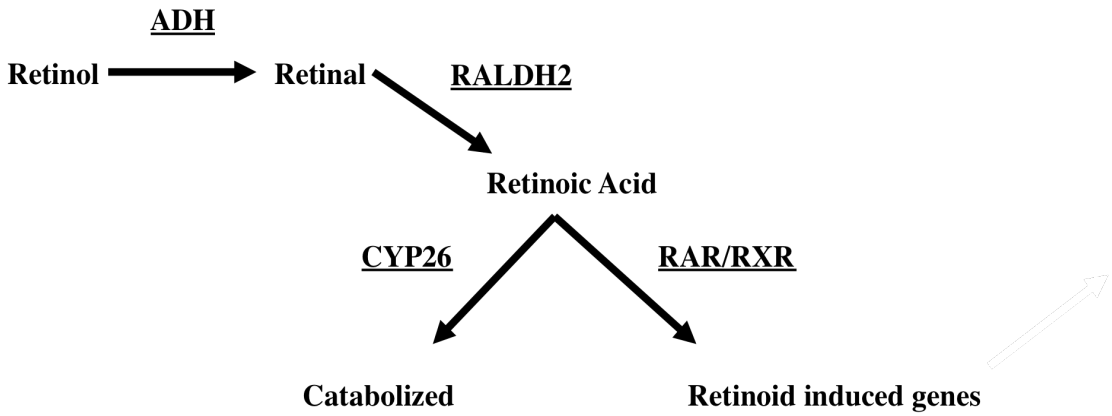

AGN 193109

Pan-RAR antagonist

**B**




**C**



developing embryo would receive extremely low levels of vitamin A from the maternal source. Offspring demonstrated a high level of abnormalities in the development of the eye, the respiratory system, the heart and major arteries, and the uro-genital tract (Warkany and Schraffenberger, 1946; Wilson et al., 1953; Wilson and Warkany, 1947a; Wilson and Warkany, 1947b; Wilson and Warkany, 1948; Wilson and Warkany, 1949; Wilson and Warkany, 1950). Although cardiac defects were not among the most frequent of those seen in later embryos or in those pups which survive until birth, a high degree of embryonic lethality was observed between embryonic day 10 and 15 (E10-15), presumably due to major defects in cardiovascular development (Wilson and Warkany, 1949; Wilson and Warkany, 1950). Finally, it was subsequently demonstrated that the majority of defects seen in the vitamin A deficiency syndrome of rats could be rescued if the mother was given an oral vitamin A supplement by E10, with decreasing effectiveness if given at later times (Wilson et al., 1953). The only developmental abnormalities of the VAD syndrome that could not be rescued were cardiac defects, which is understandable since heart development has already commenced well before E10.

### 1.5.2 Knock-out Mouse Models of Retinoic Acid Signalling

With the advent of targeted gene knockouts in the latter part of the 20$^{th}$ century, a new approach could be taken to understand the importance for vitamin A for early development. One obvious approach to study the RA signalling system is to target the retinoic acid receptors, however this has been difficult to analyze due to the extensive redundancy between the RARs. All of the single RAR knockouts survive

until birth in mouse, although both RARβ and RAR γ null embryos display growth retardation (Ghyselinck et al., 1997; Lohnes et al., 1993), and RARα and RARγ null embryos have a higher post natal mortality rate than control embryos (Lohnes et al., 1994; Lufkin et al., 1993). However, while no single receptor knockout displayed the entire VAD phenotype, the double knockout mouse embryos were able to better recapitulate the spectrum of disorders associated with VAD. All of the double knockouts were lethal: RARα$^{-/-}$/RARβ$^{-/-}$ and RARβ$^{-/-}$/RARγ$^{-/-}$ mice died within 24 hours of birth (Ghyselinck et al., 1997), while RARα$^{-/-}$/RARγ$^{-/-}$ mice died *in utero* (Lohnes et al., 1993; Mendelsohn et al., 1994). Interestingly, in each case the mice had major defects in the cardiovascular system, suggesting that RA signalling is essential for proper heart formation.

No single RAR knockout was able to recapitulate the full spectrum of the VAD syndrome, and while double RAR mutants were closer, there was still a large degree of variation between the phenotypes. However, a targeted deletion of *Raldh2* has been very useful in examining the consequences of an RA signalling loss. In all cases the *Raldh2*$^{-/-}$ mutation was lethal by E10.5 (Niederreither et al., 1999). The *Raldh2*$^{-/-}$ embryos were smaller, with the entire posterior region being severely shortened, and they had not undergone axial rotation that normally occurs at approximately E8.5 (Niederreither et al., 1999). There was also a dramatic loss of extra-embyonic vessels in the yolk sac membranes and the heart was dilated with no obvious left-right asymmetry (Niederreither et al., 1999; Niederreither et al., 2001). This was the first definitive model of a nearly complete loss of RA signalling, and the severe phenotype demonstrates that an *in vivo* source of RA signalling is absolutely

required for a number of embryonic processes, including early cardiovascular development.

All of the previously discussed knockout mouse models have focused on a loss, either partial or complete, of RA signalling. However, shortly after the *Raldh2* null mouse embryo was described, a *Cyp26A1* null mutant was also created to study the effects of an increase in embryonic RA. Cyp26A1 is the enzyme predominantly responsible for RA catabolism in the embryo. The *Cyp26A1* phenotype was embryonic lethal, although highly variable in severity, with mild cases dying at approximately E18.5, while more severely affected embryos were developmentally arrested at approximately E8.5 (Abu-Abed et al., 2001). All embryos demonstrated some form of posterior truncations, with a very high level of neural tube closure defects, as well as kidney abnormalities and posterior gut tube defects, such as a complete absence of the rectum (Abu-Abed et al., 2001). Some of the more severely affected embryos arrested during early development and displayed severe heart defects including a dilated heart cavity and imperfectly looped hearts, suggesting a cause of early lethality (Abu-Abed et al., 2001). These effects of loss of *Cyp26A1* function demonstrates the fine balance normally required of RA signalling in the embryo, and a role for *Cyp26* in restricting RA signalling during early embryogenesis.

### 1.5.2 Retinoic Acid Signalling in Development

As demonstrated by the mouse mutant studies, retinoic acid is involved in many developmental processes. An in depth examination of RA signalling

throughout development is not within the scope of the current discussion, I will therefore focus on only those effects that are pertinent to the topic of this thesis.

During early development, increased RA signalling before or during early gastrulation (stages 8-10) in frog embryos leads to inhibited development anterior (head) end of the embryo in a dose-dependant manner (Durston et al., 1989; Koide et al., 2001; Papalopulu et al., 1991). The mechanism responsible for the RA-induced anterior truncation remains undefined, and a number of possibilities remain, including altered cell movements during gastrulation, or more likely, altered inductive signals passed between the mesoderm and ectoderm (Lloret-Vilaspasa et al., 2010). However, to study RA dependent patterning after gastrulation, it is necessary to delay application of RA to avoid compounding results with the effects during gastrulation.

Following gastrulation, RA has been implicated in a number of patterning processes including the patterning of the somitic mesoderm (Kawakami et al., 2005; Moreno and Kintner, 2004), pronephric fate in the kidney (Cartry et al., 2006), neurectoderm (Sharpe, 1991), as well as for specifying a number of endodermal cell fates (Zeynali and Dixon, 1998), including:, endocrine fate in the pancreas (Chen et al., 2004), and the lung primordium in mouse (Chen et al., 2010). Interestingly, the unliganded form of the RARs has also been shown to a be necessary in anterior-neural patterning acting to suppress transcription (Koide et al., 2001). These studies demonstrate that the function of the RARs is dynamic and context dependent, required as activators in some contexts when bound to ligand, while being equally

important as repressors in other tissues in which the RA ligand is normally not present (for further discussion see: (Weston et al., 2003)).

Over the past two decades there has also been a keen interest in RA signalling during heart development, where RA clearly has a complex function that is highly stage dependent. During early heart specification in zebrafish, RA acts as a negative input to heart development by restricting the number of cardiac progenitors (Keegan et al., 2005). This is in line with studies in frog that show that *raldh2* is not normally expressed in the early heart field (Chen et al., 2001), and that up regulated RA signalling is capable of blocking differentiation of the myocardium (Drysdale et al., 1997). However, retinoic acid signalling is also necessary for later morphogenesis of the heart, as the heart fails to form a tube when RA signalling is antagonized (Collop et al., 2006). In mouse and chick, RA signalling has further been implicated in anterior-posterior patterning of the heart tube (Hochgreb et al., 2003; Sirbu et al., 2008). Finally, there is some recent evidence that RA is also necessary for secondary heart field differentiation (Li et al., 2010; Lin et al., 2010). Therefore, RA signalling seems to be restrictive to early heart specification, but later required for multiple aspects of morphogenesis. While there is clearly a substantial amount of information pertaining to the role of RA signalling in specification of a number of mesodermal lineages during later development, a role in early patterning of the LPM during the neurula stage embryo has thus far remained undefined.

Although little is known in *Xenopus* regarding the role of retinoic acid during endothelial cell development, studies in amniote embryos have suggested a role. Using the *Raldh2*$^{-/-}$ knockout mouse it has been shown that RA is necessary to

restrict proliferation within the endothelial progenitors and for proper endothelial cell maturation (Bohnsack et al., 2004; Lai et al., 2003), although the vascular plexus was still present. Conversely when RA signalling was increased, extra-embryonic vasculature was perturbed, with no identifiable intact blood vessels (Ribes et al., 2007). However, at this point the role of RA in endothelial specification and development remains poorly understood.

## 1.6 The Fibroblast Growth Factor Signalling Pathway

The FGF pathway is an essential signalling pathway in development. The Fgf family comprises at least 22 family members in higher vertebrates, and signals through a smaller family of four receptor tyrosine kinase receptors (FGFR1-4) to regulate a diverse array of cellular processes including proliferation, differentiation, apoptosis and migration (For thorough reviews see (Bottcher and Niehrs, 2005; Eswarakumar et al., 2005)).

All of the FGF ligand family members share a conserved region of 120 amino acids with 16-65% sequence homology in the 'core domain' that is responsible for interacting with the FGFRs. FGFs are diffusible peptides and appear to have different effects at different concentrations (Green et al., 1992; Kengaku and Okamoto, 1995) suggesting a possible role as a morphogen during early development. In addition to binding the FGFRs, FGF ligands also interact with heparan sulfate proteoglycans (HSPGs) that facilitate ligand-receptor binding (Chuang et al., 2010; Kan et al., 1993; Kato et al., 1998; Lanner et al., 2010; Steinfeld et al., 1996).

The FGFRs are single pass transmembrane proteins that function as dimers. The receptors contain extracellular ligand binding domains (Immunoglobin (Ig)-like domains) and an intracellular tyrosine kinase domain (Figure 3B). As previously noted there are four FGFRs (FGFR1-4) and several splice variants of each with altering affinities for FGF ligands (Lee et al., 1989; Pasquale, 1990). The extracellular Ig-like domains regulate ligand specificity and binding affinity. Between Ig-like domains 1 and 2 exists a heparin-binding domain that allows the receptor to interact with the extracellular matrix and bind HSPGs and cell adhesion molecules. The tyrosine kinase tails reside on the intracellular side of the molecule, as well as binding sites of various interacting proteins, such as protein kinase C and FRS2 (for a review of FGFR structure see (Eswarakumar et al., 2005)). Upon ligand binding, the tyrosine kinse tails undergo autophosphorylation leading to the activation of a downstream signalling cascade (Ullrich and Schlessinger, 1990; Yarden and Ullrich, 1988).

Downstream of FGFR dimerization and autophosphorylation, signal transduction can occur via three distinct pathways: The MAPK pathway (the canonical FGF signalling pathway: Figure 3C), the PLCγ/$Ca^{2+}$ pathway and the PI3 kinase/Akt pathway. In general, the canonical FGF signalling pathway is the predominant pathway controlling FGF induced transcription through a MAP kinase phosphorylation cascade (Ras/Raf/Mek/Erk) (Besser et al., 1995). The canonical FGF signalling pathway leads to the induction of a number of FGF inducible genes, including members of the Fgf synexpression group that includes *sprouty2* (Chambers et al., 2000; Hacohen et al., 1998; Minowada et al., 1999). The PLCγ/$Ca^{2+}$

is upstream of cytoskeletal organization (Williams et al., 1994a; Williams et al., 1994b) and the PI3 kinase/Akt pathway has anti-apoptotic functions in some tissue (Debiais et al., 2004; Lenhard et al., 2002).

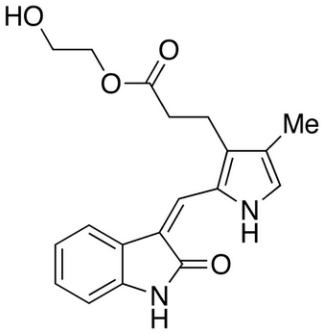
### 1.6.1 Fibroblast Growth Factor Signalling in Development

The FGF signalling pathway is essential in early development, with a number of functions throughout different tissues at different developmental stages, and many of the functions defined for FGF signalling have been conserved throughout evolution. Before gastrulation, FGF signalling is required as a competence factor for mesoderm induction (Amaya et al., 1991; Amaya et al., 1993; Cornell et al., 1995). During gastrulation FGF signalling is also directly required for convergent extension movements, and without this the embryos fail to gastrulate properly (Nutt et al., 2001). The early role of FGF signalling both before and during gastrulation creates an added level of complexity in studying later roles of FGF signalling during organogenesis.

Recently, FGF signalling has also been demonstrated to be necessary for the induction of the heart field in *Xenopus* (Keren-Politansky et al., 2009; Samuel and Latinkic, 2009) as it is in both chick (Alsan and Schultheiss, 2002) and zebrafish (Marques et al., 2008). There is some evidence that FGF signalling through Fgfr1 may be, at least in part, responsible for the role of Fgf in heart induction (Dell'Era et al., 2003). Furthermore FGF signalling is required in the mouse after heart field induction for the development of the secondary heart field

**Figure 3.** The fibroblast growth factor signalling pathway. A) Chemical structure of SU5402, the synthetic FGFR inhibitor which binds the intracellular domain of the FGFR and inhibits autophosporylation of the receptor tyrosine kinase tails of the receptor dimer. B) A generalized structure of the fibroblast growth factor receptors (FGFRs), showing the three extra cellular domains (D1, D2, and D3), the acid box (AB), the transmembrane domain (TM) and the tyrosine kinase tail. C) A schematic of the canonical Fgf signalling pathway.

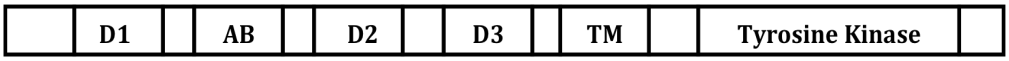
**A**



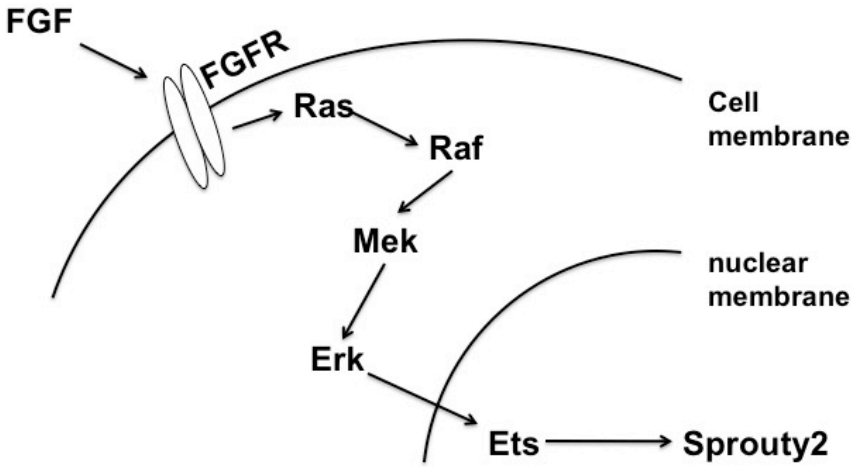

SU5402

FGFR inhibitor

**B**




**C**



(Ilagan et al., 2006; Park et al., 2006). While a role distinct for FGF in the secondary heart field has yet to be defined in *Xenopus*, *fgf8* is expressed in the anterior heart field during the mid-tailbud stages (Gessert and Kuhl, 2009) suggesting this role may be conserved. Thus, FGF signalling in heart, as in gastrulation, is required for multiple processesfrom induction to morphogenesis in a context dependent manner.

While there is little evidence that FGF signalling is involved in vasculogenesis, there is a wealth of data indicating Fgfs in angiogenesis. Fgf1 (acidic Fgf) was initially identified as endothelial cell growth factor due to its mitogenic activity in umbilical vein endothelial cells (Maciag et al., 1979). The angiogenic activity of FGF signalling seems to function through VEGF signalling (Magnusson et al., 2004; Seghezzi et al., 1998), and blocking VEGF signalling blocks the angiogenic activity of Fgf's in embryoid bodies (Magnusson et al., 2004). However, one study in *Xenopus* suggests that Fgfs are involved in determining the balance of blood versus endothelial marker expression in ventral marginal zone explants (Iraha et al., 2002). Therefore, while there has been a great deal of focus placed on Fgf's role in angiogenesis, it may have additional roles in vasculogenesis.

### 1.6.2 Interaction of the Fibroblast Growth Factor and Retinoic Acid Signalling Pathways

Generating polarized tissues within the embryo is a common process that often requires the input of two opposing signalling pathways specifying each end of the tissue. The RA and FGF signalling pathways have been described as opposing pathways in generating polarized tissue in a number of patterning processes. During heart

development in mice RA acts at the atrial (posterior) end of the heart tube in opposition to FGF signalling at the ventricular (anterior) end to pattern the anterior-posterior axis of the heart tube (Sirbu et al., 2008). During somite patterning, the RA signalling pathway in the anterior end of the embryo is opposed by FGF signalling from the posterior end (Diez del Corral et al., 2003; Moreno and Kintner, 2004; Sirbu and Duester, 2006). Similarly, during body axis extension, RA is necessary to restrict FGF signalling to the tailbud (Zhao and Duester, 2009).

Furthermore, both the RA and FGF pathways are known to modulate the signalling levels of the other. FGF signalling restricts RA signalling by up regulating the expression of *cyp26*, the RA metabolizing enzyme (Goncalves et al., 2009; Moreno and Kintner, 2004; White et al., 2007). However, the effect of RA on FGF signalling appears much more complex. ChIP experiments have demonstrated that the RARs (all of RARα, β and γ) are able to bind the *Fgf8* promoter suggesting that *Fgf8* is directly regulated by RA signalling (Zhao et al., 2009). The ChIP data is further supported by the finding that RA signalling modulates the level of Fgf signalling, and in most reports RA represses *Fgf8* expression (Diez del Corral et al., 2003; Ribes et al., 2009; Sirbu and Duester, 2006; Sirbu et al., 2008; Zhao et al., 2009), although there is also data that RA can up regulate *fgf8* expression in some contexts (Moreno and Kintner, 2004; Stavridis et al., 2010).

## 1.7 Early Lateral Plate Mesoderm Gene Expression

Traditionally, the LPM has been pictured as a homogenous tissue with minimal patterning following gastrulation in *Xenopus*. The first described pattern of regionally expressed genes occurred during the mid to late neurula with the

expression of the heart field marker *nkx2-5* (Tonissen et al., 1994) in the anterior-ventral LPM and markers of the myeloid lineage (*mpo*) just posterior to the heart field (Smith et al., 2002) (Figure 1C). However, further patterning of the LPM in the neurula stage embryo has thus far not been reported.

Following a microarray on frog embryos treated with RA and an RA antagonist in a four hour time window during neurulation a number of genes were identified whose expression was significantly altered under one or both of the treatments as compared to control embryos (Drysdale, personal communication). Known targets of RA signalling showed significant changes in gene expression suggesting that the microarray could identify novel genes regulated, at least in part, by RA signalling. Several genes were identified that were not previously recognized as being regulated by RA including: *hand1*, *foxf1*, and *sall3*. These three genes encode transcription factors that are expressed in the LPM and necessary for heart development at later stages, although their expression had not been thoroughly analyzed at earlier stages. Of the three genes identified by microarray analysis, *hand1* was the most highly studied, and will be discussed below in its own section (see section 1.7.1).

*Foxf1* is a member of the large family of fork head box (Fox) transcription factors. The defining characteristic of the Fox family is that they all contain a winged helix DNA binding domain that resembles a helix-turn-helix motif (Brennan, 1993). *Foxf1* has previously been characterized in the anterior LPM beginning in the early tailbud stage embryo (stage 26), and throughout most of the LPM by the late tailbud stage (stage 36), although no expression pattern was described during neurula

stages (Koster et al., 1999). In both *Xenopus* and mouse, *foxf1* has been implicated in the patterning of the endoderm by the lateral plate mesoderm (Ormestad et al., 2006; Tseng et al., 2004). Furthermore, in mice, *FoxF1* has been implicated in early mesoderm formation (Mahlapuu et al., 2001b), vascular development (Astorga and Carlsson, 2007) and lung development (Mahlapuu et al., 2001a).

The posterior LPM marker *sall3* is much less studied. *Xenopus sall3* was originally cloned as *Xsal-1*, and described as a homolog of the *Drosophila* gene *spalt* (Hollemann et al., 1996), but was later renamed *sall3* to be consistent with the human nomenclature (Kohlhase et al., 1999). *Sall3* is a zinc finger transcription factor with a characteristic arrangement of four double zinc finger domains. S*all3* expression was originally described in neural tissues during early *Xenopus* development, as well as in the limb buds during later development (Hollemann et al., 1996), however no expression was demonstrated in the early LPM.

In *Drosophila*, *spalt* is necessary for determining the head-trunk, and trunk-tail boundaries (Frei et al., 1988; Jurgens, 1988). For the vertebrate *sall3*, there is very little functional data available. In mice, loss of *Sall3* causes defects in the organs necessary for feeding, such as the palate, tongue and epiglottis and thus causes perinatal lethality due to inability to feed (Parrish et al., 2004). In humans, the *SALL3* gene has been correlated with 18q deletion syndrome characterized by a combination of mental retardation, growth retardation, developmental delay, as well as eye, nose, mouth and limb defects among others (Dostal et al., 2009; Kohlhase et al., 1999).

### 1.7.1 *Hand1* Gene Expression and Function

One gene of significant interest to this study is *hand1*. *Hand1* is a basic helix-loop-helix transcription factor, whose expression was initially characterized in a broad domain within the LPM at the mid-tailbud stage in *Xenopus* (Sparrow et al., 1998). The first report in *Xenopus* characterized an asymmetric left-right expression pattern within the early LPM, with stronger expression on the left that right side of the embryo (Sparrow et al., 1998), although I find no evidence of this asymmetric expression (see results). This left-right asymmetric expression of *hand1* has also been characterized during heart development of mice (Biben and Harvey, 1997). A recent report in mice has shown that *hand1* expressing cells contribute to a vast array of mesodermally derived structures including the placenta, limbs, heart and vasculature (Barnes et al., 2010). In zebrafish, *hand* (the lone homologue of vertebrate *Hand1* and *Hand2* genes) is expressed in the early LPM and is described as a pan-LPM marker (Keegan et al., 2005; Yelon et al., 2000).

Unlike the previously described genes, *Hand1* function has been thoroughly studied in mouse, with most of the focus on heart morphogenesis. Complete loss of *Hand1* in mice is lethal between E8.5 and E9.5 due to defects in extraembryonic vasculature and heart development (Firulli et al., 1998; Riley et al., 1998). In the extra embryonic vasculature, *Hand1*$^{-/-}$ embryos undergo vasculogenesis, however the expression of several angiogenic genes is perturbed, suggesting a role for *Hand1* in placental angiogenesis (Morikawa and Cserjesi, 2004). Furthermore, a loss of *Hand1* during the early stages of heart development leads to a delay in heart tube formation (Smart et al., 2002), and morphogenesis of the left ventricle (McFadden et

al., 2005), whereas mutants overexpressing Hand1 have defects in ventricular septation (Togi et al., 2004). This suggests that *Hand1* is necessary for morphogenesis of the left ventricle but must be inhibited from the region correctly fated to form the septum.

Since *hand1* is expressed in a broad region of the early frog embryo that will give rise to both the vasculature and heart (Sparrow et al., 1998), and *hand1* expression is RA responsive, this gene is of particular interest to this study. Furthermore, that *hand1* has been implicated in both heart and vascular development in some contexts in mouse suggests that it may play a pivotal role in early cardiovascular specification. Finally, since *hand1* mutant mice display defects in heart tube closure (Smart et al., 2002), a phenotype which closely resembles a loss of RA signalling in *Xenopus* (Collop et al., 2006), I hypothesize that this gene may be downstream of RA signalling in early specification and morphogenesis of cardiovascular lineages.

## 1.8 Experimental Rationale

The purpose of this thesis is to examine the role of RA and FGF signalling in regional patterning within the early lateral plate mesoderm of *Xenopus laevis*. While some pattern has been described by the early tail bud stage, there has been no comprehensive analysis to gain an understanding of when the LPM is patterned, and how this pattern is established. Patterning within the LPM has been demonstrated in the neurula stage embryo, but focus has been placed on the ventral cardiovascular precursors, while the more dorsal LPM has been ignored. Furthermore, the RA and FGF signalling pathways have been demonstrated to oppose one another in a delicate balance leading to patterning across a number of tissues during development. The expression domains of *raldh2* and *fgf8* strongly suggest that these two signalling systems may be responsible for patterning the anterior and posterior ends of the LPM respectfully.

I hypothesize that early patterning of the LPM occurs, at least in part, under the control of the RA and FGF signalling pathways shorty after gastrulation. Furthermore, I predict that RA will pattern the anterior-dorsal end of the LPM corresponding to the expression pattern of *raldh2*, and FGF signalling the anterior-ventral and posterior ends, corresponding to the expression domain of *fgf8*.

In the first aim of my thesis, I demonstrate that the LPM has significant pattern during the neurula stage embryo. Furthermore, I will show that RA signalling is necessary for properly specifying the anterior-dorsal end of the LPM during early development. When RA signalling is lost, both the anterior-dorsal, and middle LPM markers are restricted. In increased RA conditions, both the anterior-dorsal and middle LPM markers are expanded posteriorly, while posterior markers are lost.

The second aim of my thesis will demonstrate that FGF signalling is necessary to specify both the posterior tail bud domain and the anterior-ventral heart field. When FGF signalling is inhibited, markers of both the heart field in the anterior-ventral LPM, and the tail bud domain in the posterior LPM are lost. Furthermore I demonstrate that the FGF and RA signalling pathways are antagonistic, and that they have opposing function on LPM marker domains.

For the third aim of my thesis I will describe a role for both RA and FGF in specifying the early cardiovascular precursors. By increasing RA signalling, the early heart progenitors are severely reduced, while the expression of vascular markers is increased. Conversely, by reducing FGF expression, markers of the early heart field are lost and vascular markers are also increased. These results implicate proper RA and FGF signalling in subdivision of the early mesodermal precursors that will give rise to the cardiac and endothelial lineages.

Finally, for my fourth objective I will present preliminary evidence that Hand1 function is required for the proper morphology of the heart, and specification of the endothelial lineage. A reduction in Hand1 function mirrors the phenotype of a loss of RA signalling on heart development. Since RA signalling regulates *hand1*, this may provide a link connecting RA signalling to heart tube formation, and may be of interest to future studies on cardiovascular development.

# Chapter 2 Methods

## 2.1 *Xenopus laevis* Embryo Generation

Female *Xenopus laevis* frogs were injected in the thigh muscle with 500-700 IU of human chorionic gonadotropin the evening before collecting eggs. Eggs were collected by 'squeezing' females and fertilized *in vitro* by adding minced testis collected from sacrificed males to the 80% Steinberg's solution in which the eggs were being cultured. Embryos were flooded with 20% Steinberg's solution following fertilization. Embryos were dejellied in 2.5% cysteine, pH 8.0, and cultured in 20% Steinberg's solution. Embryo staging was based on the Normal Table of *Xenopus laevis* (Nieuwkoop and Faber, 1994). The Steinberg's solution was kept as two stock solutions at a 2000% concentration, and mixed 1:1 to yield a 1000% stock (for 1L of each, Steinberg's Stock A: 68g NaCl, 1g KCl, 4.09g $MgSO_4$-$7H_2O$, 1.58g $Ca(NO_3)_2$-$H_20$; Steinberg's Stock B: 11.2g Tris-HCl pH 7.4). The 1000% Steinberg's solution stock was then diluted 1:5 for the 200% Steinberg's solution with distilled $H_2O$, 1:12.5 for the 80% Steinberg's solution, or 1:50 for the 20% Steinberg's solution. All embryos were fixed in MEMPFA (4% paraformaldehyde, 1mM $MgSO_4$, 2mM EDTA (ethyleneglycol-bis-(β-aminoethyl ether) N',N',N',N'-tetra-acetic acid) pH 8.0), 0.1M MOPS pH7.4 for 2 hours at room temperature, or overnight at 4°C and then stored in 100% at -20°C until assayed.

## 2.2 Experimental Treatments

All treatments were accomplished by adding the exogenous chemical of interest to the solution that the embryos were developing in. Embryos were cultured in 20%

Steinberg's solution unless otherwise noted, and cultured at varying temperatures (between 12° C and room temperature) as required to control the rate of development and obtain embryos at a desired stage.

### 2.2.1 Retinoic Acid Treatments

Embryos were treated with 1μM *all-trans* RA (Sigma) (Collop et al., 2006), an RARα antagonist (allergan; AGN 194301; (Teng et al., 1997)) or a pan RAR antagonist (RAA) (Allergan #193109; (Agarwal et al., 1996)) at stage 14, and cultured until the desired stage. Stock solutions for both RA and RAA were stored at a 1 mM concentration in DMSO, and therefore a control treatment was performed with 0.1% DMSO in 20% Steinberg's solution. To alter the bio-availability of endogenous RA ligand, ketoconazole was used to at a concentration of 50 μM to inhibit Cyp26 (Lutz et al., 2001; Van Wauwe et al., 1988), the enzyme primarily responsible for RA catabolism and thereby increasing the levels of RA. Raldh2, the enzyme primarily responsible for synthesis of RA, was inhibited with either 120 μM citral (Cartry et al., 2006) or 20 μM diethylaminobenzaldehyde (DEAB) (Begemann et al., 2004) to create an endogenous reduction of RA ligand. Finally, to determine if RA was directly regulating gene expression, embryos were treated with RA in conjunction with cycloheximide to inhibit protein synthesis. Cycloheximide treatments were performed at a concentration of 10 μg/mL (Moreno and Kintner, 2004) at stage 14 and cultured for 90 minutes before being fixed. Experimental embryos were exposed to cycloheximide 15 minutes prior to treatment with RA.

To study the effets of a restricted time window of RA signalling during neurulation in embryos that were allowed to develop past stage 20, I added an equal concentration of RAA (where embryos were first treated with RA at stage 14) or RA (where they had been treated with RAA) for 30 minutes, before being transferred to 20% Steinberg's solution without exogenous RA. The neutralization treatments with both RA and RAA were necessary since simple washes were found to be insufficient to eliminate exogenous RA from the embryo.

### 2.2.2 FGF Inhibitor Treatments

The FGF inhibitor experiments were performed by adding 10 μM SU5402 (Calbiochem) to embryo cultures in conjunction with 0.1 mM ATP. The stock solution for SU5402 was 10 mM dissolved in DMSO, and the stock solution for ATP was 0.1 M dissolved in distilled $H_2O$. Therefore, a control treatment was performed with 0.1% DMSO and 0.1 mM ATP in 20% Steinberg's solution. Note that treatments with 10 μM SU5402 in conjunction with 0.1 mM ATP has yielded a phenotype comparable to other studies using as much as ten fold higher concentrations when ATP is not added (Walmsley et al., 2008; Wills et al., 2010). To remove the inhibitor when needed, the petri dishes in which the embryos were being cultured were flushed five times with 'fresh' 20% Steinberg's solution without ATP, and the embryos were then moved to a clean dish with 20% Steinberg's solution.

## 2.3 Obtaining Ventral Explants

Embryo explants were generated at stage 12.5-13 in 1x Modified Barth's Saline (MBS) by making an incision through the center of the neural plate into the achenteron.

The resulting flaps of tissue were folded back and removed at the point at which the walls of the archenteron met the archenteron floor, effectively isolating tissue from the floor of the archenteron to the ventral pole of the embryo. Explants were then treated with 1μM RA, RAA or control DMSO for 90 minutes before being fixed. MBS was made as a 10x stock (for 1L of 10x stock: 51.43g NaCl, 0.75g KCl, 2.02g $NaHCO_3$, 23.83g HEPES, 0.99g $MgSO_4$, 0.78g $Ca(NO_3)_2$-$4H_2O$, pH to 7.5) and diluted 1:10 in $dH_2O$ with 0.7% 0.1M $CaCl_2$ for 1x MBS working solution.

## 2.4 Microinjections

An antisense morpholino oligomer was designed to recognize the translational start site of the *Hand1* transcript (GeneTools Inc. suggested sequence: GTTGGTAGCTCCCAATCAGGTTCAT). Control morpholino oligos were also used with a random sequence that did not correspond to any known EST in the *Xenopus* genome. In each case the morpholinos were fluorescently tagged with carboxyflourescein on the 3' end of the oligo. The antisense morpholinos were reconstituted in distilled $H_2O$ to a concentration of 1 mM and aliquots were stored at -80°C until use, as per the recommendation of GeneTools Inc. Injections were accomplished by using a Drummond Nanoject (Drummond Scientific; Broomall, Pa) to inject either 4.6 nL, or 9.2 nL into a dejellied one cell embryo, or into one blastomere of a two cell embryo. Injections were accomplished in 1x MBS with 2% ficoll. Embryos were incubated in 1x MBS with ficoll for roughly four hours following injection and then transferred to 20% Steinberg's Solution. Successful injections were assayed and sorted the following day by fluorescence. Successfully injected embryos were allowed to develop until the desired

stage and were fixed in MEMPFA for further analysis by whole mount *in situ* hybridization.

## 2.5 Antisense Probe Synthesis for *in situ* Hybridization

Digoxygenin-labelled antisense riboprobes were synthesized from cDNA constructs (For a full list please see Table 1). DNA constructs were transformed into competent *E. coli* bacteria to amplify the plasmid. Once the plasmid had been amplified to a suitable concentration, it was digested at the 5' end of the gene of interest and an *in vitro* transcription reaction was performed to synthesize the DIG (Digoxygenin-11-UTP; Roche) anti-sense ribonucleotide probe.

### 2.5.1 Competent Cell Culture and Transformation of cDNA Constructs

*E. coli* competent cells (*Xl1 Blue* strain) were grown in approximately 4mL of liquid Luria Broth (LB; for 1L: 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl, pH 7.0) overnight agitating at 37°C. The following morning 0.2mL of the overnight culture was used to inoculate a 50 mL culture of LB and agitated at 37°C for 4 hours, or until the culture had reached log phase growth. The cultures were then chilled on ice for 20 minutes, and pelleted at 4000 RPM for 5min at 4°C. Cells were then resuspended in 20 mL ice cold 0.1 M $CaCl_2$, followed by centrifugation at 4000 RPM for 5 min at 4°C. Finally the cells were resuspended in 2 mL ice cold 0.1 M $CaCl_2$, 0.1% glycerol and aliquots were stored at -80°C until used.

To transform cDNA constructs into competent *E.coli* cells, 2 μL of cDNA template was added to 50 μL of ice cold competent cells (or 10 μL into 100 μL for

constructs which were more difficult to transform). The competent cell/DNA solution was kept on ice for 20 minutes, before being heat shocked at 37° for 90 seconds. Following heat shock, the competent cells were then incubated on ice for a further 20 minutes before being plated on solid LB plates (for 500 mL: 5 g bacto-tryptone, 2.5 g bacto-yeast extract, 5 g NaCl, 7.5 g bacto-agar) containing the appropriate antibiotic (in each case ampicillin was used for positive selection). The LB plates were then cultured overnight at 37°C. The following afternoon, the colonies were picked with a heat sterilized metal loop and were used to inoculate a 4 mL culture of liquid LB with 1 μL/mL of 50 mg/mL ampicillin (one colony per culture), and these cultures were agitated overnight at 37°C. The following morning the cDNA plasmids were isolated from the *E.coli* cells following the tabletop microcentrifuge protocol of the QIAprep Spin Miniprep Kit (Quiagen).

### 2.5.2 Restriction Digest of cDNA Constructs

Plasmid templates were digested with the appropriate restriction endonuclease enzyme. For the digest reactions, approximately 15 μg of DNA was digested in a 50 μL total reaction (15 μL or 1 μg/μL DNA, 28 μL $dH_2O$, 5 μL appropriate Buffer, 2 μL restriction endonuclease) for a minimum of 2 hours, or a maximum of overnight at 37°C. Following the restriction digest reaction, the cDNA was purified by adding 50 μL $dH_2O$ and 100 μL phenol, mixed thoroughly and centrifuged in a tabletop microcentrifuge at 14000 RPM for 10 min. The aqueous layer was then removed to a new tube and the plasmid DNA was precipitated by adding 0.1 volumes of 3 M NaAcetate, 2.5 volumes of cold 95% ethanol, mixed thoroughly and centrifuged for 10 min at 14000 RPM. Following centrifugation, the supernatant was poured off and the pellet allowed to dry at

room temperature, before being resuspended in 10 μL $dH_2O$. 1 μL of the resulting cDNA template was loaded into a 1% Agarose-TAE (6.73 mM Tris-HCl pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA pH 8.0) gel with Ethidium Bromide (1.5 μL per 100 mL Agarose-TAE) to visualize the product of the restriction digest reaction.

### 2.5.3 *In vitro* Transcription of Antisense Ribonucleotide Probes

Antisense riboprobes for *hand1* (Sparrow et al., 1998), *sall3* (Hollemann et al., 1996), *foxf1* (Koster et al., 1999), *nkx2-5* (Tonissen et al., 1994), *hoxc10* (Christen et al., 2003), *cardiac Troponin I* (Drysdale et al., 1994), *apeline receptor* (*aplnr*) (Devic et al., 1996), *etv2* (Salanga et al., 2010), *globin* (Knochel et al., 1987), *tal1* (Mead et al., 1998), *spib* (IMAGE 7023083), *mpo* (IMAGE 5336501), *fgf4* (Isaacs et al., 1992), *fgf8* (Christen and Slack, 1997), *cyp26* (Hollemann et al., 1998) and *raldh2* (Chen et al., 2001) were labelled with digoxygenin (DIG)-labelled UTP (Roche Diagnostics) following the protocol by (Harland, 1991) except that incorporating $P^{32}$ labelled nucleotides was omitted. The transcription reaction was assembled at room temperature (except for the RNase Inhibitor and RNA Polymerase enzyme which were kept on ice until added) with the following components in this order: 2-4 μL of template DNA (from digest reaction above), $dH_2O$ (sufficient to bring total reaction volume to 20 μL), 4 μL NTPs (2.5 mM ATP, CTP and GTP; 1.625 mM UTP; 0.875 mM DIG-11-UTP), 4 μL 10 mM DTT, 0.5 μL RNaseOUT (RNase Inhibitor: Invitrogen #10777-019), 4 μL 5x Transcription Buffer (Invitrogen) and 2 μL of the appropriate RNA polymerase (table 1; Invitrogen), and was incubated for 2 hrs at 37°C. Once the incubation was complete 1 μL DNase1 (Invitrogen

**Table 1.** List of the reagents used to generate antisense RNA probes from the cDNA constructs. In each case the vector that each construct had been cloned into is listed, the restriction enzyme necessary to digest the cDNA construct at the 5' end of the insert, and the proper RNA polymerase corresponding to the promoter located at the 3' end of the insert.

| Construct | Vector | Restriction Enzyme | Polymerase |
|---|---|---|---|
| *Bra* | pSP73 | *Eco*RV | T7 |
| *CTnI* | pBluescript SK | *Not*I | T7 |
| *Cyp26* | pCS2 | *Eco*RI | T3 |
| *Etv2* | pGEM-T Easy | *Sal*I | T7 |
| *Fgf4* | pBluescript | *Eco*RI | T3 |
| *Fgf8* | pCS2 | *Xho*I | T7 |
| *FoxF1* | pBluescript II | *Eco*RI | T7 |
| *Globin* | pCS107 | *Hind*III | T3 |
| *Hand1* | pKRX | *Bam*HI | T7 |
| *Hoxc*10 | pGEM-T Easy | *Not*I | T7 |
| *Isl*1 | pBluescript SK | *Eco*RI | T7 |
| *Mpo* | pSport1 | *Sal*I | T7 |
| *Nkx2*-5 | pGEM 3Z | *Kpn*I | T7 |
| *Pitx2c* | pBluescript II | *Not*I | T7 |
| *Raldh2* | pCS2 | *Bam*HI | T3 |
| *Sall3* | pBluescripts KS | *Hind*III | T7 |
| *Tal1* | pGEM7 | *Xho*I | SP6 |
| *Sprouty2* | pCS2 | *Xho*I | SP6 |
| *Spib* | pCMV-Sport6 | *Sal*I | T7 |
| *Aplnr* | pBluescript SK- | *Bam*HI | T7 |

#AM2222) was added to digest the template DNA and incubated for a further 10 min at 37°C. 1 μL of the transcription product was loaded into a 1% agarose gel (as above) to check the reaction yield. The remainder of the transcription reaction was then precipitated by adding 4 volumes of 1% SDS in TE (10 mM Tris pH7.4, 1 mM EDTA), 0.5 volumes of 5M ammonium acetate and 2.5 volumes of 95% ethanol, mixing thoroughly and centrifuging at 14000 RPM for 10 min. The reaction was the dried and resuspended in RNA Hybridization buffer. The RNA hybridization buffer was: 50% formamide, 5x SSC (from a 20x SSC stock: 3 M NaCl, 0.3 M sodium citrate, pH7.0), 5 mM ethylenediaminetetraacetic acid (EDTA) pH 5.0, 1 mg/ml Yeast RNA extract (Boehringer), 1 M Denhart's solution (2% bovine serum albumin, 2% polyvinylpyrrolidone (PVP-40), 2% ficoll 40), and 0.1% Tween-20. Probes were then transferred to a 15 mL screw cap tube and diluted to a working concentration of approximately 0.5 μg/ml (using approximated yield estimates from the agarose gel), and stored at -20°C until used.

### 2.6 Whole Mount *In Situ* Hybridization

Whole mount *in situ* hybridizations were performed following established protocols (Harland, 1991) with several modifications as described below. Procedure was carried out in borosilicate glass vials with phenolic screw cap (1.5 dram, VWR; Cat. No. 66011-063). Each vial was labelled with riboprobe identity, treatment, and date via permanent marker, and labels were covered with a strip of transparent tape to avoid labels being removed if exposed to alcohols. Embryos stored in methanol were rehydrated through a graded methanol to aqueous series (5 minute washes in 75%, 50%, 25%

methanol) in tris buffered saline with 0.1% Tween-20 (Ttw). The proteinase K step was omitted and embryos were washed twice in Triethanolamine (0.125M), and 5μL acetic anhydride was added to the second wash of triethanolamine, twice, for two further washes of 5 minutes each. The embryos were then rinsed in Ttw, 4 washes for five minutes each, before being moved into RNA hybridization buffer, one wash of 10 minutes in 1:1 Ttw to RNA hybridization buffer, followed by one further wash of 10 minutes in 100% RNA hybridization buffer. The pre-hybridization step was then completed in hybridization buffer while the embryos were agitated at 65°C for a minimum of 1 hour, although performing this step for 2-3 hours tended to reduce background. The probe was then added and embryos were left agitating in probe solution over night at 65°C.

The following morning the probe solution was removed from the vials, and replaced with 10 minute washes of each of 100% RNA hybridization buffer, 50% hybridization buffer in 2x SSC, 25% hybridization buffer in 2x SSC all performed at 65°C. At this juncture, the RNase A step was omitted without an appreciable increase in background. Low stringency washes were then performed to remove unbound probe consisting of two washes in 2x SSC at 37°C for 30 minutes each, followed by three high stringency washes of 0.2x SSC at 65°C for 45 minutes each. Embryos were then washed in Ttw for ten minutes, followed by a 10 minute wash in TBT (Ttw with 2mg/ml bovine serum albumin). A blocking step was then performed preceding antibody addition in blocking solution (TBT with 20% heat-treated sheep serum). Finally, alkaline phosphatase conjugated anti-digoxygenin antibody (Anti-digoxygenin-AP, Fab Fragments; Roche Applied Science, Cat. No. 11 093 274 910) was diluted in blocking solution 1:5000. Embryos were agitated overnight at 4°C.

After an overnight incubation, the antibody solution was removed and embryos were thoroughly washed for thirty minutes in TBT for twelve washes, before a 10 minute wash in alkaline phosphatase buffer (100mM NaCl, 100mM Tris pH 8.4, 50mM $MgCl_2$, 0.1% Tween-20). The colour reaction was accomplished by adding 0.5 ml of BM Purple AP Substrate (Roche; Cat. No. 11 442 074 001). The colour reaction was allowed to proceed until the desired intensity had been reached, at which time the embryos were then dehydrated and subsequently rehydrated in a graded methanol series (in 25% steps) to stop the reaction. Once rehydrated embryos were re-fixed in Mempfa for 20 minutes, thoroughly washed in Ttw (4 washes for 5 minutes each) and bleached to remove pigment in the embryos yielding better contrast for imaging. Bleaching solution was 5% formamide, 0.5% SSC, 0.3% $H_2O_2$). The bleaching reaction was allowed to continue until the embryos had reached an acceptable level of contrast was present between the stain and embryo colour, embryos were washed and stored in 100% methanol until being imaged. Stained embryos were imagined on a Leica MZ12 dissecting microscope and images were captured using Northern Eclipse software (Empix Imaging, Mississauga, Ontario, Canada).

### 2.6.1 Double Whole Mount *In Situ* Hybridization

Double whole mount *in situ* hybridizations were performed according to recently published protocols (Koga et al., 2007) with some further modifications. The probes were synthesized separately, one probe was labelled with DIG-11-UTP as described above, while a second flourescein labelled probe was synthesized using the same protocol as for DIG labelled probed except that Fluorescein-12-UTP (Roche; Cat. No. 11 427 857 910)

was substituted for DIG-11-UTP. The product of the transcription reaction was diluted in RNA hybridization buffer to yield a 3x more concentrated probe than used for single *in situ* hybridizations and the two concentrated probes were mixed 1:1.

The same *in situ* hybridization protocol was used for double *in situ* hybridizations as for single *in situ* hybridizations as described above, except that the double probe (probe containing the 1.5x concentrated mixture of Dig labelled and Fluorescein labelled probes) was added at the end of the first day in place of a single *in situ* hybridization probe. The second day of the double *in situ* hybridization protocol was identical to the single hybridization protocol except that anti-fluorescein-AP Fab Fragments (Roche; Cat. No. 11 426 338 910) were added in place of anti-DIG-AP Fab Fragments at 1:4000 diluent. Washing excess antibody from the embryo was accomplished as above and the first colour reaction was carried out using BM-Purple AP substrate.

Following the first colour reaction, the flourescein antibody was inactivated in 0.1M glycine pH 2.0 as previously described (Sive et al., 2000) for 40 minutes followed by four ten minute washes in maleic acid buffer (100mM Maleic acid, 150mM NaCl, pH 8.0). Embryos were then washed for ten minutes in Ttw followed by ten minutes in TBT, and then blocked for 90 minutes in TBT with 20% sheep serum. The Anti-DIG antibody was then added at a 1:2000 dilution in Blocking Solution and the embryos were incubated at 4°C overnight. The following day the embryos were washed thoroughly in TBT (12 washes of 30 minutes) to remove the excess antibody. The embryos were then washed for 10 minutes in AP Buffer before being stained in 5-Bromo-4-chloro-3-indolyl phosphate (BCIP: 0.5mg/ml in AP Buffer).

## 2.7 Statistical Analysis

To quantify the area of *hand1* expression in explants, the area of staining was measured using Northern Eclipse software (Empix Imaging; Mississauga, ON, Canada). Total staining area was then compared between treatments by virtue of a one way ANOVA with a Tukey's post hoc test (GraphPad Prism; La Jolla, CA). The confidence interval was set at 95% in order to determine statistical significance.

The length of the LPM expression domains at stage 36 was measured using Northern Eclipse software. The posterior border of the anterior LPM domains was measured as the distance from the front of the cement gland to the posterior edge of the staining domain at the point at which the LPM met the axial mesoderm (I have denoted this distance *x*), divided by the distance from the front of the cement glad to the point at which the posterior edge of the body axis met the somites (denoted *y*; fraction *x/y*). The exception was the *hoxc10* domain that was instead measured as a fraction of the length from the most anterior point of the expression domain to the point at which the body wall met the somites (distance denoted *z*) divided by *y* (fraction *z/y)*. These values were then compared by a one-way ANOVA with a confidence interval of 95% with a Tukey's post hoc test. A minimum of 10 embryos was used for each experiment.

# Chapter 3 Results

## 3.1 Restricted Expression Domains in the Early Lateral Plate Mesoderm

Immediately following gastrulation, the LPM is generally considered to have only a rudimentary pattern, with the heart field and early hemangioblast lineages occupying the anterior-ventral and middle-ventral domains respectively, while the rest is considered homogenous. The homogeneity of the LPM can be exemplified by the expression of *Hand* that has been used as a pan LPM marker in zebrafish (Keegan *et al.*, 2005). The *Xenopus* homologue, *hand1*, is expressed in the anterior and middle LPM in *Xenopus*, with a clear domain in the posterior LPM that is *hand1* negative (Figure 4B). There also exists a region in the anterior-ventral mesoderm that is devoid of expression creating a hole in the staining pattern just posterior to the heart region after stage 18. This hole has previously been shown in the expression domain of *foxf1* (Tseng et al., 2004) and *scl2* (Kumano et al., 2006) and at early stages it correlates well with early myeloid lineage markers.

Expression of *Xenopus sall3* has previously been described in dorsal neural tissue (Hollemann et al., 1996), but I now extend the expression pattern to include a region of the posterior LPM that corresponds to the region that is *hand1* negative, although some overlap is likely at stage 20 (Figure 4C). The *sall3* expression domain in the posterior LPM is transient and lost shortly after neural fold closure (stage 20). Subsequent sectioning of embryos assayed for either *sall3* or *hand1* confirmed expression in the LPM. While I cannot rule out overlap between the *hand1* and *sall3* domains, a significant portion of the *Xenopus* LPM is *sall3* positive and *hand1* negative.

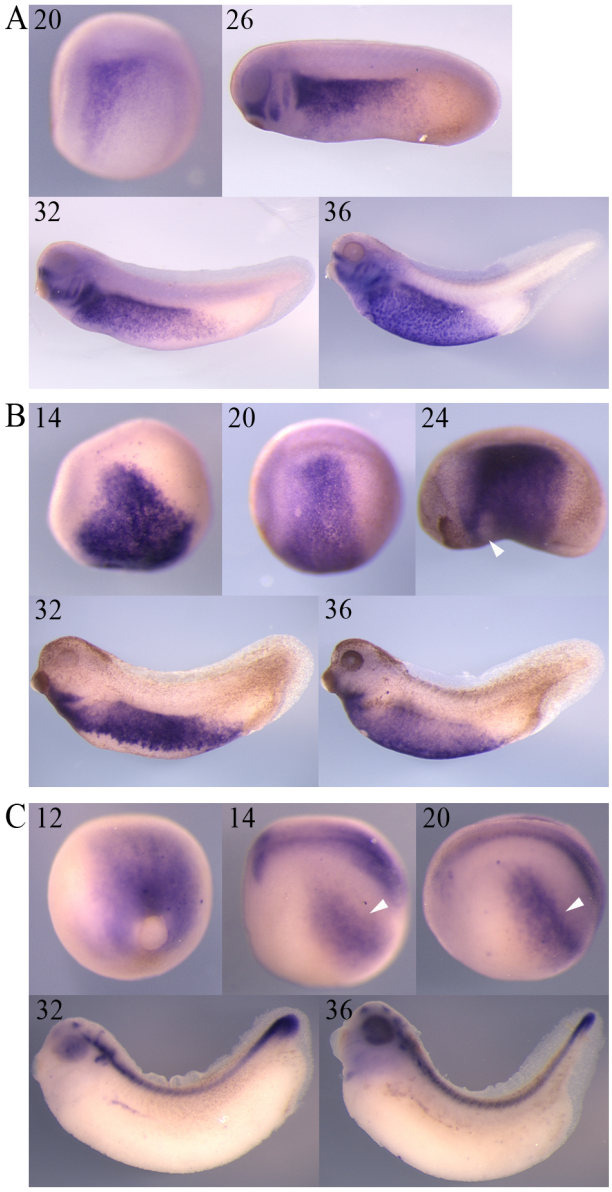
I subsequently examined LPM expression patterns of multiple genes looking for additional genes whose expression patterns display a restricted domain in the early LPM. Two other transcription factors, *foxf1* (Figure 4A) and *nkx2-5* (Tonissen et al., 1994) have restricted expression domains that mark anterior-dorsal and anterior-ventral LPM (myocardium) respectfully. Furthermore the enzyme responsible for retinoic acid synthesis, *raldh2*, was also found to show a restricted expression domain within the LPM along the anterior and dorsal edges (previously described (Koster et al., 1999)). However, *raldh2* expression is not restricted to the LPM as it is also expressed dorsally in the somites.

Patterning in the early LPM has been poorly described, with the exception of the early cardiovascular progenitors in the ventral LPM. However the domains of *nkx2-5*, *foxf1*, *hand1* and *sall3* clearly demonstrate that this tissue has a complex pattern in both the anterior-posterior and dorsal-ventral axes by the end of neurulation.

### 3.1.1 The Lateral Plate Mesoderm is RA Responsive

The RA synthesizing enzyme *raldh2* is differentially expressed along the anterior and dorsal borders of the LPM suggesting an asymmetric distribution of ligand within the LPM (Chen et al., 2001). Also, *foxF1*, *hand1* and *sall3* were identified to be RA responsive in a microarray at the same stage of development (Drysdale, personal communication). Therefore, I predicted that RA signalling would be involved in generating the early LPM pattern. I exposed post-gastrula embryos to exogenous RA, RAA, or a DMSO carrier control for an approximate four-hour time window throughout neurulation (stage 14-20) and assayed LPM marker gene expression. I observed a marked

**Figure 4.** Expression of *foxf1*, *hand1* and *sall3* in the early *Xenopus* embryo. *Foxf1* (A) is expressed in the mid-late neurula stage embryo in the anterior-dorsal LPM. As the tail bud elongates, the *foxf1* expression domain elongates to encompass the anterior two thirds of the LPM by the late tailbud stage. Expression of *hand1* (B) is first detectable immediately following gastrulation and continues into the swimming tadpole stage in the medial LPM. At stage 20 it is clear that the *hand1* staining does not extend to the posterior end of the LPM. *Sall3* (C) is expressed during late gastrulation in the presumptive neural plate, and by stage 14 in the posterior LPM (arrows). Expression in the posterior LPM disappears by the end of neurulation, while expression in the neural tube persists into the late tailbud stage. The black numbers indicate the developmental stage of the embryo. The embryos are viewed from the side with dorsal up and the anterior end to the left, with the exception of the stage 12 *sall3* staining where the embryo is viewed from the posterior end looking at the dorsal surface.


A
20
26
32
36
B
14
20
24
32
36
C
12
14
20
32
36

expansion of our anterior-dorsal marker *foxf1* (Figure 5E-F) along the anterior-posterior axis, particularly at the dorsal most region of this domain in the RA treatment when compared with control embryos. In contrast, embryos exposed to RA and assayed for *nkx2-5* expression demonstrated a reduction in expression domain size (Figure 5B-C). When embryos were exposed to RAA, the anterior-dorsal *foxf1* domain was severely reduced (Figure 5D) while the anterior-ventral *nkx2-5* domain remained unchanged (Figure 5A).

The middle LPM marker *hand1* is normally expressed in an inverted saddle shape and this domain is expanded towards both anterior and posterior poles of the embryo under exogenous RA conditions (Figure 5H-I). Conversely, the *hand1* domain is restricted when RA signalling is blocked by addition of RAA (Figure 5G). Furthermore, the hole normally observed in the anterior-ventral end of the *hand1* pattern in DMSO controls is absent in embryos treated with RA (Figure 5K-L). Finally, when embryos are exposed to RA, the posterior and dorsal most edge of the *hand1* domain stains darker in all cases when compared to the rest of the domain, suggesting that *hand1* is being more highly expressed along the posterior-dorsal border of the domain. There was no marked difference in *hand1* staining intensity within the *hand1* expression domains of RAA treated embryos.

In contrast to *hand1*, when RA signalling is increased the posterior *sall3* expression domain is lost in the early LPM (Figure 5O). I also noted that the previously described *sall3* expression pattern (Sparrow et al., 1998) in the neural tube was also changed, as staining in the basal ganglia was much darker with increased RA signalling (Figure 5R). Conversely, treatment with RAA has no obvious effect on the *sall3* expression domain in the LPM (Figure 5M).

To test if these expression domains were directly adjacent to one another in the LPM, I performed a double *in situ* hybridization with DIG probes against either, both *foxf1* and *hand1*, or both *hand1* and *sall3*. In control treated embryos (Figure 6H, Q) there was no gap between either of the *foxf1* and *hand1* domains, or the *hand1* and *sall3* domains, although a region of overlap could not be ruled out.

To confirm the results of the exogenous RA treatments, which rely on concentrations of RA ligand well above physiological levels, I altered the endogenous RA concentrations by inhibiting the function of Cyp26 with ketoconazole, thereby inhibiting RA catabolism and presumably increasing the availability of endogenous RA ligand. In addition, inhibition of Raldh2 by treating embryos with citral and DEAB, presumably decreasing RA signalling by inhibiting RA synthesis, was done to test whether endogenous RA signalling plays a role in patterning the LPM. Both *foxf1* and *hand1* domains were reduced in size when the embryos were treated with either DEAB (Figure 7G, K) or citral (Figure 7H-L) as would be expected if endogenous levels of RA were decreased. However, no consistent changes were observed in the expression pattern of *foxf1* or *hand1* when the embryos were treated with ketoconazole (Figure 7E, I) and compared to controls (Figure 7F, J). No obvious changes in the expression domains of either *nkx2-5* or *sall3* were observed under any of those conditions (Figure 7A-D, M-P). Therefore, decreasing the availability of endogenous RA ligand by inhibition of Raldh2 causes a change in the size of the anterior *foxf1* and middle *hand1* expression domains supporting a role of endogenous RA in defining these LPM domains. However, increasing the endogenous concentrations of RA through inhibition of Cyp26 has no discernable effect on the expression domains within the LPM.

**Figure 5.** The expression domains of *nkx2-5, foxf1, hand 1* and *sall3* are regulated by RA signaling. Embryos are treated with RAA (left panel), RA (right panel) or DMSO (center panel) at stage 14 and were allowed to develop until stage 20. Whole mount *in situ* hybridization for *nkx2-5* (A-C) demonstrates the reduced expression domain under the RA treatment (C), as compared to the DMSO control (B). In contrast, the expression domain of the anterior-dorsal marker *foxf1* (D-F) is extends further to the posterior end of the embryo under exogenous RA conditions (F) and reduced under treatment with the RAA (D) as compared to the DMSO control (E). Similarly, the middle marker *hand1* (G-L) demonstrates a reduced expression domain under the RAA treatment (G), while the domain is expanded when treated with RA (I) as compared to the DMSO control (H). Note that when viewed from the side (G-I), the greatest expansion in the *hand1* domain is on the dorsal edge, where there is also increased staining intensity (arrowhead), when treated with RA. In contrast, when viewed from the side (M-O), the lateral *sall3* domain is virtually absent under treatment with RA (O) when compared to either RAA-treated (M) or control embryos (N). When viewed from the anterior end (Q-S), other areas of *sall3* expression are also affected by RA. The banded expression in the developing brain is reduced and there is increased expression in staining in presumptive ganglia (arrowhead) when compared to RAA-treated (Q) or control embryos (R). ant: anterior view, llv: left lateral view, ven: ventral view.

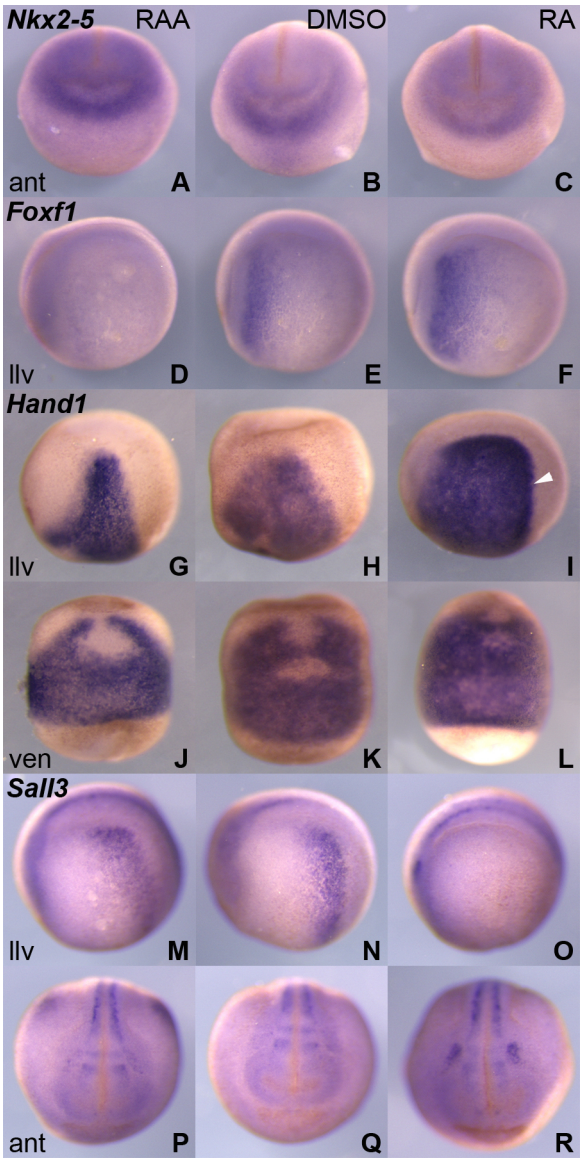

Nkx2-5
RAA
DMSO
RA
ant
A
B
C
Foxf1
llv
D
E
F
Hand1
llv
G
H
I
ven
J
K
L
Sall3
llv
M
N
O
ant
P
Q
R

**Figure 6.** The expression domain of *hand1* overlaps with both *foxf1* and *sall3*. Embryos were treated with RAA (left panel), DMSO (center panel) or RA (right panel) at stage 14 and assayed for expression of *foxf1* (A-C), *hand1* (D-F, J-L), *sall3* (M-O), or both *foxf1* and *hand1* (G-I) or *hand1* and *sall3* (P-R) at stage 20. Probes for *foxf1* and *hand1*, or *hand1* and *sall3* were Digoxygenin labeled, and detected simultaneously with an alkaline phosphatase conjugated anti-DIG antibody.

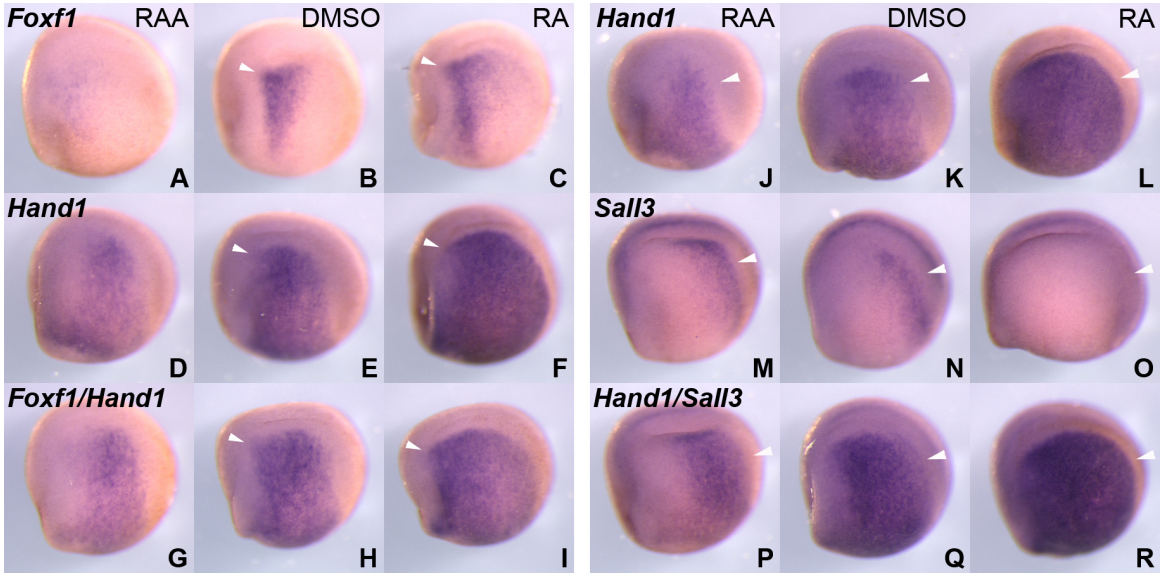
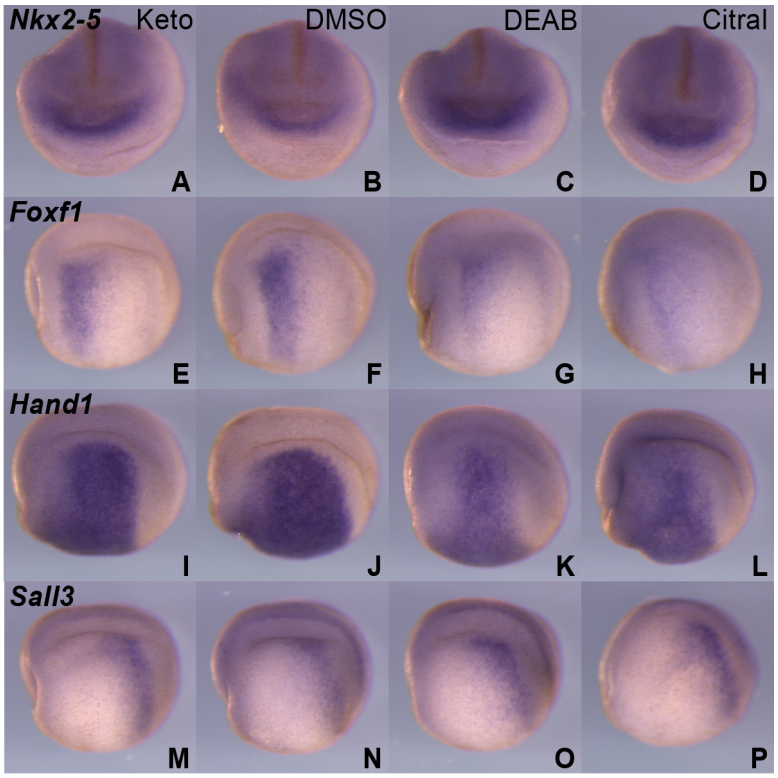

Foxf1
RAA
DMSO
RA
A
B
C
Hand1
D
E
F
Foxf1/Hand1
G
H
I
Hand1
RAA
DMSO
RA
J
K
L
Sall3
M
N
O
Hand1/Sall3
P
Q
R

**Figure 7.** Decreasing endogenous RA levels altered the expression of *hand1* and *foxf1* but does not affect the expression of *nkx2-5* or *sall3*. Embryos were treated at stage 14 with ketoconazole (left column) to increase endogenous RA levels by inhibiting Cyp26 and DEAB or citral was used to lower endogenous RA levels by inhibiting RALDH2. The expression domain of *nkx2-5* (A-D) was not noticeably altered by any of the treatments when compared to DMSO controls (B). In contrast, the expression of *foxf1* (E-H) was not obviously altered by treatment with ketoconazole (E) but both DEAB and citral (G, H) treatments reduced the expression domain of *foxf1* as compared to the DMSO control embryos (F). Similarly, the size of the *hand1* (I-L) domain was not clearly altered by ketoconazole (I) when compared to controls (J), but both DEAB and citral (K, L) diminished the expression domain. The expression domain of *sall3* was not altered by any of the treatments (M-P).

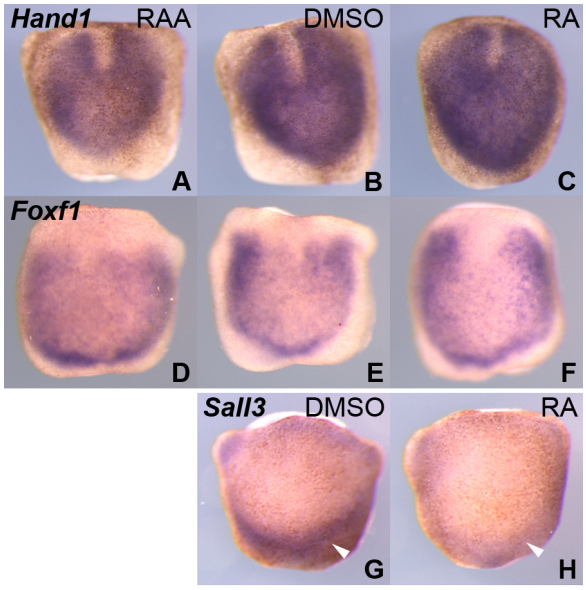

Nkx2-5
Keto
DMSO
DEAB
Citral
A
B
C
D
Foxf1
E
F
G
H
Hand1
I
J
K
L
Sall3
M
N
O
P

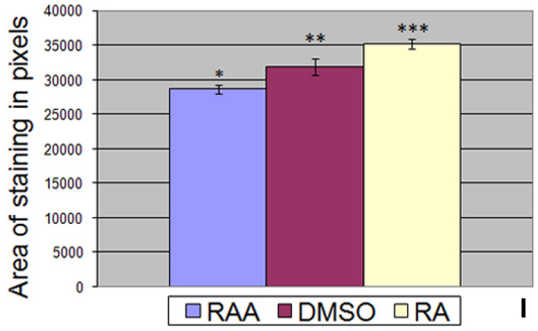
### 3.1.2 Retinoic Acid Directly Regulates *Hand1* Transcription

It has been previously established in *Xenopus*, that the lateral plate mesoderm receives instructive signals from the dorsal mesoderm, as signals establishing the left-right axis originating from the node are passed onto the LPM during this stage of *Xenopus* development (Schweickert et al., 2007). Therefore, it was a distinct possibility that RA could act on the dorsal mesoderm and that the observed changes in LPM expression domains were a secondary effect to RA activities within the dorsal mesoderm. To test whether RA directly alters the LPM, or at least the ventral pole of the embryo, independent of dorsal axial mesoderm and neural plate, I created ventral tissue explants lacking all dorsal tissue (Figure 8). Once isolated, I treated the explants with RA or RAA, and looked for changes in the expression domains of *foxf1*, *hand1* and *sall3*. Both *hand 1* (Figure 8A-C) and *sall3* (Figure 8G-M) LPM expression domains are altered under exogenous RA treatments in the absence of dorsal tissue implying that RA is directly affecting the ventral side of the embryo. As the explants could be laid flat after staining, I was able to measure the total area of the *hand 1* expression domain. I found that total area of expression was significantly different ($p<0.05$) between all treatments (Figure 8I), with the expression domain in RA treated explants significantly larger, and RAA treated explants significantly reduced when compared to control explants, mirroring the effect seen in whole embryo treatments.

The *sall3* domain present in the DMSO control explants and absent in RA treated explants similar to whole embryo treatments (Figure 8G-H). These explant results demonstrate that RA can pattern the LPM independent of signals from the dorsal side of the embryo. An interesting result was observed when explants were assayed for *foxf1* expression (Figure 8D-F). In untreated ventral explants, *foxf1* was detectable

**Figure 8.** RA signalling affects the expression of *hand1* and *sall3* in the absence of dorsal tissue. As in the whole embryo, the *hand1* expression domain area was found to be enlarged under RA treatment (A) and reduced with RAA (C) when compared to controls (B). Measurements and comparison of the total staining area confirmed that this change was statistically significant (I). Explants lacking dorsal tissue show an expansion of *foxf1* (D-F) expression throughout the LPM in all treatments. Ventral explants also show the same lack of *sall3* expression in the LPM (arrows) when treated with RA (F) when compared to control explants (E). In each panel, ventral explants are positioned with the anterior end toward the top of the image.


Hand1
RAA
DMSO
RA
A
B
C
Foxf1
D
E
F
Sall3
DMSO
RA
G
H


Area of staining in pixels
40000
35000
30000
25000
20000
15000
10000
5000
0
*
**
***
RAA
DMSO
RA
I

throughout the entire LPM. This suggests that an inhibitory factor controlling *foxf1* expression was removed in isolation from dorsal tissue.

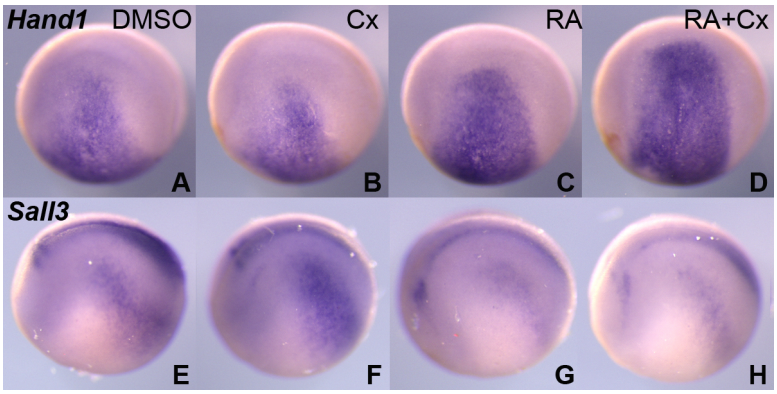
As the RARs function as ligand activated transcription factors, I wished to determine if RA signaling was directly regulating *hand1* and *sall3* expression. Embryos were treated with cycloheximide to inhibit protein synthesis 15 minutes prior to treatment with RA (Cascio and Gurdon, 1987). If RA is not acting through RARs directly bound to the promoter regions of *hand1* or *sall3*, their expression should not be altered. Following *in situ* hybridization, I observed that addition of RA altered the *hand1* domain even in the presence of cycloheximide (Figure 9 A-D), suggesting that RA is acting directly on *hand1* expression.

In contrast, the *sall3* domain was not completely absent in either the RA or RA and cycloheximide treatment (Figure 9G-H), although it does seem to be consistently reduced from controls (Figure 9E). The reduction in *sall3* domain is not as obvious as the previous RA treatements (section 3.1.1, Figure 5) because this treatment was limited to one hour before fixation due to the toxic effects of cycleheximide. However, since the *sall3* domain is detectable in each treatment, drawing definite conclusions regarding *sall3* being a direct target of RA signalling is difficult.

### 3.1.3 The RA Induced Changes of LPM Patterning are Permanent

Since the observed alterations to the LPM are occurring within a relatively short developmental time window, I wished to test if these changes are permanent or, if RA signalling is returned to normal, the LPM pattern is able to recover. I exposed early neurula embryos to RA or RAA, and cultured them until stage 20

**Figure 9.** RA directly regulates the middle *hand1* domain. Embryos treated with cycloheximide at stage 14 had no change in the expression of either *hand1* (B) or *sall3* (F) when compared to control embryos (A, E) when assayed 90 minutes later. Expansion of the *hand1* domain, when treated with RA (C), was also observed in embryos treated with both RA and cycloheximide (D). When the embryos were treated with RA for one hour, there was a reduction in the *sall3* expression domain although not the severe loss seen in the 4-5 hour treatments (G). In the presence of cycloheximde, the reduction in the *sall3* domain was not observed (H). In contrast, the expression domain of *sall3* under treatment with RA and cycloheximide is not reduced to the extent seen in RA alone (H). In each panel the left lateral side is shown with the anterior end to the left, dorsal to the top. Cycloheximide (Cx).


Hand1
DMSO
Cx
RA
RA+Cx
A
B
C
D
Sall3
E
F
G
H

when the neural tube is sealed. In order to then restrict abnormal RA levels to a defined developmental time window, equimolar RAA was added to the embryos that had previously been treated with RA, and RA was added to RAA treated embryos effectively neutralizing the effect of either treatment. This neutralization was necessary since simply flushing the embryos was unable to remove the lipophillic RA ligand and insufficient to return the level of RA signalling to normal (Figure 10). Furthermore, a 1:1 concentration of RA to RAA was found to be sufficient to neutralize the effect of either on RA signalling as assayed by *cyp26* expression, a direct target of RA signalling (Figure 11M-O). The embryos were then cultured for one hour in the combination of RA and RAA before being thoroughly flushed and moved into a new culture solution without exogenous RA or RAA. The embryos were fixed at the tailbud stage (Stage 34-36).

Both *foxf1* and *hand1* but not *sall3* expression persists in the LPM after the early tailbud stage. Therefore, I could only assay for permanent alterations in the *foxf1*and *hand1* expression domains. To extend the observations of altered patterning within the posterior LPM, I also assayed for the expression of two additional transcription factors that are expressed in the LPM at later stages with restricted expression domains: *hoxc10* and *pitx2*. *Pitx2* is expressed in the anterior to medial LPM during the late tailbud stages (Figure 12B) on the left side of the embryo (Campione et al., 1999). *Hoxc10* is normally expressed in the posterior half of the LPM (Figure 12K) during the mid to late tail-bud stage (Christen et al., 2003).

**Figure 10.** Exogenous RA cannot be flushed from the embryo. Embryos were treated with RAA (A-C), DMSO (D-F) and RA (G-I) at stage 14 and assayed for *cyp26* as a measure of active RA signalling at stage 20. To determine if RA signalling could be washed from the embryo, embryos were further treated with RA for 90 minutes beginning at stage 14, flushed thoroughly at the end of the 90 minute time interval (roughly stage 16) and then washed at 30 minute intervals untile stage 20 (J-L). Finally, a last treatment was performed where embryos were treated with RA at stage 14 for 90 minutes, and then an equal concentration of RAA was added for a further 30 minutes, before the embryos were flushed thoroughly, and washed at 30 minute intervals until stage 20 (M-O). ant: anterior view (left panel), dor: dorsal view (center panel), pos: posterior view (right panel).

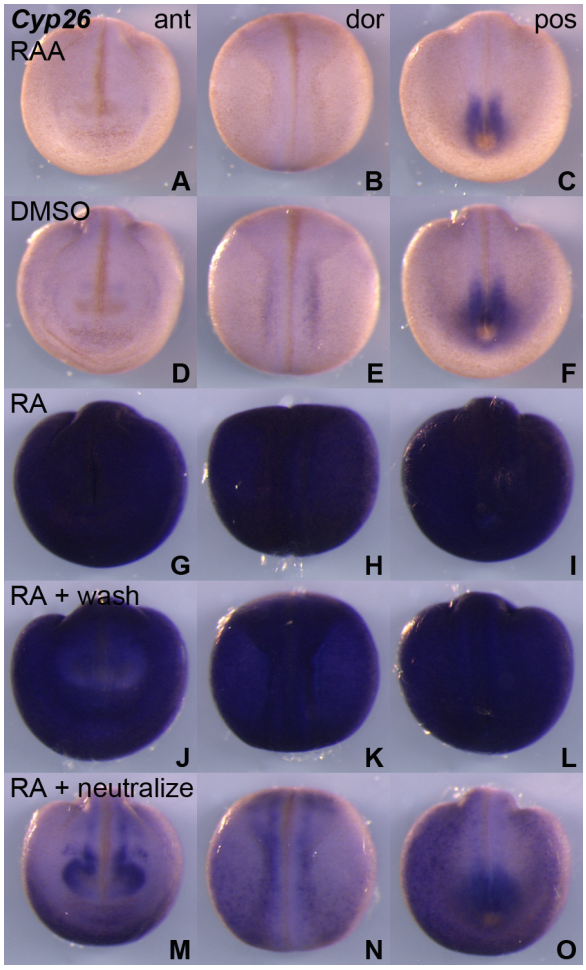
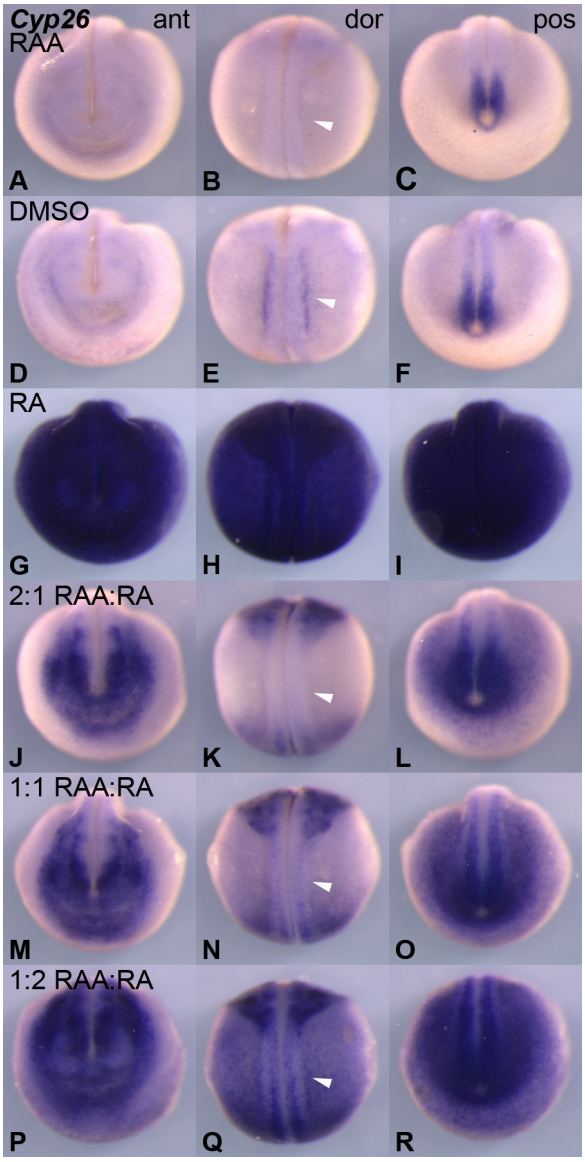

Cyp26
ant
dor
pos
RAA
A
B
C
DMSO
D
E
F
RA
G
H
I
RA + wash
J
K
L
RA + neutralize
M
N
O

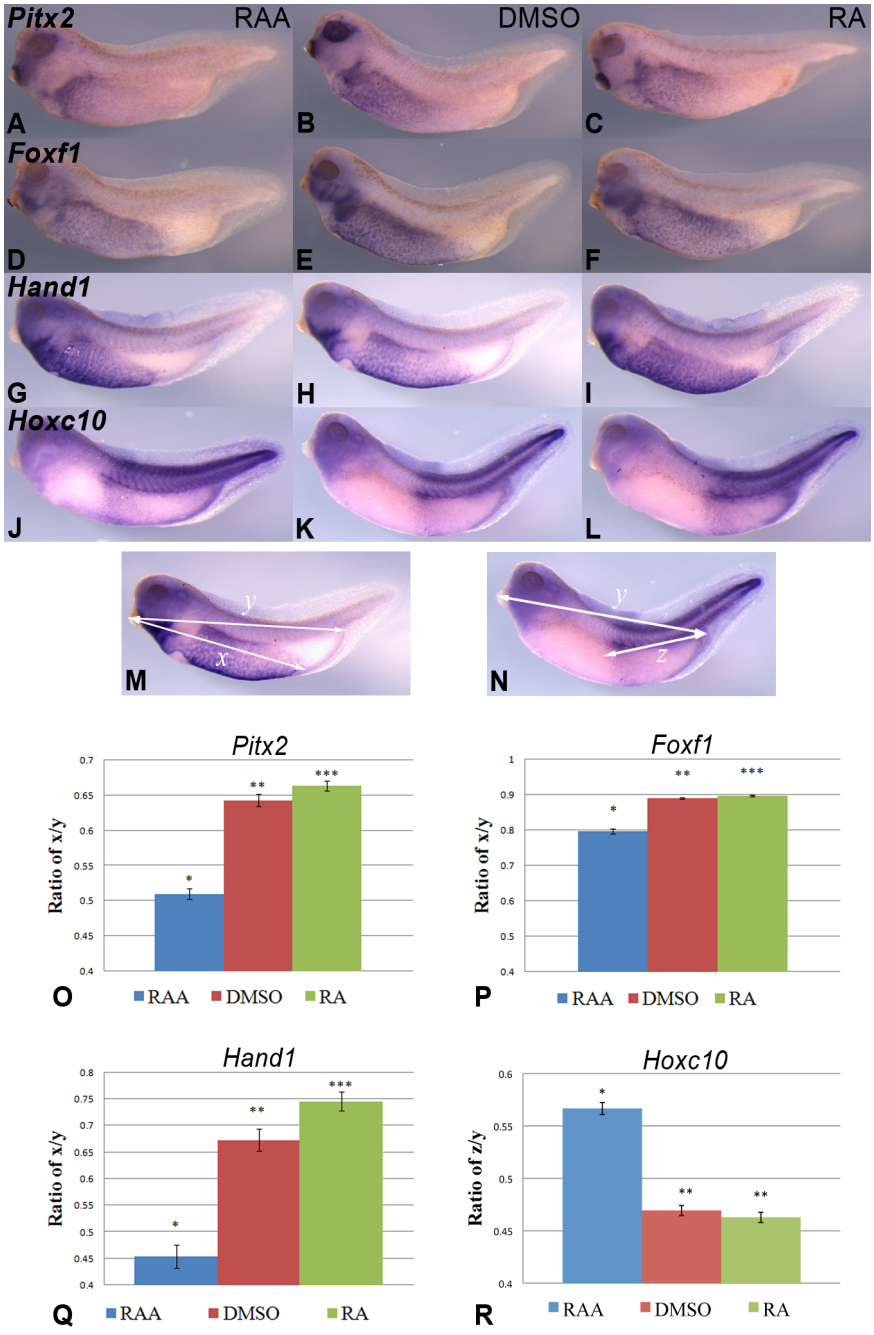
**Figure 11.** Embryos treated with 1:1 RA:RAA demonstrate a proper dorsal expression domain of *cyp26*. Embryos were treated with RAA (A-C), DMSO (D-F), RA (G-I), 2:1 RAA:RA (J-L), 1:1 RAA:RA, 1:2 RAA:RA at stage 14 and assayed by whole mount *in situ* hybridization to determine the proper dosage of RA:RAA required to neutralize abnormal RA signaling. The dorsal neural tube domain of *cyp26* expression was used to assay for the proper dosage in the medial embryo (arrow heads in middle column). Interestingly, although the medial LPM showed signs of rescue, the expression domains in both the anterior and posterior pole were not rescued. Anterior views of embryos are shown in the left column, dorsal views in the center column, and posterior in the right column.


*Cyp26*
ant
dor
pos
RAA
A
B
C
DMSO
D
E
F
RA
G
H
I
2:1 RAA:RA
J
K
L
1:1 RAA:RA
M
N
O
1:2 RAA:RA
P
Q
R

The *foxf1*, *pitx2* and *hand1* domains extended further posterior in RA treated embryos (Figure 12C, F, I) when compared to DMSO control treated embryos (Figure 12B, E, H). Exposure to RAA during the same time window demonstrated a restriction of the expression domains of these three transcription factors towards the anterior end of the embryo (Figure 12A, D, G). To quantify these results, the lengths of the expression domains from the cement gland to the posterior extent of the expression domains was compared to the length from the cement gland to the point where the posterior trunk meets the somites. Significant changes were found for the lengths of the *foxf1*, *pitx2*, and *hand1* domains (Figure 12O, P, Q), confirming my earlier qualitative observations.

The anterior edge of the *hoxc10* expression domain was displaced anteriorly under treatment with RAA (Figure 12J). This displacement was quantified by taking a ratio of the length from the back of the body axis to the front of the expression domain to the length of the body axis (cement gland to posterior trunk/somite junction) and the difference was found to be statistically significant between the RAA treated embryos and the DMSO control (Figure 12R). However, RA had no significant effect on the expression domain of *hoxc-10*. Therefore, altered RA signalling during a restricted four hour time window between stages 14 and 20, was sufficient to permanently alter the expression domains of our anterior LPM markers, extending their expression domains further posterior. Conversely decreased RA signalling restricted the expression domains of all of the anterior-middle markers, and extended our posterior marker, *hoxc10*, further anterior.

**Figure 12.** Changes to the LPM patterning persist after time restricted RA treatments. Experimental changes in RA signaling were limited to between stages 14-20 and the embryos were fixed at stage 34-36 for *in situ* hybridization for *pitx2* (A-C), *foxf1* (D-F), *hand1* (G-I), and *hoxc10* (J-L). The expression domain is reduced under treatment with RAA (A,D, and G), and expanded under RA (C, F, and I) as compared to the DMSO controls (B, E, and H) for all of *foxf1*, *pitx2*, and *hand1*. While the domain of the posterior marker *hoxc10* was anteriorly displaced under RAA (J) as compared to DMSO controls (K), the domain is unaffected under treatment with RA. In each case the lengths of the domains were measured as a ratio of the length from the cement gland to the posterior LPM staining (x) to the length from the cement gland to the back of the body (y) for the anterior *foxf1*, *pitx2*, and *hand1*. The differences in the length of the staining domain were found to be significant (M, N and O) between all treatments in embryos assayed for these three genes. *Hoxc10* was similarly measured (P) using the length of the body axis from the cement gland to the back of the body axis (y), however the length of the domain was measured from the back of the body axis to the anterior edge of the domain (z), and was found to be significantly different between the DMSO controls and the RAA treatment however no significant difference was found between RA treatments and controls. In all embryos the left lateral side is shown, with the anterior pole to the left, dorsal to the to


Pitx2
RAA
DMSO
RA
A
B
C
Foxf1
D
E
F
Hand1
G
H
I
Hoxc10
J
K
L
y
x
M
y
z
N
Pitx2
Ratio of x/y
0.7
0.65
0.6
0.55
0.5
0.45
0.4
*
**
***
O
RAA
DMSO
RA
Foxf1
Ratio of x/y
1
0.9
0.8
0.7
0.6
0.5
0.4
*
**
***
P
RAA
DMSO
RA
Hand1
Ratio of x/y
0.8
0.75
0.7
0.65
0.6
0.55
0.5
0.45
0.4
*
**
***
Q
RAA
DMSO
RA
Hoxc10
Ratio of z/y
0.6
0.55
0.5
0.45
0.4
*
**
**
R
RAA
DMSO
RA

## 3.2 FGF Signalling Contributes to LPM Patterning

While proper RA signalling is clearly important for patterning the early LPM, RA signalling alone cannot explain all aspects of the observed of the LPM pattern. The effect of dorsal tissue on *foxf1* expression suggests that a second signalling mechanism is required to inhibit its expression in the posterior LPM. I hypothesized that the FGF signalling pathway is a good candidate since *fgf4* is expressed in the posterior pole of the embryo, while *fgf8* is expressed both in the posterior pole of the embryo as well as at the anterior end of the LPM in the heart region. To test if FGF signalling was necessary for patterning of the LPM after gastrulation, I used a chemical FGFR inhibitor, SU5402, which binds to the intracellular ATP binding domain of the FGFRs (Mohammadi et al., 1997) and inhibits autophosphorylation between the RTK tails. This approach was used because it allowed greater control over the timing of FGF inhibition, and allowed me to avoid compounding the results with earlier effects of FGF inhibition on early mesoderm formation (Amaya et al., 1993) (see section 1.6.1).

### 3.2.1 FGF Signalling is Essential for LPM Patterning

To test if FGF signalling was necessary post gastrulation for proper patterning of the LPM we treated embryos at stage 12.5-13, immediately following blastopore closure and as soon as the neural plate is evident, and fixed embryos at stage 20. While SU5402 has been used at concentrations of up to 100 μM in *Xenopus* (Fletcher and Harland, 2008), we found that by adding 0.1 mM ATP, as per instructions (CalBiochem) that it was possible to see a full loss of FGF signalling phenotype including loss of the tail outgrowth (Figure 13), loss of the heart field (Figure 14), and loss of *sprouty2* expression,

a member of the FGF synexpression group (Furthauer et al., 2004) at a 10 μM concentration (Figure 15). Although SU5402 has been described as a specific FGFR1 inhibitor, the original paper did not test its effectiveness at inhibiting the other FGFRs (Mohammadi et al., 1997) and it has since been shown to inhibit both FGFR2 (Bernard-Pierrot et al., 2004; Moftah et al., 2002) and FGFR3 (Grand et al., 2004). It is therefore likely that the SU5402 treatments show at least some inhibition of all of the FGFRs.

*Foxf1*, expressed in the anterior-dorsal LPM, was expanded toward the ventral pole of the embryo when FGF signalling was blocked with 10 μM SU5402 (Figure 16 A-B). In addition, *foxf1* is normally restricted to the anterior half of the LPM at stage 20, but when FGF signalling is inhibited *foxf1* expression is up regulated at the posterior-ventral end of the LPM on the ventral side of the closed blastopore (Figure 16C-D). Blocking FGF signalling also resulted in a posterior expansion of the middle *hand1* domain, particularly evident in the dorsal half of the LPM (Figure 16E-F). The posterior border of the *hand1* expression domain was also extended further posterior, approaching the neural folds more closely in the SU5402 treatment than in the DMSO control (Figure 16G-H).

In the posterior LPM, the *sall3* expression domain was displaced further posterior when FGF signalling was inhibited, in a domain reminiscent of the ectopic *foxf1* domain next to the closed blastopore (Figure 16I-L). Furthermore, the *bra* domain, normally present in the most posterior mesoderm (the tail bud domain), was completely undetectable after treatment with 10 μM SU5402 (Figure 16M-N) as has been described (Fletcher and Harland, 2008). Therefore, over the entire LPM there is a coordinated shift of all expression domains towards the posterior end of the embryo when FGF signalling is lost.

**Figure 13.** ATP is necessary for full efficacy of SU5402 at a 10 μM concentration. Embryos were treated with DMSO+ATP (A), 10 μM SU5402 (B) or 10 μM SU5402 in conjunction with 0.1 mM ATP (C) at stage 12.5. Embryos treated with SU5402 elongated similar to DMSO controls, while embryos treated with SU5402 in conjunction with ATP demonstrate the truncated phenotype characteristic of a loss of FGF signalling.

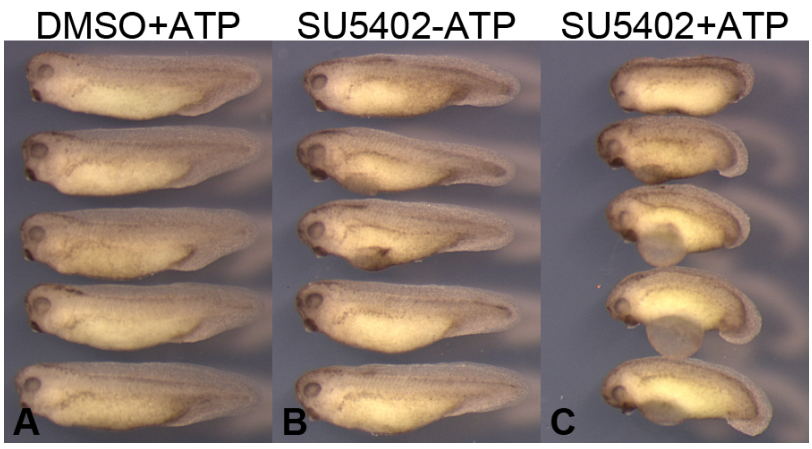

DMSO+ATP
SU5402-ATP
SU5402+ATP
A
B
C

**Figure 14.** ATP is required in conjunction with SU5402 at a 10 μM concentration to block heart specification. Embryos that were treated with 10 μM SU5402 at stage 12.5 show proper patterning of the heart tube (C-D) shown by expression of *cardiac Troponin I* (*cTnI*) at stage 34, whereas embryos treated with 10 μM SU5402 and 1mM ATP fail to specify and form the heart tube (E-F); a known effect resulting from a loss of FGF signalling (Keren-Politansky et al., 2009; Samuel and Latinkic, 2009).

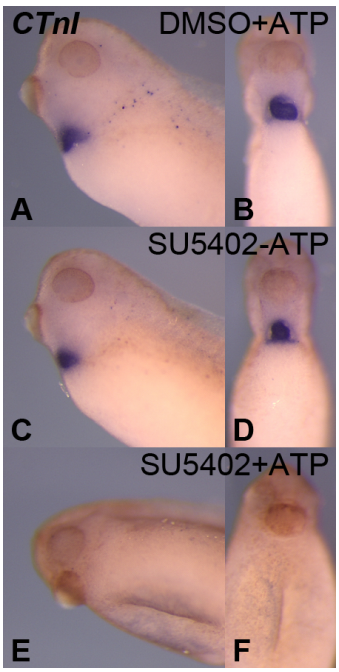

CTnI
DMSO+ATP
A
B
SU5402-ATP
C
D
SU5402+ATP
E
F

**Figure 15.** 10 μM SU5402 in conjunction with ATP yields the full loss of Fgf signalling phenotype. Effectiveness of SU5402 is based on absence of *sprouty2* (A-L) expression, absence of heart development shown by *cTnI* expression (M-X), and a loss of tail bud outgrowth. A dose of 1μM SU5402 at stage 12.5 was sufficient to completely block heart development (Q-R), but not *sprouty2* expression (E-F) or tailbud outgrowth. Doses of 10 μM SU5402 higher were required to fully block all of *sprouty2* expression (I-J), heart development (U-V) and tailbud outgrowth (I). Higher doses of 20 μM SU5402 (P-R) also blocked both *sprouty2* expression (K-L), heart development (W-X) and tail bud outgrowth (K), but also led to an increase in lethality by the mid tailbud stage.

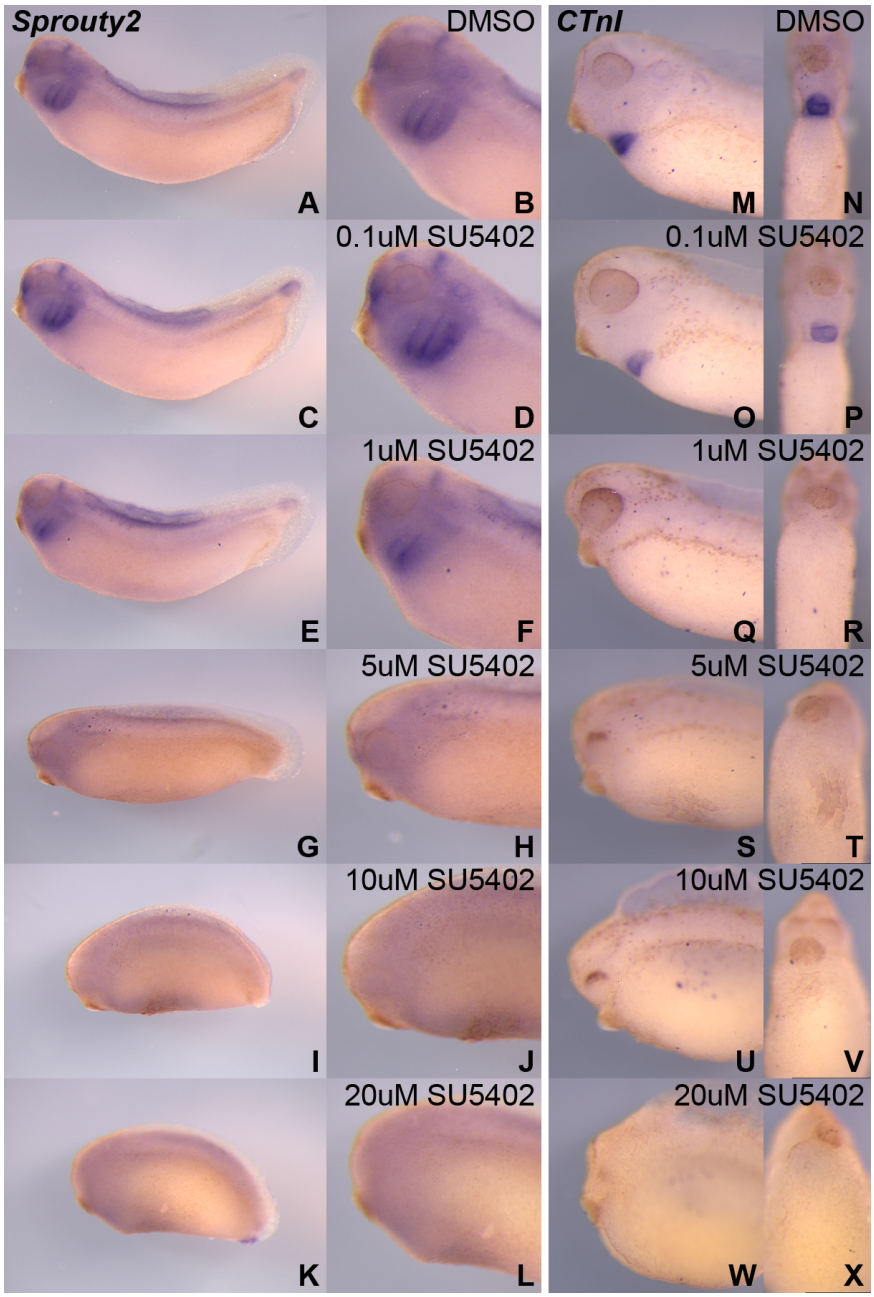
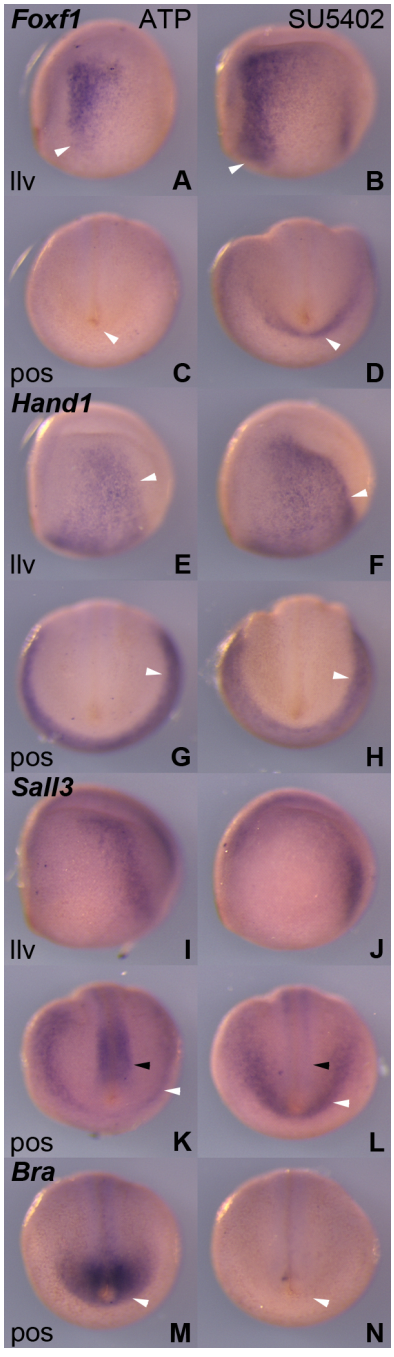

Sprouty2
DMSO
CTnI
DMSO
A
B
M
N
0.1uM SU5402
0.1uM SU5402
C
D
O
P
1uM SU5402
1uM SU5402
E
F
Q
R
5uM SU5402
5uM SU5402
G
H
S
T
10uM SU5402
10uM SU5402
I
J
U
V
20uM SU5402
20uM SU5402
K
L
W
X

**Figure 16.** Fibroblast Growth Factor signalling is necessary for proper LPM patterning. The expression domains of *foxf1*, *hand1*, *sall3* and *bra* were assayed at stage 20 for changes when FGF signalling was inhibited with SU5402 at stage 12.5. The anterior domain of *foxf1* (A-D) was expanded toward the ventral side of the embryo (arrowheads in A and B), and was upregulated at the posterior end of the LPM just ventral to the blastopore (arrowheads in C and D). The posterior border of *hand1* (E-H) is displaced further posterior, particularly at the dorsal edges of the domain (arrowheads in E-H). The LPM domain of *sall3* (I-L) is displaced posterior with a loss of FGF signalling (white arrowheads in K-L) while the posterior neural tube domain is undetectable (black arrowheads in K-L). Expression of *bra* in the tail bud domain is completely lost in the absence of FGF signalling (arrowheads in M-N). llv: left lateral view, embryos oriented with the anterior pole toward the right of the image, dorsal at top. pos: posterior view, embryos oriented with dorsal at top of image.

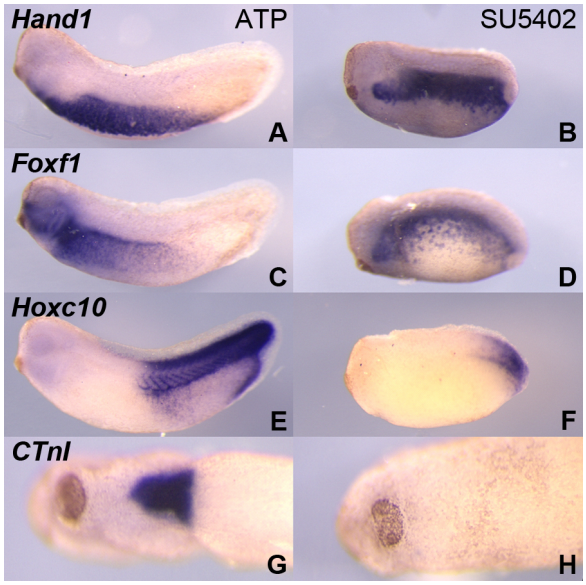

Foxf1
ATP
SU5402
llv
A
B
pos
C
D
Hand1
llv
E
F
pos
G
H
Sall3
llv
I
J
pos
K
L
Bra
pos
M
N

Since early LPM patterning requires FGF signalling, I also wanted to confirm that later LPM patterning was dependent on FGF. To test this possibility, FGF signalling was inhibited from the early neurula to the tailbud stage with either 10 μM SU5402 in conjunction with ATP, or a DMSO and ATP control. Treated embryos were then assayed for the expression of *hand1*, *foxf1*, *hoxc10* and *cTnI* which all have restricted expression domains within the LPM at later stages. A loss of FGF signalling from shortly after gastrulation until the late tail bud stages (stage 32-34) resulted in a stunted embryo phenotype, as has been previously described (Amaya et al., 1991; Amaya et al., 1993; Fletcher and Harland, 2008). As I have previously shown in the RA experiments, both *hand1* and *foxf1* are normally expressed in the anterior two thirds of the LPM at the early tailbud stage, with a defined area free of staining in the posterior LPM in both cases. However, the expression of both markers was detectable in the dorsal LPM over the entire anterior-posterior axis, from the anterior end to the closed blastopore when FGF signalling was blocked (Figure 17A-D). Conversely, *hoxc10* expression, that is normally restricted to the posterior half of the LPM, was entirely absent when FGF signalling is lost (Figure 17E-F). Lastly, the expression of *cTnI* was inhibited under treatment with SU5402 (Figure 17G-H).

### 3.2.2 FGF and RA Signalling Interact in Patterning the LPM

Since both RA and FGF are necessary to pattern separate areas of the LPM, and have been previously shown to form mutually antagonistic gradients in other tissues, I wished to test if the two pathways interacted in patterning the LPM. To determine if the RA signalling pathway directly impacts FGF signalling, embryos were treated with 1 μM

**Figure 17.** FGF is required for the patterning of late LPM markers. *Hand1* (A, B) and *foxf1* (C, D) are normally restricted in the anterior and middle LPM with a clear posterior domain free of expression in both cases. When embryos were treated with SU5402 at stage 12.5 both *hand1* (B) and *foxf1* (D) were observed along the entire anterior-posterior axis by stage 32. Conversely, the LPM expression domains of both *hoxc10* (E-F; normally expressed in the posterior half of the LPM) and *cTnI* (G-H; marker of cardiac differentiation) were completely undetectable when FGF signalling is inhibited, although expression of *hoxc10* in the somites and neural tube is still observed. A-F: lateral view of the embryos is shown, with anterior toward the left, dorsal at top. G-H; ventral view of the heart region is shown, with anterior toward left.

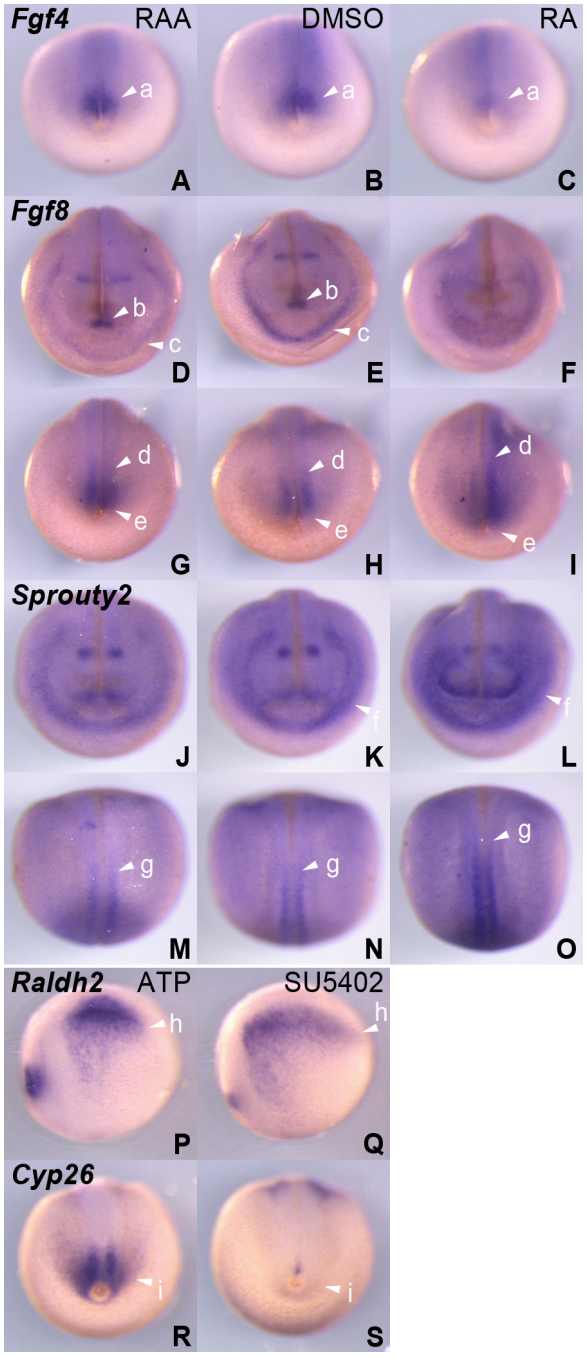

Hand1
ATP
SU5402
A
B
Foxf1
C
D
Hoxc10
E
F
CTnI
G
H

RA, RAA or a DMSO control and assayed for both *fgf4* and *fgf8* expression at the end of neurulation. When RA signalling was antagonized, the anterior trunk domain of *fgf8*, located just posterior to the cement gland, was decreased in intensity in comparison to staining in the pituitary anlagen (Figure 18D-E). However, the posterior domains of both *fgf4* and *fgf8* remained unaffected. Increasing RA signalling by treatment with *all-trans* RA resulted in an increase in the *fgf8* expression domain in anterior trunk (Figure 18E-F), becoming much thicker and surrounding the entire cement gland rather than residing posterior to it. *Fgf8* expression in the posterior neural tube was also extended much further anterior along the neural folds (Figure 18H-I), which is in agreement with previous results (Moreno and Kintner, 2004). Conversely, the expression domain of *fgf4* was decreased in the posterior neural tube to essentially background levels (Figure 18B-C).

Finally, I used *sprouty2* expression as an assay of active FGF signalling. *Sprouty2* is normally expressed in both anterior and posterior domains overlapping with the *fgf8* expression domains (Figure 18K, N). When RA signalling is increased the anterior domain of *sprouty2* becomes much broader in both the heart region and the pituitary anlagen (Figure 18L). Expression of *sprouty2* is also increased in the neural tube (Figure 18O), as it is normally present in the posterior half of the neural folds, but is displaced much further anterior with increased RA signalling.

To test if FGF signalling impacted the RA signalling system, I treated embryos at stage 12.5-13 with 10 μM SU5402 and assayed at the end of neurulation for both *raldh2*, the enzyme predominantly responsible for synthesizing *all trans* RA *in vivo*, and *cyp26*, the enzyme responsible for RA catabolism. *Raldh2* is normally expressed in the anterior half of the somites and dorsal LPM, as well as the anterior trunk along the anterior border

**Figure 18.** The RA and Fgf pathways regulate each other. The levels of RA signalling (A-I) were altered by addition of a synthetic RA antagonist (left column) or *all-trans* RA (right column) at stage 12.5 and compared to a DMSO control (center column). Embryos were assayed for *fgf4* (A-C) and *fgf8* (D-I) expression at stage 20. The posterior domain of *fgf4* (a) is lost in RA treated embryos (C) when compared to the control (B), but unaffected in embryos treated with RAA. Expression of *fgf8* however is expanded both anteriorly (E-F) and posteriorly (H-I; compare distance between arrowheads (d) marking the anterior limits of domain, and (e) marking posterior limits of domain) under treatment with RA. Decreasing RA signalling also reduces the anterior domain of *fgf8* underlying the heart region (compare ratio of staining intensity between (b) marking the pituitary anlagen to (c)). A similar effect is seen with *sprouty2* expression (J-O), as its domain is increased with RA in both the anterior heart region (L; arrowhead f) and it extends further anterior (g) in the dorsal neural tube (O) when compared to controls (K-N). Conversely, embryos were treated with SU5402 at stage 12.5 and assayed for expression of *raldh2* (P-Q) or *cyp26* (R-S) at stage 20 to determine the effect of a loss of FGF signalling on the RA signalling pathway. The expression domain of *raldh2* was expanded posterior (Q) (arrowhead: h – marking posterior limit of expression domain) as compared to control embryos (P). *Cyp26*, normally present in the posterior LPM tailbud domain (R; arrowhead i) is undetectable when FGF signalling is inhibited (S). Ant: anterior view with dorsal at top of image. Dor: dorsal view with anterior at top. Llv: left lateral view with anterior toward left, dorsal at top of image. Pos: posterior view with dorsal at top.


Fgf4
RAA
DMSO
RA
a
A
B
C
Fgf8
b
c
D
E
F
d
e
G
H
I
Sprouty2
f
J
K
L
g
M
N
O
Raldh2
ATP
SU5402
h
P
Q
Cyp26
i
R
S

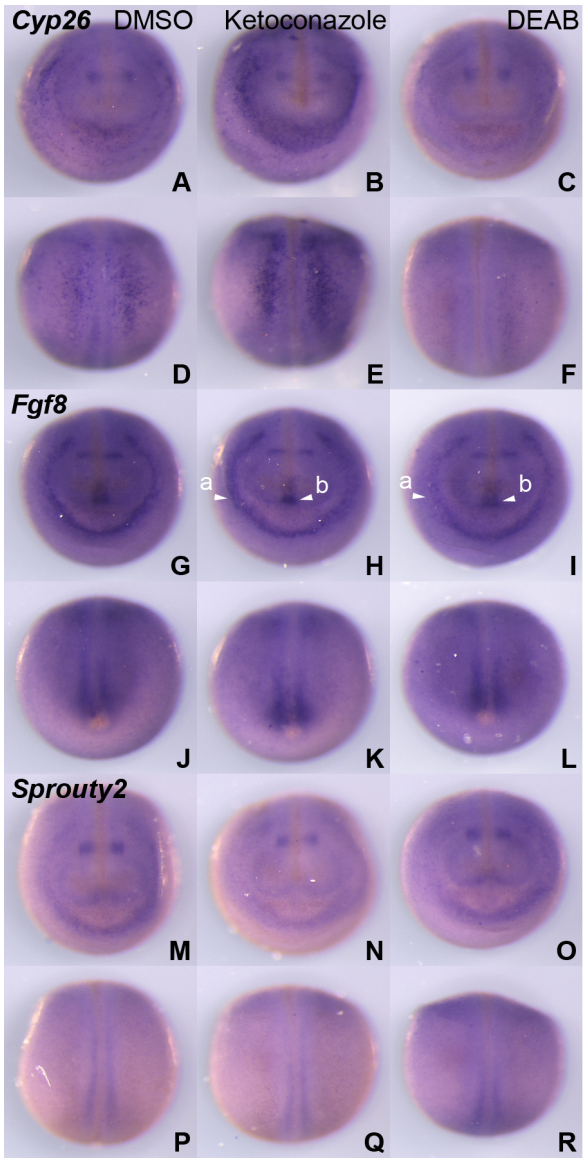
of the LPM. Inhibiting FGF signalling caused a posterior expansion of the dorsal *raldh2* domain much further posterior in the somites and LPM than in control embryos (Figure 18 P-Q). *Cyp26* is also normally expressed in the posterior neural tube and tail bud in a domain reminiscent of the *fgf4* and *fgf8* expression domains, and in the tail bud region that corresponds to the normal *bra* domain. Inhibiting FGF signalling caused a complete loss of the posterior *cyp26* domain (Figure 18R-S), which was no longer detectable in the posterior neural folds or the adjacent tail bud domain, consistent with previous studies (Moreno and Kintner, 2004).

To confirm that RA signalling was necessary for expression of the Fgf ligands, I also treated embryos with inhibitors of Raldh2 (ketoconazole) and Cyp26 (DEAB), the endogenous enzymes primarily responsible for RA synthesis and catabolism respectively. A reduction in RA levels by treatment with DEAB caused a similar reduction in *fgf8* staining in the anterior domain to that seen with RAA treatments (Figure 19 H-I), although to a lesser degree. However, increasing endogenous RA signalling by treating with ketoconazole did not seem to have an obvious effect on *fgf8* expression (Figure 19G-H). Neither an endogenous increase or decrease in RA signalling led to obvious changes of the *sprouty2* domain (M-R).

### 3.2.3 Synergistic Effects Between RA and FGF Signalling on LPM Patterning

Since the RA and FGF signalling pathways seem to be antagonistic in patterning the LPM, I wished to test if up-regulating RA signalling and inhibiting FGF signalling would show synergistic effects on LPM patterning. I treated embryos at stage 12.5-13 with RA, SU5402 or both and assayed for the expression of LPM markers at stage 20. As

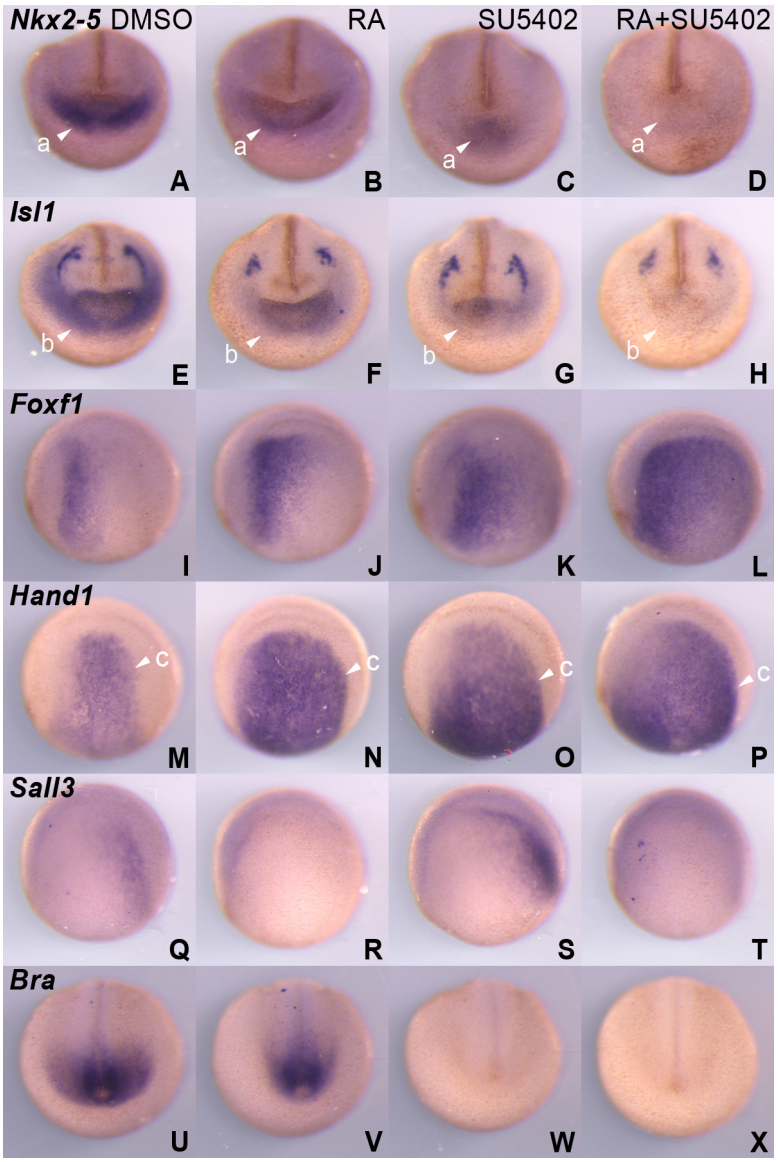
**Figure 19.** Altering the availability of endogenous RA ligand affects FGF signalling. Expression of *cyp26*, a biomarker of RA signalling, is slightly increased under treatment with ketoconazole (B, E), and decreased with DEAB (C, F) as compared to control embryos (A, D). The anterior *fgf8* expression domain (arrowhead a) was unchanged with a moderate increase in RA signalling when treated with ketoconazole (H) was also reduced in comparison to expression in the pituitary anlagen (arrowhead b) under treatment with DEAB (I) when compared to controls (G). However consistent changes were seen with either ketoconazole or DEAB on the posterior domain of *fgf8* expression (J-L). Also, no obvious and consistent changes were seen on the size and position of the *sprouty2* domain (M-R).


Cyp26
DMSO
Ketoconazole
DEAB
A
B
C
D
E
F
Fgf8
a
b
a
b
G
H
I
J
K
L
Sprouty2
M
N
O
P
Q
R

expected, treating with either RA or SU5402 individually drastically reduced the expression domain of *nkx2-5* when compared to control embryos (Figure 20 A-C). However, when embryos were treated with both RA and SU5402 expression of *nkx2-5* was completely abolished (Figure 20 D). Furthermore, *Isl1*, a marker of the secondary heart field (Cai et al., 2003; Moretti et al., 2006; Sun et al., 2007), displayed a similarly reduced expression domain as *nkx2-5* above when treated with either RA or SU5402 independently as compared to controls (Figure 20E-G). However, *Isl1* was completely undetectable in the heart domain when treated with RA and SU5402 together (Figure 20H). Conversely, the *foxf1* expression domain was expanded when either RA signalling was increased, or when FGF signalling is decreased (Figure 20I-K). However it was detectable throughout the entire LPM when embryos were treated with both RA and SU5402 (Figure 20L).

Lastly, *hand1*, *sall3* and *bra* were also assayed for changes in expression domain (Figure 20M-X). However additive effects between treatments were difficult to determine since the *hand1* domain changes are indistinguishable between the RA, SU5402 and RA and SU5402 treatments. The *sall3* domain is completely undetectable when treated with either RA or RA and SU5402, thus additive effects could not be demonstrated. The same issue existed with the *bra* domain, because *bra* was undetectable when treated with SU5402 or a combination of RA and SU5402.

**Figure 20.** Retinoic acid and FGF are opposing signalling molecules in patterning the LPM. Embryos were treated at stage 12 with RA, SU5402 or both RA and SU5402 and assayed by whole mount *in situ* hybridization for expression of *nkx2-5* (arrowhead a), *Isl1* (arrowhead b) and *foxf1* at stage 20. Treating embryos with RA reduces the expression domain of both *nkx2-5* (B) and *Isl1* (F) compared with controls (A, E). Significantly reduced domains of *nkx2-5* and *Isl1* were also present in SU5402 treated embryos (C, G), however when embryos are treated with both RA and SU5402 neither marker is detectable (D, H). The expression domain of *foxf1* was expanded in both the RA (J) and SU5402 (K) treatments when compared to controls (I), however when embryos were treated with both RA and SU5402 *foxf1* expression was detectible across the entire LPM although expression was graded. The expression of *hand1* (M-P), *sall3* (Q-T) and *bra* (U-X) was also analyzed, however the RA+SU5402 treatment was indistinguishable with one or more of the single treatments, thus I was unable to assess an additive effect between a gain of RA and loss of Fgf signalling. Ant: anterior view with dorsal at top of image. Pos: posterior view with dorsal at top of image.


*Nkx2-5* DMSO
RA
SU5402
RA+SU5402
a
A
B
C
D
*Isl1*
b
E
F
G
H
*Foxf1*
I
J
K
L
*Hand1*
c
M
N
O
P
*Sall3*
Q
R
S
T
*Bra*
U
V
W
X

## 3.3 Early Cardiovascular Progenitors

To determine relevance for the early LPM patterning process I have described, and the RA and FGF induced alterations on this pattern, I decided to examine how altering RA and FGF signalling would impact the early cardiovascular system. The cardiovascular system, one of the earliest organ systems to develop, is derived from the LPM. Furthermore, previous evidence demonstrating that both RA and FGF signalling were important to proper specification of one or more of the cardiovascular lineages (see sections 1.5.2 and 1.6.1) suggests that my manipulations of the early patterning event (see sections 3.1 and 3.2) could be correlated with altered specification of the early mesodermal precursor cells that will give rise to the cardiovascular lineages.

### 3.3.1 FGF Signalling is Necessary for Heart Field Specification and Maintenance

I have shown that during the neurula stage of embryogenesis FGF signalling is necessary for early heart field specification, in line with previous results (Keren-Politansky et al., 2009; Samuel and Latinkic, 2009), while RA signalling during this same satge is inhibitory as assayed by either *nkx2-5* or *isl1* expression (see section 3.2.3). Interestingly, while RA signalling is detrimental to the heart field into the early tail bud stages (stages 20-24) (Drysdale et al., 1997), it is also required for proper morphogenesis (Collop et al., 2006), suggesting tight control over this pathway is necessary throughout cardiac development. Therefore, since the role of RA in heart development is well documented in *Xenopus*, I wished to further characterize the requirement of FGF signalling on heart field specification, in particular to determine the temporal requirements of FGF signalling.

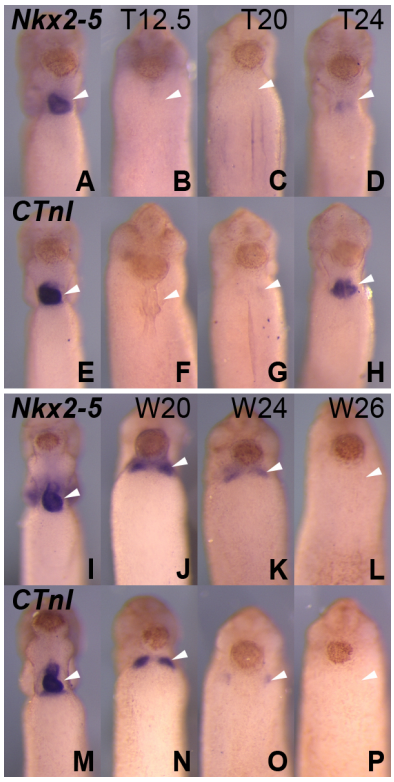
Embryos were treated with 10 μM SU5402 at successively later stages and assayed for effects on gross heart morphology and differentiation using the expression of *nkx2-5* and *cTnI* as markers. If FGF signalling is inhibited immediately after gastrulation (stage 12.5), early expression of *nkx2-5* is lost and later differentiation is blocked as assayed by *cTnI* expression (Figure 21B, F). Similar results were also obtained if FGF signalling was blocked after stage 20 (Figure 21C, G). If FGF signalling was inhibited after stage 24, expression of both *nkx2-5* and *cTnI* was detected, but the heart did not form a tube, nor loop (figure 21D, H).

Since it is possible to flush the inhibitor out of the embryo and thus restore normal FGF signalling (Marques et al., 2008) I could narrow down the temporal requirement for FGF signalling in cardiogenesis. To test if heart development is able to recover after FGF signalling is restored, I treated embryos at stage 12.5, and then restored FGF signalling by removing SU5402 and thoroughly flushing. When SU5402 was removed by stage 20, expression of both *nkx2-5* and *cTnI* recovered by stage 32 (Figure 21J, N). However, heart morphology was clearly abnormal. *Nkx2-5* and *cTnI* expression was present in two bilateral patches, with no indication of tube formation. If the inhibitor was removed at the early tail bud stage (Stage 22) both *nkx2-5* and *cTnI* domains were present in similar bilateral domains but the size of these domains was further reduced compared to controls (Figure 21K, O). However, if SU5402 was not removed until stage 26, neither the expression of *nkx2-5* nor *cTnI* was observed (Figure 21L, P).

### 3.3.2 RA Signalling Affects the Boundaries of the Vascular System

I have previously demonstrated that RA signalling is necessary for both properly patterning the LPM, as well as early heart specification (Figure 20). Therefore, I tested

**Figure 21.** FGF signalling is necessary to maintain heart specification and for morphogenesis. Embryos were treated with SU5402 at stages 12.5 (T12.5), 20 (T20), or 24 (T24) to determine when FGF signalling is necessary for heart development and assayed for expression of *nkx2-5* (A-D) or *cTnI* (E-H). FGF signalling was inhibited at successively later stages (top panel: A-H) and compared to control embryos (A and E). Embryos treated with SU5402 at either stage 12.5 (B and F) or stage 20 (C and G) demonstrate a complete loss of heart marker expression by stage 32, while embryos treated at stage 24 (D and H) show expression of both *nkx2-5* and *cTnI* but no discernable heart tube. FGF signalling was also inhibited at stage 12.5 and restored at later stages by removing the inhibitor at stages 20 (W20), 24 (W24) and 26 (W26) (bottom panel: I-P) and compared to control embryos (I and M) and assayed for *nkx2-5* (I-L) or *cTnI* (M-P). When FGF signalling is restored by either stage 20 (J and N) or stage 22 (K and O) both heart markers are expressed however a normal heart tube is not formed. If signalling is not restored until stage 26 (L and P) neither *nkx2-5* or *cTnI* are detectable. White arrows mark the heart region in each panel.


Nkx2-5
T12.5
T20
T24
A
B
C
D
CTnI
E
F
G
H
Nkx2-5
W20
W24
W26
I
J
K
L
CTnI
M
N
O
P

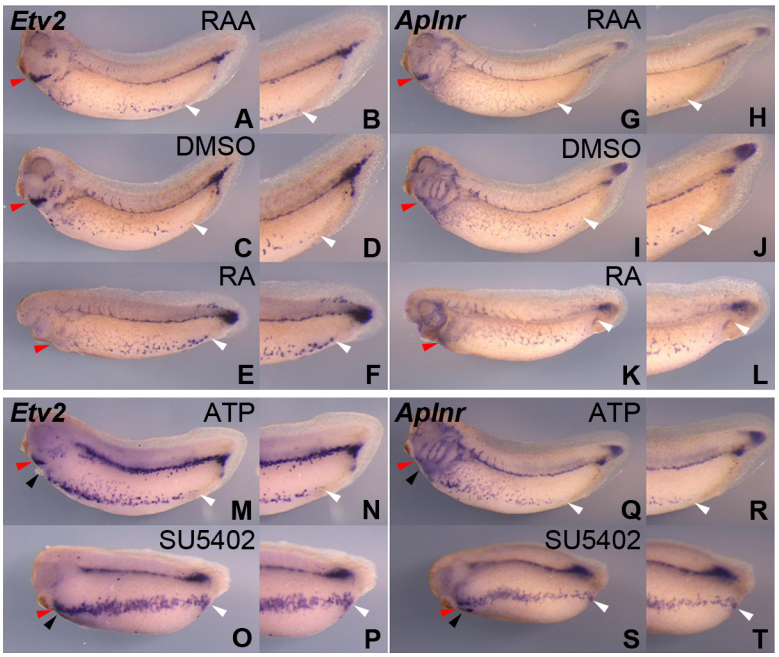
whether altering RA signalling also affects the specification of the vascular system. To visualize the developing vascular system I used two transcription factors essential for the endothelial lineage: *etv2* and *aplnr*. In control embryos there is a vascular free region in the posterior trunk of the embryo where neither of the *etv2* or *aplnr* domains extend to the posterior limits of the trunk. This vascular free zone is evident in expression patterns of various vascular markers demonstrated by others (Cox et al., 2006; Meadows et al., 2009; Salanga et al., 2010; Xu et al., 2009).

Embryos were treated with RA or RAA at stage 14 and fixed and assayed at stage 32-34. When RA signalling was blocked with an RA antagonist, there were no obvious changes in the expression domains of either *etv2* or *aplnr* when compared to control embryos (Figure 22 A-B, G-H). However, increasing RA signalling by treating the embryos with exogenous *all trans* RA caused a posterior expansion in the expression domains of both *etv2* and *aplnr* (Figure 22E-F, K-L), and an overall reduction in the vascular free zone. At the anterior end of the embryo, expression in the rostral lymph sac is also significantly decreased with increased RA signalling (Figure 22E, K). This suggests that overall vascular specification is not dependent on RA signalling, as a loss of RA does not significantly affect vascular specification, however RA may be involved in defining the boundaries of the vascular plexus.

### 3.3.3 FGF Signalling is Necessary to Restrict Early Vascular Specification

Since FGF signalling was required for patterning the LPM and for differentiation of the heart, I examined the role of FGF signalling in patterning the vascular system. Embryos were treated with 10 μM SU5402 at stage 12.5, and allowed to develop until the tail bud stage (stage 32) when they were fixed and assayed for expression of the vascular

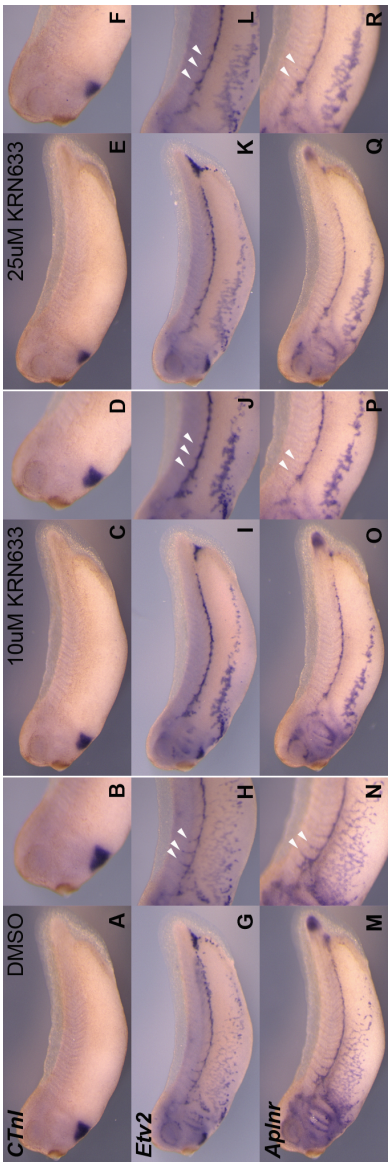
**Figure 22.** RA and FGF signalling are required for defining boundaries of the vascular plexus. Embryos treated at stage 14 with a synthetic RA antagonist (RAA), *all- trans* RA or a DMSO control (Top panel: A-L) and assayed for *etv2* (A-F) or *aplnr* (G-L). While no obvious changes are present after treating embryos with RAA (A-B and G-H), the posterior limit of the LPM vasculature (white arrowheads) is extended further posterior in RA treated embryos (E-F and K-L) when compared with control embryos (C-D and I-J). The staining seen in the rostral lymph sac (RLS; red arrows in C and I) is also absent in embryos treated with RA (red arrows in E and K). To inhibit FGF signalling, embryos were treated with DMSO or SU5402 at stage 12 (Bottom panel: M-T) and allowed to develop until stage 32 when vascular pattern was assessed by expression of *etv2* (M-P) and *alpnr* (Q-T). Both *etv2* (M-N) and *aplnr* (Q-R) are normally restricted from the posterior end of the LPM. However, when FGF signalling is lost the expression domains of both *etv2* (O-P) and *aplnr* (S-T) are extended to the posterior end of the trunk (white arrows). Also, the anterior gap (black arrows) in vasculature between the RLS (red arrows) and trunk vasculature corresponding to the location of the developing heart is absent in SU5402 treated embryos.


Etv2
RAA
A
B
DMSO
C
D
RA
E
F
Aplnr
RAA
G
H
DMSO
I
J
RA
K
L
Etv2
ATP
M
N
SU5402
O
P
Aplnr
ATP
Q
R
SU5402
S
T

markers *etv2* and *aplnr*. The vascular free zone, shown as an absence of *etv2* and *aplnr* expression in control embryos, was lost when FGF signalling was inhibited, and both *etv2* and *aplnr* were subsequently expressed along the entire anterior-posterior axis in the LPM (Figure 22 O-P, S-T). In addition, the gap in staining between the rostral lymph sac and the trunk vasculature, seen in the controls and normally occupied at least in part by the heart, is absent (Figure 22O, P). In the SU5402 treated embryos the trunk vasculature is continuous with staining in the rostral lymph sac.

Since SU5402 is known to cross-react with the Vegf receptor (Mohammadi et al., 1997), albeit with much less affinity, I tested whether the effects of SU5402 on the early cardiovascular system were due to a loss of Vegf signalling. Embryos were treated with a VEGFR specific inhibitor, KRN633 (Nakamura et al., 2004), after gastrulation and assayed for both cardiac and vascular marker expression. The overall phenotype of the embryos was compared between KRN633 treated embryos and controls, as well as the effects on both vascular and heart development. Doses of 10 μM, and 25 μM of KRN633 (Figure 23) were sufficient to block angiogenesis, the sprouting of vascular precursors to form endothelial tubes and networks. The inhibition of angiogenesis was demonstrated using *etv2* (Figure 23G-L) and *alpnr* (Figure 23M-R) expression to mark endothelial cells. Both the vascular plexus and intersomitic vessels fail to form in the presence of KRN633 (Figure 23; white arrows). However, there was no detectable effect on defining the vascular free zone, on heart differentiation as assayed by *cTnI* expression, or on tail bud outgrowth (Figure 23 A-F), suggesting that the effects of SU5402 on the heart, vascular free zone and tail bud are due to specific inhibition of the FGFRs, and not due to cross reactivity of SU5402 on the Vegf signalling pathway.

**Figure 23.** Inhibiting VEGF signalling with KRN633, a VEGFR specific inhibitor, does not recapitulate the SU5402 phenotype. Neither a concentration of 10 μM or 25 μM KRN633 added at stage 12.5 was able to block expression of *cTnI* (A-F), alter the posterior vascular free region or to create posterior truncations as seen in the Fgf inhibitor treatments. However, treating embryos with both10 μM or 25 μM KRN633 leads to a loss of angiogenesis when embryos are assayed for either *etv2* (G-L) or *alpnr* (M-R) as demonstrated by a loss of vascular budding in the ventral trunk, and a loss of intersemitic vessels (white arrow heads).


DMSO
10uM KRN633
25uM KRN633
CTnI
Etv2
Aplnr
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R

### 3.3.4 RA and FGF Signalling in Primitive Haematopoiesis

Since proper levels of FGF and RA signalling are necessary for both heart and vascular development, I wished to test if altering the levels of these signals would impact the expression of the ventral blood islands domains. Previous reports have suggested that FGF signalling is required for haematopoiesis in both zebrafish (Yamauchi et al., 2006) and *Xenopus* at earlier time points (Walmsley et al., 2008), however I wished to test if RA and FGF signalling during the neurula stage embryo is essential for proper blood development.

By the late tailbud stage, after specification of the endothelial lineage, *tal1* is expressed solely in the erythroid progenitors, while *globin*, a marker of erythroid differentiation, begins to be expressed in an anterior-posterior wave as erythroid cells differentiate. The expression domain of erythroid progenitor marker, *tal1*, remains unaltered in either RAA or RA treated embryos (Figure 24A-F) compared with the DMSO control treatment. However, an obvious decrease in the *globin* domain is seen after increasing RA signalling (Figure 24 I, L), in line with previous results (Bertwistle et al., 1996). Conversely, a loss of RA signalling has no effect on the boundaries of the *globin* domain (Figure 24 G, J) suggesting that RA is not normally required for defining the erythroid lineage.

To examine the effects of a loss of FGF signalling on the ventral blood islands, embryos were treated with SU5402 at stage 12.5 and allowed to develop until stage 32. In control embryos the erythroid progenitor marker *tal1* is expressed along the ventral midline in the LPM to the closed blastopore (Figure 25A). Inhibiting FGF signalling causes a restriction of the progenitor domain from the posterior half of the LPM, as the *tal1* domain does not approach the closed blastopore as closely in SU5402 treated

embryos (Figure 25D) as in control embryos. *Globin*, a marker of erythroid differentiation is significantly decreased in the posterior ventral LPM (Figure 25J) when compared to controls (Figure 25G). To further characterize the effect of a loss of FGF signalling on erythroid development I wished to test if the loss of *globin* was simply a delay, or a true reduction in the domain size when embryos were treated with SU5402. Embryos were treated with SU5402 at stae 12.5, and fixed at stages 34 and 36 and assayed from both *tal1* and *globin* expression. At all stages it appeared that the *tal1* domain did not extend as far posterior in the SU5402 treated embryos as in the control treated embryos (Figure 25 A-F). While the *globin* domain would eventually expand to encompass a similar domain as *tal1*, the *globin* domain also seemed to be displaced anteriorly, as it did not approach the closed blastopore nearly as closely in SU5402 treated embryos (Figure 25J-L; distance between white and dark arrows) as in control embryos (Figure 25 G-I). These results suggest that there is both a delay in erythroid differentiation, shown by a delay in the onset of *globin* expression, as well as a reduced erythroid domain, shown in the anterior displacement of both *tal1* and *globin*.

Finally, I also wished to examine the early myeloid lineage, which had been shown to be Fgf responsive during gastrulation (Walmsley et al., 2008). Embryos were treated with RA, RAA or a DMSO control, as well as SU5402 and an ATP and DMSO control at stage 12.5 and assayed for the expression of *mpo* and *spib* at stage 20. Both *mpo*, and *spib* are markers of the anterior blood island that form the myeloid lineage. However, neither of the anterior blood island marker domains were different in any of the altered RA or FGF conditions (Figure 26).

**Figure 24.** Abnormal levels of RA or FGF signalling lead to impaired erythroid differentiation. Altering levels of RA signalling by addition of either RA (right column), or RAA (left column) at stage 12.5 does not lead to any consistent changes in *tal1* expression (A-F) when compared to DMSO treated embryos (center column), while increasing RA signalling reduces expression of *globin* (G-L) particularly in the posterior-most limits of its normal expression domain (I, L; arrowhead a).

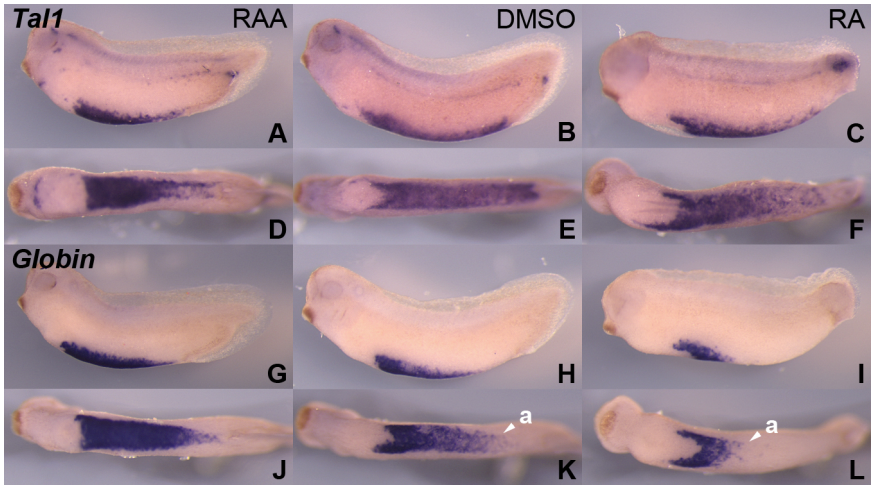

Tal1
RAA
DMSO
RA
A
B
C
D
E
F
Globin
G
H
I
J
K
L
a
a

**Figure 25.** Decreased erythroid differentiation is not due to a delay in development when FGF signalling is lost. FGF signalling was inhibited after gastrulation at stage 12.5 and embryos were fixed and assayed at stage 32 (left column), stage 34 (center column) or stage 36 (right column). When Fgf signalling is lost, the posterior end (white arrows) of the *tal1* expression domain was restricted further anterior (D-F) as it did not approach the closed blastopore (black arrows) as closely as in control embryos (A-C). When FGF signalling is inhibited *globin* expression was only weakly detectable by *in situ* hybridization at stage 32 (J) but does recover to become more easily detectable at later stages. However, the length of the *globin* domain remains reduced as the posterior edge (white arrowhead) does not approach the proctodaeum (black arrowheads) as closely in the SU5402 treated embryos (K-L) as in the control treated embryos (H-I).

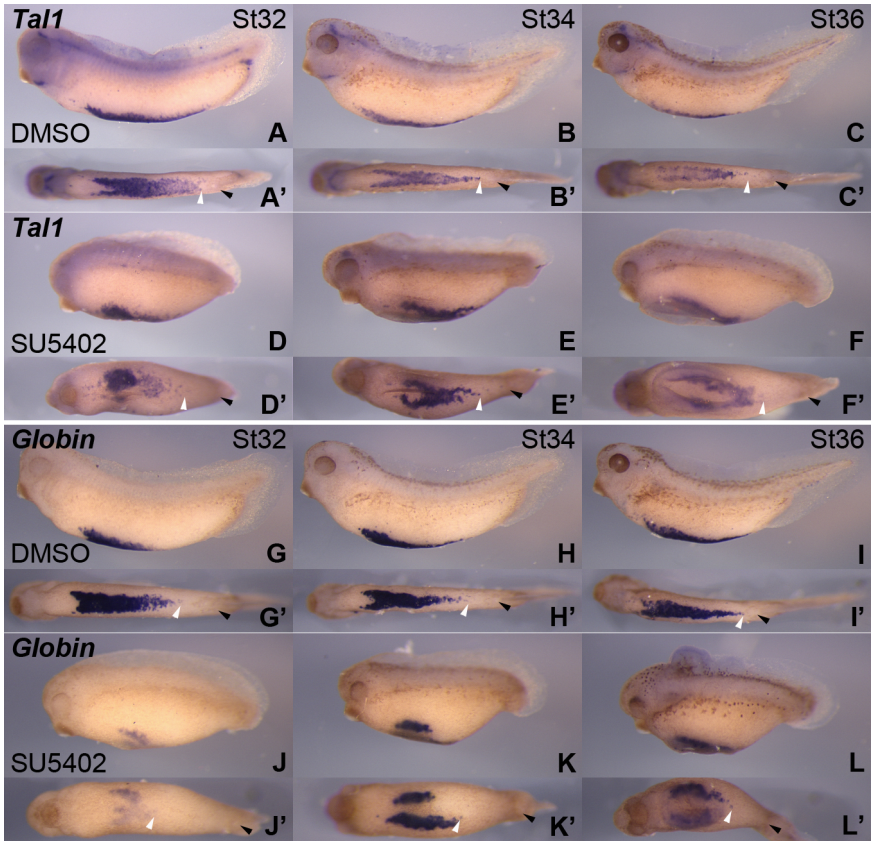
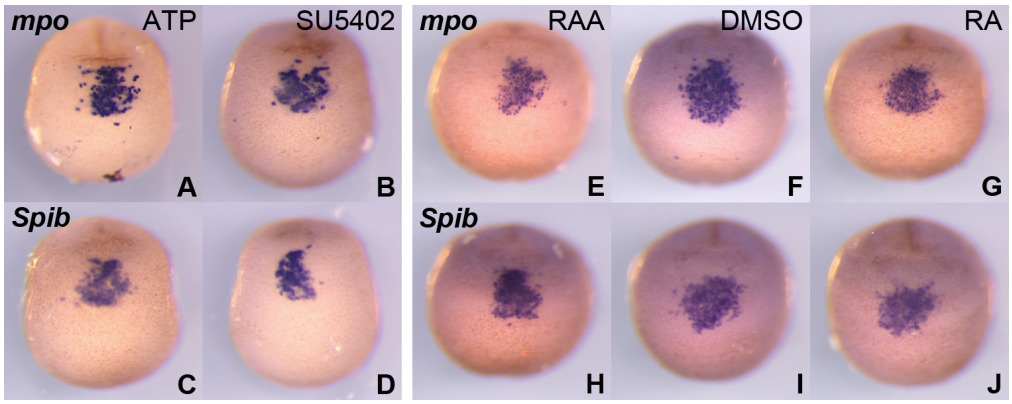

Tal1
St32
DMSO
A
St34
B
St36
C
A'
B'
C'
Tal1
SU5402
D
E
F
D'
E'
F'
Globin
St32
DMSO
G
St34
H
St36
I
G'
H'
I'
Globin
SU5402
J
K
L
J'
K'
L'

**Figure 26**. Neither RA nor FGF signalling is required after gastrulation for early myeloid marker expression. Anterior ventral blood markers *mpo* (A-B, E-G), and *spib* (C-D, H-J) expression domains (myeloid cell markers) remain unaffected when FGF signalling is inhibited (A-D), or when RA signalling is altered (E-J) at stage 12.5 and expression of markers is assayed at stage 20.

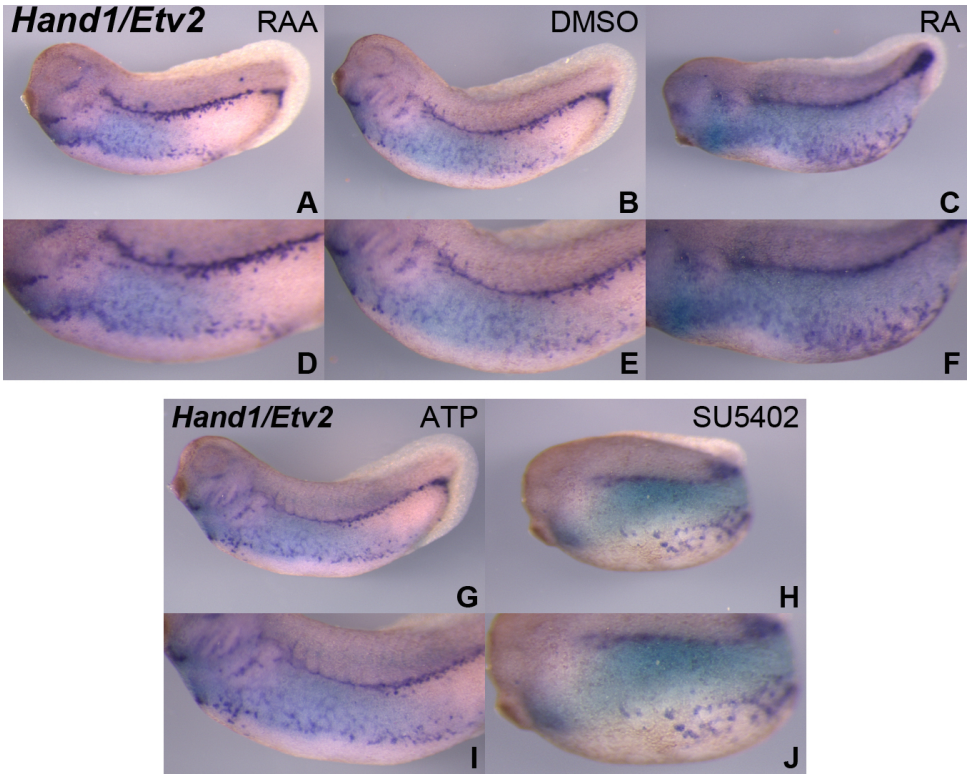

mpo
ATP
SU5402
A
B
Spib
C
D
mpo
RAA
DMSO
RA
E
F
G
Spib
H
I
J

### 3.4 Examining the Role of *Hand1* in Early Cardiovascular Progenitors

The previous experiments with markers of the developing vasculature demonstrated a region in the posterior of the LPM in which no vasculature marker was expressed (see 3.3.2 and 3.3.3). In comparing this vascular free region with the late anterior-middle markers of the LPM, *hand1* and *foxf1*, whose expression is also restricted in the posterior LPM, it was noted that the vascular free region was roughly similar to the posterior limits of the *hand1* and *foxf1* domains. To better test if this vascular free region and the *hand1* negative domain in the posterior LPM were directly correlated I performed a double *in situ* hybridization in embryos treated with RA, RAA, the FGFR inhibitor SU5402, or control embryos (Figure 27).

In control embryos the *hand1* and *etv2* were closely correlated, particularly in the posterior limits of the two domains (Figure 27 B, E). When retinoic acid signalling was inhibited by treatment with the retinoic acid antagonist there was little change in either domain (Figure 27 A, D). However, when retinoic acid signalling was increased by addition of the agonist, both the *hand1* and *etv2* domains were extended much further toward the posterior pole of the embryo (Figure 27 C, F), with the posterior limits again corresponding very closely. If FGF signalling was inhibited both domains were extended to the posterior limits of the LPM, similar to the RA treatment (Figure 27 H, J). While this is similar to individually stained embryos examined earlier, the high degree of overlap of the two domains, along with results previously discussed (see section 1.7.1) strongly suggests a possible role for *hand1* in early cardiovascular specification.

**Figure 27.** The LPM marker *hand1* and vascular marker *etv2* expression domains overlap and respond similarly to either altered RA signalling, or a loss of Fgf signalling. Antisense probes for *hand1* (Flourescein, light blue) and *etv2* (DIG, purple) were used to visualize the overlap in domains. Embryos were treated with RAA (A, D), RA (C, F), and compared to DMSO controls (B, E) or SU5402 (H, J) and compared to a DMSO and ATP control (G, I). The two domains were highly correlated in each treatment, as each domain expanded similarly in both RA and SU5402 treatments when compared to control embryos.


Hand1/Etv2
RAA
DMSO
RA
A
B
C
D
E
F
Hand1/Etv2
ATP
SU5402
G
H
I
J

### 3.4.1 *Hand1* is Necessary for Cardiac Morphogenesis, but not Early Specification

To determine a role for *hand1* in heart development, I designed an antisense morpholino directed to the translational start site of the *hand1* transcript to disrupt translation. Blocking Hand1 translation should lead to a loss of function phenotype. The antisense morpholinos were injected into one-cell embryos to attain a partial loss of *hand1* activity throughout the entire embryo, at a volume of either 4.6 nL or 9.2 nL. To determine if *hand1* was necessary for early heart field specification embryos were fixed at stage 20 and assayed for expression of *nkx2-5* and *Isl1*. Upon inhibiting *hand1* function, there was no discernable effect of the morpholino at either volume in the expression of *nkx2-5* or on *Isl1* (Figure 28), suggesting that *hand1* is not required for expression of early heart field markers.

Reports of *hand1* loss of function in both mouse (Smart et al., 2002) and zebrafish (Garavito-Aguilar et al., 2010) have suggested that *hand1* may be involved in later heart morphogenesis. Therefore, I wished to test if *hand1* had any obvious effects on later heart development in *Xenopus*. Embryos were injected at the one cell stage with 4.6 nL of morpholino and fixed and assayed for the expression of *nkx2-5* and *cTnI* during late tailbud stages. Interestingly, embryos injected with 9.2 nL of *hand1* morpholino displayed a greatly increased rate of death during early tail bud stages when compared to control morpholino injected embryos, and thus this concentration of morpholino was not used further for analyzing later development. At stage 32, *hand1* morpholino injected embryos express both *nkx2-5* and *cTnI*, supporting the conclusion that *hand1* is not necessary for heart specification or differentiation (Figure 29 E-O). However, morphogenesis of the heart tube, in particular formation of the tube itself is inhibited

**Figure 28.** *Hand1* function is dispensable for early heart specification. Embryos were injected at the one cell stage with 4.6 nL (C and G) or 9.2 nL (D and H) of the *hand1* morpholino, or 9.2 nL of a control morpholino (B and F) and compared to uninjected embryos (A and E). Neither the expression domain of *nkx2-5* or *Isl1* was altered in any condition. In all cases, the anterior-ventral end of the embryo is viewed, with dorsal toward the top of the image. Un: uninjected, Cont: Control morpholino injected, *Hand1-MO*: *Hand1* morpholino injected.

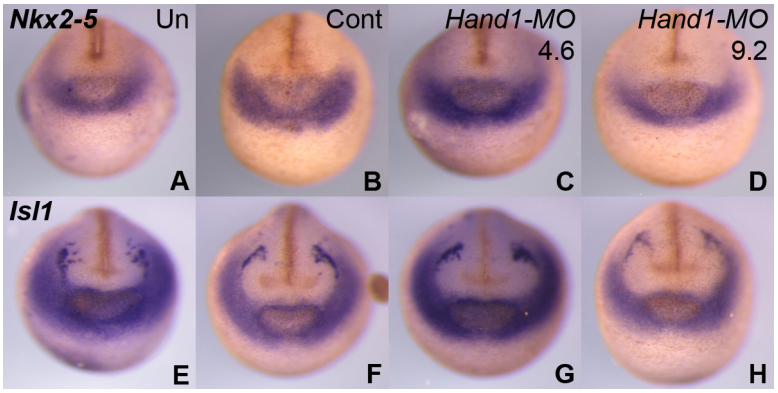
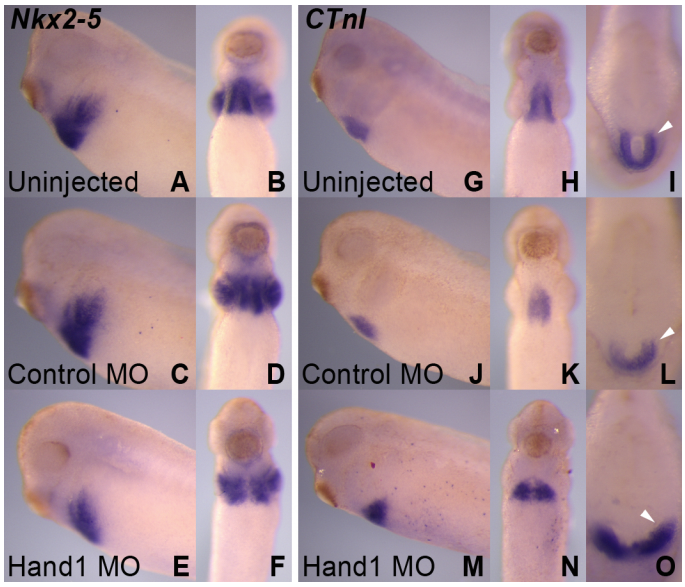

Nkx2-5
Un
Cont
Hand1-MO
4.6
Hand1-MO
9.2
A
B
C
D
Isl1
E
F
G
H

**Figure 29.** Loss of Hand1 leads defects in myocardial trough formation at stage 30. Embryos were injected with 4.6 nL of *Hand1* morpholino (Bottom panel) and compared to either control injected morpholino (middle panel) embryos or unjectected controls (top panel). Embryos were assayed for expression of *nkx2-5* (A-F) and *cTnI* (G-O) to visualize the heart. Both of the uninjected embryos and control injected morpholinos had formed a myocardial trough (arrows), whereas none of the *Hand1* morpholino injected embryos had formed a trough.


Nkx2-5
CTnI
Uninjected
A
B
Uninjected
G
H
I
Control MO
C
D
Control MO
J
K
L
Hand1 MO
E
F
Hand1 MO
M
N
O

(Figure 29F, O), reminiscent of embryos in which RA signalling is lost (Collop et al., 2006), or Notch signalling has been inhibited (Rones et al., 2000).

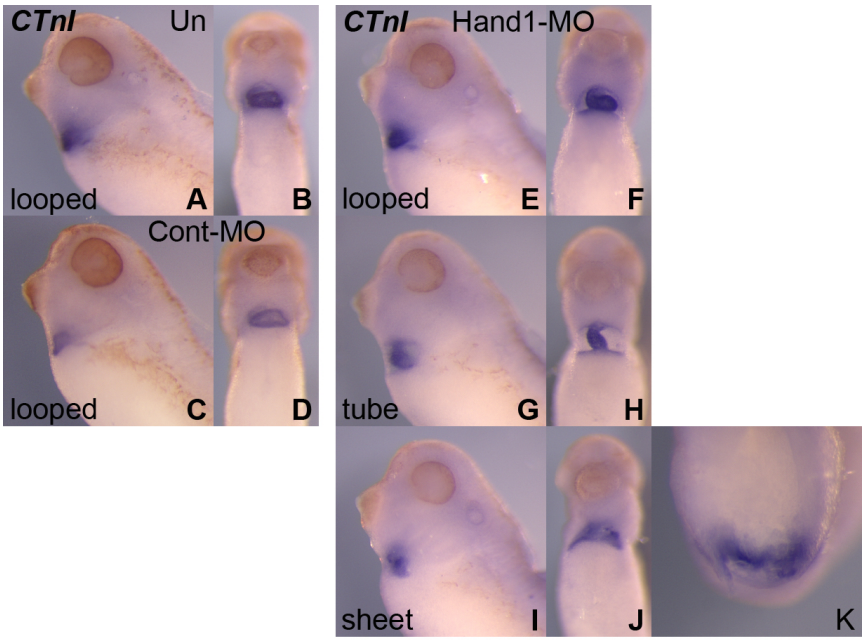
To test if *hand1* morphant hearts were simply delayed in heart tube formation, or if they were unable to form a heart tube, I fixed and assayed embryos at stage 36, long after the heart tube should have closed and looped (Figure 30). At this time, I assayed for *cTnI* to visualize the heart and scored the number of embryos that had successfully formed a heart tube. Of the morphant embryos assayed (n=14) 57% of embryos had apparently normal hearts, while 36% of morphant hearts remained as an open sheet of cells (Figure 30E-L). In control morpholino injected embryos (n=10), 100% of embryos had hearts that had formed a tube and looped (Figure 30A-D). These results suggest that there is a significant delay, and in some cases a complete block of heart tube formation.

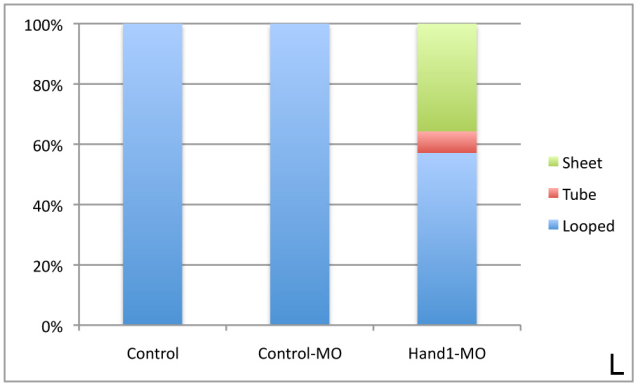
### 3.4.2 *Hand1* is Necessary for Vascular Specification

Due to the high degree of overlap in the *hand1* and *etv2* domain (see section 3.4.0), I hypothesized that a functional relationship may exist between *hand1* and vascular specification. Embryos were injected with the *hand1* morpholino at either the one cell stage, yielding an embryo wide distribution of the morpholino, or one cell of a two cell embryo, yielding embryos where *hand1* was blocked only on one side (left or right) of the embryo. If *hand1* is required for the endothelial lineage, *hand1* function would provide a link between the early LPM patterning I have described and the later effects of RA and FGF on vascular specification.

When Hand1 function was blocked in the whole embryo by injection of the anti-sense morpholino into a one-cell embryo the formation of the trunk vascular plexus is

**Figure 30.** Loss of Hand1 leads to a delay in heart tube formation. Embryos were injected with 4.6 nL of *Hand1* morpholino (E-K) and assayed with *cTnI* to visualize the heart field at stage 36. Heart morphology was then divided into three groups: the heart had looped (E-F), the heart had formed a tube (G-H), or the myocardium remained a sheet of cells (I-K). Both uninjected control embryos (A-B) and control morpholino injected embryos (C-D) had formed a tube with 100% occurance, where as the *Hand1* morpholino injected embryos had a severly reduced frequency of heart tube formation (L).


CTnI
Un
looped
A
B
Cont-MO
looped
C
D
CTnI
Hand1-MO
looped
E
F
tube
G
H
sheet
I
J
K


100%
80%
60%
40%
20%
0%
Control
Control-MO
Hand1-MO
Sheet
Tube
Looped
L

clearly altered (Figure 31 C-D, G-H). Both endothelial markers *etv2* and *aplnr* are expressed in the LPM, however the density of the vascular plexus is reduced (Figure 31 D, H). The reduced density of the vascular plexus could be due to either a reduced number of endothelial cells differentiating (reduced vasculogenesis), or reduced migration into the vascular networks (reduced angiogenesis).

To further characterize the role of *hand1* in endothelial development I performed unilateral injections, injecting the *hand1* morpholino into one blastomere of a two-cell embryo (Figure 32). This effectively created embryos that were wild type on one half of the embryo, and were *hand1* morphants on the other side. In embryos assayed for either *etv2* or *alpnr* the vascular plexus was extremely perturbed, being almost undectectable at the anterior-end of the LPM (Figure 32D, H). In addition, in both cases the anterior half of the posterior cardinal vein was also absent. This suggests that *hand1* is required for proper development of the endothelium, and a loss of *hand1* greatly perturbs formation of the vascular plexus and posterior cardinal vein.

### 3.4.3 *Hand1* is Dispensable for Embryonic Blood Formation

Since *hand1* is necessary for both heart and vasculature development, I also wished to test if it was involved in blood differentiation. While *hand1* is initially expressed in the ventral LPM at the neurula stage, it is excluded from both the myeloid cells (anterior blood islands) in the neurula, and future erythrocytes (ventral/posterior blood islands) by the mid tailbud stages (stage 26), long before *globin* expression. The exclusion of *hand1* from the early blood-forming region suggests that *hand1* may not normally be involved in haematopoiesis. I therefore hypothesized that a loss of Hand1 would not affect primitive blood development.

**Figure 31.** Vascular plexus density is decreased when Hand1 is lost in the whole embryo. One-cell stage embryos were injected with 4.6 nL of *Hand1*, and compared to a control morpholino. Embryos were assayed with *etv2* (A-D) or *alpnr* (E-H) to visualize the vasculature. In each case the *hand1* morpholino injected embryos demonstrated a reduced density of the vascular plexus. Left panel: whole embryo view with anterior toward the right, dorsal at top. Right panel: magnified view of LPM showing the vascular plexus.

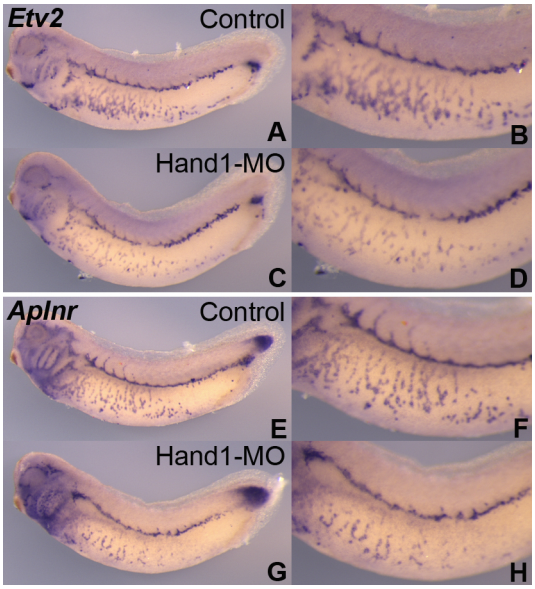
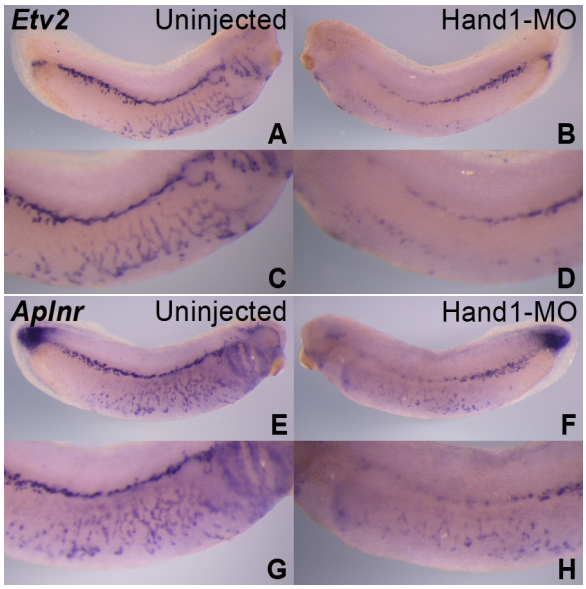

Etv2
Control
A
B
Hand1-MO
C
D
Aplnr
Control
E
F
Hand1-MO
G
H

**Figure 32.** Loss of Hand1 leads to severely reduced vascularization in contralateral injected embryos. Two-cell embryos were injected with 4.6 ηL of *hand1* morpholino into the left blastomere (right panel), and compared to the contralateral uninjected side (left panel). Embryos were assayed with either *etv2* of *alpnr* to visualize the vasculature. In each case, the morphant side displayed dramatically reduced staining when compared to the uninjected side. Top panels (A-B, E-F): whole embryo views. Bottom panels (C-D, G-H): magnified view of LPM showing vascular plexus.


Etv2
Uninjected
Hand1-MO
A
B
C
D
Aplnr
Uninjected
Hand1-MO
E
F
G
H

Embryos were injected with either 4.6 nL or 9.2 nL of *hand1* morpholino and assayed for the expression of *mpo* and *spib*, the anterior blood island markers and *etv2*, a marker of the hemangioblast lineage. There was no consistent detectible difference found in expression domains of either *mpo* or *spib* (Figure 33 A-H), suggesting that *hand1* is not involved in the anterior blood island lineage. Furthermore, there was no difference in the early expression of *etv2* (Figure 33I-L), suggesting that the early hemangioblast lineage is not dependent of Hand1 function.

To determine if *hand1* has a role in the posterior blood island (erythroid lineage) I injected whole embryos with the *hand1* morpholino and assayed for *tal1*, a marker of erythrocyte precursors, and *globin*, a marker of differentiated erythrocytes. *Hand1* morphants were indistinguishable from either uninjected or control morpholino injected embryos when assay for either *tal1* or *globin* at all stages assayed (Figure 34), suggesting that Hand1 is also dispensable for erythroid lineage development.

**Figure 33.** Hand1 is dispensable for the myeloid and early hemangioblast lineages. Embryos were injected with either 4.6 ηL (C, G, K) or 9.2 ηL (D, H, L) of *hand1* morpholino, or a control morpholino (B, F, J) and compared with uninjected control embryos (A, E, I). Embryos were assayed for *mpo* (top panel) and *spib* (middle panel), markers of the myeloid lineage, or *etv2*, a marker of the hemangioblast lineage (bottom panel). No difference was discernable in any of the conditions examined.

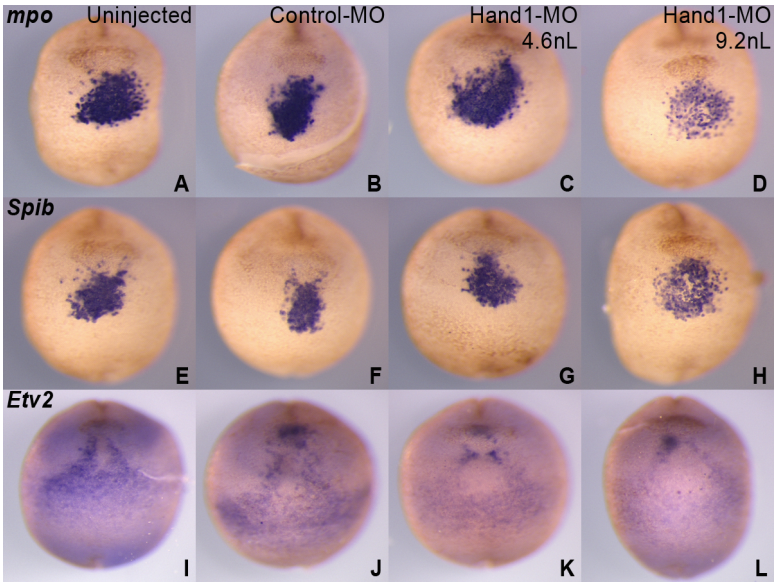
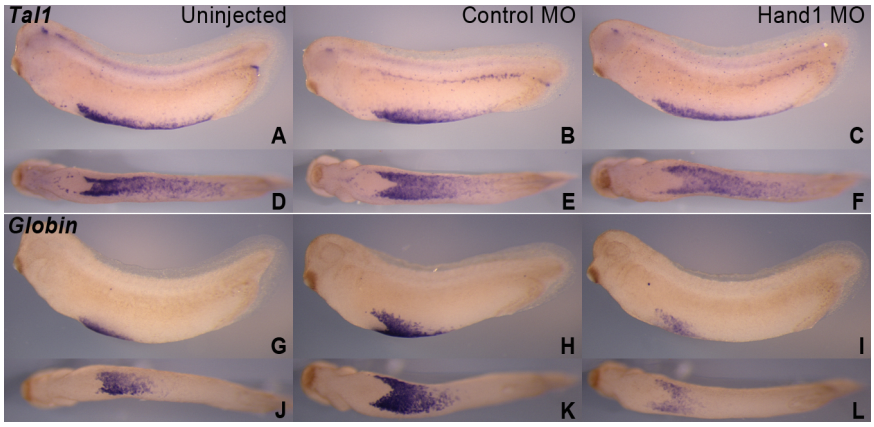

mpo
Uninjected
Control-MO
Hand1-MO 4.6nL
Hand1-MO 9.2nL
A
B
C
D
Spib
E
F
G
H
Etv2
I
J
K
L

**Figure 34.** Hand1 is dispensable for erythrocyte differentiation. Embryos were injected with 4.6 nL of *hand1* morpholino, or a control morpholino and compared to uninjected control embryos. Embryos were assayed for expression of *tal1* (top: A-F), a marker of erythrocyte progenitors, or *globin* (bottom: G-L), a marker of differentiated erythrocytes. No consistent differences were detectable in any of the conditions. Top panels (A-C, G-I): whole embryo views with anterior toward the left, dorsal at top. Bottom panels (D-F, J-L): ventral views showing the ventral blood islands.


Tal1
Uninjected
Control MO
Hand1 MO
A
B
C
D
E
F
Globin
G
H
I
J
K
L

# Chapter 4 Discussion

Patterning within the early LPM has not been well described. While the early cardiovascular progenitors are present along the ventral side of the LPM, patterning throughout the early LPM, particularly toward the dorsal LPM has been underappreciated. Here, I demonstrate that LPM is clearly subdivided into specific domains based on the expression of specific transcription factors at the neurula stage of development. I describe four distinct domains: an anterior-ventral *nkx2-5* expression domain, an anterior-dorsal *foxf1* domain, a middle *hand1* domain and posterior *sall3* domain. Although these domains are not mutually exclusive of each other, this pattern suggests that significant anterior-posterior patterning exists within the LPM much earlier than previously appreciated. This patterning event is likely not limited to *Xenopus* development, but rather may be an important process in all vertebrates as homologues of all of these genes are present in similar domains in other vertebrates (Lints et al., 1993; Mahlapuu et al., 2001b; Srivastava et al., 1995; Sweetman et al., 2005).

## 4.1 Retinoic Acid and Patterning of the Early LPM

I have demonstrated that RA is necessary for defining the anterior-dorsal end of the LPM during early LPM patterning. When RA signalling is lost, the domains residing along the anterior and dorsal borders of the LPM are most affected. The *foxf1* domain is almost completely lost in LPM, while the dorsal part of the *hand1* domain is severely restricted. However, the anterior-ventral, and posterior domains are independent of RA signalling and are unaffected when RA signalling is lost. The ability of RA signalling, to pattern the anterior and dorsal LPM correlates with the expression pattern of *raldh2*, and a loss of *raldh2* function mirrors the effect of treatment with an RA antagonist. These

results suggest that RA, synthesized by Raldh2 in the anterior and dorsal mesoderm (LPM and somites), is required to establish proper polarity within the early LPM by defining the anterior and dorsal ends of the LPM. However, while RA is important for the middle *hand1* domain, it is clearly not the only signal establishing that domain as *hand1* is still expressed in the middle LPM, albeit highly restricted, in the absence of RA signalling.

The evidence I have presented here supports the hypothesis that retinoic acid is not normally a posteriorizing factor at this stage (Maden, 1999). Instead, high concentrations of RA ligand are present in the anterior trunk of the embryo corresponding to expression of *raldh2* (Chen et al., 2001) and that RA diffuses toward the posterior of the embryo. However, the expression pattern of *raldh2* suggests a much more complex model than a direct linear gradient from anterior to posterior. Rather, any gradient of RA present in the LPM would be highest at the anterior-dorsal end of the LPM, which is bordered on two sides by *raldh2*, and decrease toward the posterior-ventral end.

The RA metabolizing enzyme *cyp26* is strongly expressed at the posterior-ventral end of the embryo around the closed blastopore, and is not normally detectable via *in situ* hybridization within the LPM. This could account for the inability of ketoconazole to significantly alter the expression domains of *foxf1* and *hand1*. The extent of the RA signal at any point within the LPM may simply be a result of diffusion away from the source, in the anterior-ventral corner of the LPM. Since ketoconazole inhibits cyp26, and *cyp26* is not normally expressed within the LPM, intuitively RA degradation by cyp26 would not be altered and therefore the levels of RA within the LPM would be unaltered.

In contrast to anterior LPM patterning, addition of RA causes a loss of the posterior *Sall3* domain. However, when RA signalling is decreased, the posterior domain remains unchanged suggesting that RA is not normally required for patterning the

posterior LPM. Since the posterior LPM is responsive to exogenous treatments of RA, one or more of the RARs should be expressed in this region, which is indeed the case as both *RARα* and *RARγ* are expressed in the posterior LPM (Escriva et al., 2006; Koide et al., 2001). It is possible that repression of RA responsive genes through unliganded RARs may be necessary for proper posterior positional identity. The role of the unliganded RARs as a transcriptional repressor has been previously proposed in mid-brain patterning (Koide et al., 2001). The possibility that RARs act as a repressor in the posterior LPM suggests an attractive model of RA signalling, where a high concentration of RA is present in the anterior-dorsal LPM, specifying the expression domain of *foxf1*, while more moderate levels are present in the middle of the embryo which act to define the border of the *hand1* domain. The posterior LPM, in which the RA ligand is normally absent (See Figure 36), requires the active repression of RA responsive genes by the unliganded RARs.

### 4.1.1 FGF Signalling and Early LPM Patterning

The loss of FGF signalling leads to a loss of proper identity of both the anterior-ventral and posterior LPM. When FGF signalling is inhibited, the anterior-ventral *nkx2-5* domain is lost, while the anterior-dorsal *foxf1* domain is expanded toward the ventral pole of embryo. At the posterior end of the embryo, *bra* is absent around the blastopore, while both *sall3* and *hand1* are displaced toward the posterior pole. Interestingly, both of the *foxf1* and *hand1* domains, that are expanding to 'fill in' the lost *nkx2-5* and *bra* domains respectively, are positively regulated by retinoic acid. This suggests that the Fgf pathway is acting in opposition to the RA signalling pathway along the anterior-posterior axis. I

propose that the role of the Fgf pathway is to restrict anterior pattern within the posterior LPM, and to keep these cells in an unspecified state.

Fgf functioning to restrict more anterior pattern seems likely since *bra*, the marker of the tail bud, which is lost when Fgf signalling is inhibited, is a marker of unspecified mesoderm and necessary for tail bud elongation (Conlon et al., 1996; Smith et al., 1991). Furthermore, this tail forming region, induced during this same stage in development (stage 13) (Beck and Slack, 1998; Gont et al., 1993), will normally give rise to axial mesoderm, such as the somites of the tail, but not LPM derived tissues (Gont et al., 1993). However, when FGF signalling is lost, genes normally expressed in the LPM, such as *foxf1* and *sall3*, are ectopically expressed the tail bud domain. The ectopically expressed anterior genes suggest that these cells are, at least in part, becoming specified into the LPM lineage instead of the tail bud lineage and are no longer able to undergo the characteristic convergent extension movements necessary for tail bud elongation.

In addition to a requirement for Fgf signalling in the maintenance of the tail bud domain, Fgf signalling is also necessary at the anterior-ventral end of the LPM for maintenance of the heart field. When Fgf signalling is inhibited, *nkx2-5*, a marker of the heart field is lost, while *foxf1*, the anterior-dorsal marker is expanded toward the ventral pole of the embryo. Similar to the posterior end of the embryo, when Fgf signalling is lost, an adjacent-RA regulated domain expands into the Fgf dependent domain. Interestingly, the anterior-ventral heart region is induced at roughly the same stage (Stage 13) that the tailbud domain is being specified at the posterior end of the embryo (Cleaver et al., 1996; Keren-Politansky et al., 2009; Samuel and Latinkic, 2009). Therefore, Fgf appears to be necessary in the anterior-ventral LPM to restrict the adjacent anterior-dorsal RA dependent domain, in this case allowing for the heart field to be specified. These dual

roles for Fgf signalling, in both the anterior-ventral LPM, and the posterior tail bud domain, correlates with the expression of *fgf8*. Although, it should be noted that other sources of FGF signalling cannot be excluded, as a number of Fgf ligands are expressed in proximity to the LPM at the neurula stages, such as *fgf3*, which may also be important and contribute to patterning the LPM (Keren-Politansky et al., 2009; Lea et al., 2009). Treating embryos with SU5402 would potentially inhibit all of these sources of FGF signalling. However, the anterior and posterior inputs of FGF signalling corresponding to the *fgf8* domain contrast the RA dependent domain nicely, with RA necessary for anterior-dorsal LPM, while Fgf signalling is necessary for anterior-ventral LPM and the posterior tail bud domain.

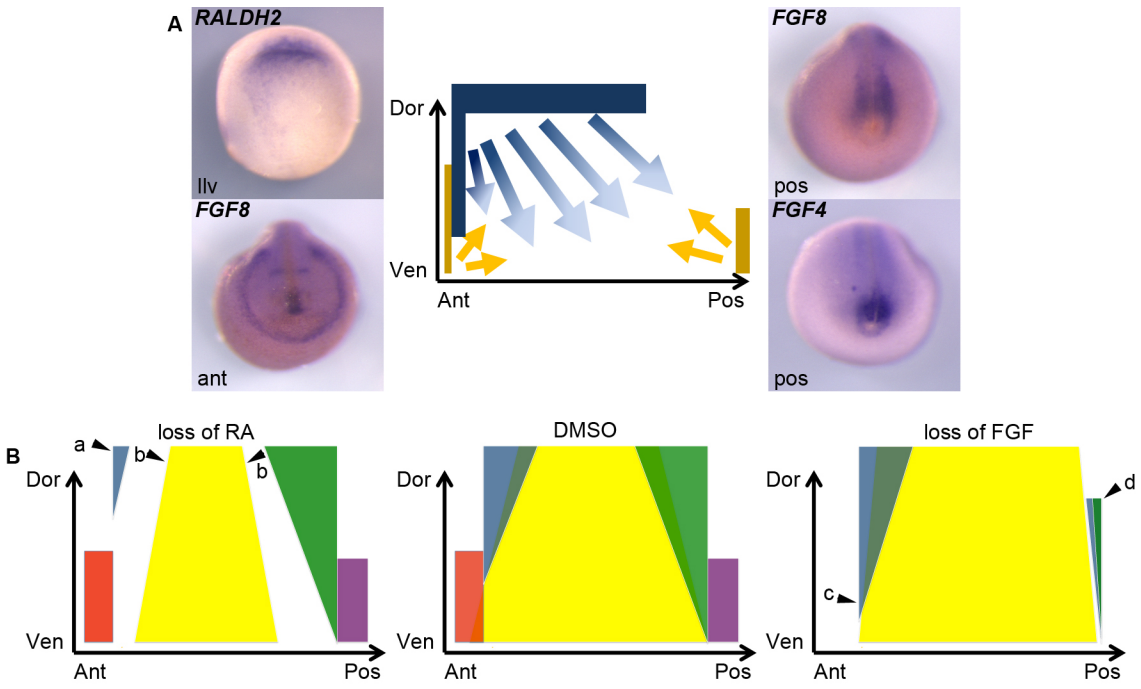
It is also possible that altered cell migration or cell survival plays a role in the altered posterior LPM patterning I observed when FGF signalling was inhibited. Previous results have demonstrated that FGF signalling is required for proper cell migration in a number of contexts (Benazeraf et al., 2010; Ciruna and Rossant, 2001; Conlon and Smith, 1999; Gisselbrecht et al., 1996; Poole et al., 2001). One area in particular which altered cell migration may play a role is in the posterior vascular free zone. It is possible that FGF signalling normally inhibits endothelial cells from migrating into the posterior LPM after they have undergone vasculogenesis. To answer this question, I propose to use the previously published *vegfr2* transgenic reporter line (Doherty et al., 2007) to visualize endothelial cells as they are specified and track the cells in real time as the undergo angiogenesis. Through the use of this reporter line I could determine if FGF signalling is necessary to restrict vascular specification in the posterior LPM, restrict migration, or both.

Lastly, If the proposed RA and FGF signalling inputs into the LPM are visualized on a diagram (Figure 35), it can be easily seen how the combinatorial input of these two pathways could yield positional identity in a two dimensional sheet of cells such as the early LPM. However, it remains likely that at least one more, as yet unidentified signalling input is required for full patterning of the LPM. In particular, this third pathway would be necessary for proper expression of the middle ventral LPM markers. This is because none of the performed treatments was able to inhibit middle and ventral domains such as *hand1* or the myeloid markers *spib*, and *mpo*. A likely candidate is the *Wnt* pathway, since at least one family member, *wnt4*, is expressed in the ventral LPM during neurula stages and has been implicated in primitive hematopoiesis (Tran et al., 2010).

## 4.2 Significance of the LPM Pattern

My results expand the role of RA and FGF signalling to include patterning of the LPM during the neurula *Xenopus* embryo. RA and Fgf signalling pathways are known to pattern the somites during this same window of time (Moreno and Kintner, 2004) and I propose that these processes are connected. Since the source of both the RA and Fgf signals responsible for patterning the somites (Moreno and Kintner, 2004), and the LPM are similar, it seems likely that the patterning of these tissues is coordinated. I also propose that this LPM patterning process is an initial step in regional specification of the LPM, leading to the subdivision of mesodermal progenitors, such as the cardiovascular lineages.

**Figure 35.** Model of early LPM patterning in the neurula stage *Xenopus* embryo. (A) A high level of RA signalling input is suggested by the expression domain of *raldh2* in the anterior and dorsal LPM. Conversely, Fgf signalling is proposed in the anterior-ventral and posterior-ventral LPM, suggested by the expression domains of *fgf8* in the anterior, and *fgf4* and *fgf8* in the posterior pole of the embryo. Dark blue and yellow bars represent the expression of *raldh2* and FGF ligands respectively, while blue and yellow arrows depict the proposed area which RA (blue) and FGF (yellow) are required for normal patterning. Model depicts a left lateral view of the LPM. Ant: anterior, Dor: dorsal, Lat: lateral view, Pos: posterior, Ven: ventral. (B) Diagrammatical representation of LPM expression domain response to decrease in either RA (left) or FGF (right) signalling when compared with the DMSO control (center). The anterior-dorsal and middle LPM domains require RA signalling for their full expression domain, whereas the anterior-ventral and posterior LPM domains are dependant on FGF signalling. Red: *nkx2-5*, blue: *foxf1*, yellow: *hand1*, green: *sall3*, purple: *bra*.


A
RALDH2
llv
FGF8
ant
Dor
Ven
Ant
Pos
FGF8
pos
FGF4
pos
B
loss of RA
a
b
b
Dor
Ven
Ant
Pos
DMSO
Dor
Ven
Ant
Pos
loss of FGF
c
d
Dor
Ven
Ant
Pos

Many studies have recently demonstrated developmental decisions between two adjacent LPM derived structures, based on the extracellular signalling molecules encountered. These reciprocal patterning processes have been described for both the myocardial and endocardial lineages (Ferdous et al., 2009; Misfeldt et al., 2009), epicardial and myocardial lineages (van Wijk et al., 2009) within the heart field, the heart field and limb bud (Waxman et al., 2008), the heart and vasculature (Schoenebeck et al., 2007), and the kidney and vasculature (Mudumana et al., 2008). In each of these examples, cells in close proximity are co-ordinately patterned leading to distinct cell lineages as a result of encountering different extracellular signals. The observations that I have provided here suggest that many of the early mesodermal lineage decisions that have thus far been examined as separate events, may be a part of a much larger, mesoderm wide patterning processes.

### 4.2.1 Relevance of LPM Pattern to Cardiovascular Development

The requirement for both FGF and RA signalling on heart development has been thoroughly studied in a number of model systems. However, this is the first report of RA and FGF signalling being required for endothelial differentiation in whole *Xenopus* embryos after gastrulation. RA has been implicated in murine endothelial development: *Raldh2*$^{-/-}$ mice fail to form organized extraembryonic vascular networks (Niederreither et al., 1999), while mouse ES cells treated with RA are inhibited from differentiating into endothelial cells (Festag et al., 2007). Here, I demonstrate that after gastrulation in *Xenopus*, RA signalling will expand the domain of vascular development. The contradiction between the early mouse ES cell work and the results reported here may simply be due to the timing and state of specification of the progenitor cells, ES cells are

isolated from the blastocyst embryo and represent a much earlier stage in development, while the cells of interest to my work are post gastrula mesodermal progenitors.

FGF signalling has been previously implicated in subdividing the hemangioblast lineage. Increasing FGF signalling before gastrulation leads to an increase in vascular markers and a corresponding decrease in blood markers (Iraha et al., 2002). Here I have shown that FGF signalling is necessary to restrict the endothelial lineage and for maintaining the posterior vascular free zone. The contradiction between previous results and those presented here is likely due to the difference in timing between experiments, as the previous experiments were performed before gastrulation. Therefore, it is likely that the earlier result on the hemangioblast lineage was compounded with earlier effects of Fgf on initial mesoderm formation, thus complicating the interpretation of those results.

Recently, the existence of the hemangioblast lineage has been called into question. It has been argued that since the endothelial and erythropoietic cells travel through the primitive streak at slightly different time points, the vascular and blood fates have been determined either prior to, or during gastrulation ((Kinder et al., 2001) and references therein). My observation that the balance between endothelial and erythropoietic lineages can be altered after gastrulation suggests that, in *Xenopus*, these cells may retain some lineage plasticity in the post gastrula embryo. While I have not shown a direct erythroid to endothelial transformation, the increase in endothelial cell markers, and corresponding decrease in erythroid differentiation in both RA, and FGF inhibitor treated embryos strongly suggest a close link between these two lineages in the *Xenopus* embryo. A cell culture approach whereby progenitor cells are differentiated into one lingeage over the other based upon the levels of active RA and FGF signalling would further support this hypothesis. The idea that the endothelial and erythroid lineages are linked after

gastrulation is difficult to test *in vivo* as these lineages share many of the defined early lineage markers, such as *etv2* and *tal1* (see section 1.4.2 and 1.4.3). However, sharing a common transcriptional regime does also support the idea that these two lineages are closely linked. I therefore propose that while endothelial and erythroid cells pass through the node at slightly different times (Kinder et al., 2001) and this is a result of being spatially distinct in the blastula marginal zone, that these two cell populations are not yet differentially specified. Rather, I suggest that the mesodermal lineages are not subdivided until later based on the positional cues received when they have reached their final location in the embryo. This model would support the existence of the hemangioblast in *Xenopus*, although only as one step in the subdivision of the mesodermal progenitor pool.

### 4.2.2 Relevance of the Early LPM Patterning to Gut Tube Development

This thesis has focused on the relevance of this patterning process to early cardiovascular lineages. However, it should be noted that the LPM has a number of functions during this stage of development, not only in relation to cardiovascular development, but it is also essential for regional specification of the gut tube (Horb and Slack, 2001). The early LPM domains of *hand1*, *foxf1* and *sall3* do not directly correlate to any previously defined structure. It is tempting to speculate that the RA/Fgf dependent patterning within the LPM during the neurula, may be important for patterning of the gut tube during the mid tail bud stage. Both RA (Zeynali and Dixon, 1998) and Fgf (Chen et al., 2003) pathways have been shown to be important for regulating endodermal development in the post-gastrula *Xenopus* embryo. Furthermore, the anterior-dorsal LPM marker, *foxf1*, is necessary for proper gut morphogenesis (Tseng et al., 2004). Since *foxf1* is a mesodermally restricted transcription factor, it is likely that it plays a key role in

regulating a diffusible factor responsible for the mesodermal-endodermal interactions. Furthermore, since RA signalling regulates *foxf1*, it is possible that at least part of the RA requirement for endodermal development is in regulating the mesodermal signals responsible for regional specification of the gut tube.

### 4.3 *Hand1* Function is Essential for Cardiac Morphogenesis

*Hand1* is essential for proper cardiac morphogenesis in the mouse embryo. *Hand1*$^{-/-}$ mice show a significant delay in the formation of the heart tube (Smart et al., 2002). This is reminiscent of the phenotype I describe here (section 3.4.1). A loss of *hand1* through injection of an antisense morpholino oligonucleotide leads to a disruption of heart tube closure in *Xenopus*. The similarity in phenotype between the mouse mutant and frog morphant provides a measure of confidence and phenotypic evidence that the *hand1* morpholino is efficiently inhibiting the translation of the *hand1* gene as intended.

The similarity in phenotype described between frog and mouse serves to further demonstrate the conserved mechanisms of heart tube formation among vertebrates. Heart tube formation is poorly understood; although a number of signalling molecules have been implicated (such as RA (Collop et al., 2006) and Notch (Rones et al., 2000)), the mechanism of heart tube formation remains unknown. However, since *hand1* is RA responsive, this may provide a link between the loss of RA, and the failure of the heart to form a tube. While *hand1* is a transcription factor, and is thus not likely to be directly involved in the cell shape changes necessary for the transition between a sheet of cells and a tube, it is possible that *hand1* regulates the expression of cellular components necessary for tissue morphogenesis, as recently shown in zebrafish (Garavito-Aguilar et

al., 2010). Therefore, further characterizing the targets of *hand1* in the heart will be of particular interest to connecting a loss of retinoic acid to heart morphogenesis. It would also be interesting to more closely compare the expression domain of *hand1* and *nkx2-5* as I would predict that a loss of RA signalling would restrict *hand1* expression out of the heart field during later stages.

### 4.3.1 *Hand1* is Essential for Vascular Development

Loss of Hand1 function severely perturbs vascular formation. Vascular plexus density was reduced when Hand1 production was inhibited in the whole embryo, as assayed by *etv2* and *alpnr*. This is similar to the phenotype of *Hand1*$^{-/-}$ mouse embryos that lacked extra embryonic vasculature (Firulli et al., 1998). Contralateral injected frog embryos demonstrated a more severe phenotype with almost a complete lack of vasculature on the injected side. The increased severity of the contralateral morphant phenotype is most likely the result of increased concentration of the morpholino on the injected side (4.6 nL of the stock morpholino oligo injected into the whole embryo, versus 4.6 nL injected on one side). Interestingly, while the vascular plexus is almost completely absent on the injected side, the posterior cardinal vein is also severely affected, particularly in the anterior half of the trunk corresponding to the region of overlap with *hand1*. Ablation of the posterior cardinal vein suggests that the endothelial defect results from a defect in vasculogenesis (Cleaver et al., 1997), as opposed to a defect in angiogenesis. However, one possibility that I have not addressed is an increase in apoptosis within the endothelial lineage. Apoptosis will need to be examined in the future to exclude the possibility that the difference between endothelial markers in the *hand1*

morphant is due to an increase in cell death rather than a specific effect on endothelial differentiation.

Both of the vascular markers *etv2* and *alpnr* are essential for endothelial development (Inui et al., 2006; Salanga et al., 2010). *Etv2* has been described to be at, or near the top of the transcriptional cascade leading to specification of the endothelial lineage (Salanga et al., 2010). In the *hand1* morphants assayed at stage 20 for early expression of vascular markers, both *etv2* and *alpnr* appear to be expressed normally. Both of these markers are expressed in precursors of the endothelial and erythrocyte lineage, or the hemangioblast lineage, at this stage. The expression of both *etv2* and *alpnr*, like *hand1*, is down regulated shortly after neural tube closure in the ventral blood islands. Since the early expression of both of these markers in the hemangioblast lineage is normal, but later expression in the endothelial cells is perturbed, this suggests that *hand1* is necessary for the maintenance of the endothelial lineage specification. Furthermore, if the down regulation of *hand1* leads to a loss of vascular markers, excluding *hand1* from the ventral blood islands in the early tail bud stage embryo may be a key step to defining the erythrocyte versus endothelial cell lineages. This could be tested by overexpressing *hand1*, and thereby not allowing it to be restricted from the hemangioblast population.

## 4.4 Future Directions

While the similarity between the results that I have discussed here and the previous results described in mouse, with regard to heart tube closure suggest that the morpholino is acting specifically through blocking Hand1 translation, a number of

experiments are still outstanding. Co-injecting a *Hand1*-RFP fusion construct to demonstrate that *Hand1* is binding specifically to the target sequence will need to be done. As well, a rescue of the phenotype, by injection of a *hand1* construct with a mutated target sequence is also needed to demonstrate specificity of the morpholino. Furthermore, overexpression of the construct to determine if it is able to ectopically induce endothelial development is of great interest in order to place *hand1* within the endothelial specification cascade. Finally, determining direct targets of Hand1 is essential to an understanding of *hand1* function in both heart morphogenesis and endothelial specification. Chromatin immunoprecipitation could be done to determine the target genes. However, the lack of a suitable *Xenopus* Hand1 antibody, in addition to the current lack of genome data, particularly promoter sequences in *Xenopus laevis*, complicates these final experiments.

## Chapter 5 Conclusions

I find here that both the RA and FGF signalling pathways are absolutely required for proper early patterning of the LPM in a mutually antagonistic fashion. This extends the role of RA and Fgf in embryonic patterning to include the whole LPM tissue. I propose that RA is released from *raldh2* expressing cells in the anterior and dorsal LPM and somites, and that it is necessary for patterning the anterior-dorsal and middle LPM domains. Conversely, Fgf8 is produced in the anterior-ventral end, while Fgf4 and 8 are produced posteriorly, corresponding to the heart domain and tail bud domain respectfully, the two areas dependent on FGF signalling. I propose that the sum of RA and FGF signalling, and additional as yet unidentified signalling molecules creates a combinatorial signalling code that yields two-dimensional positional information across the LPM. Proper establishment of this early LPM pattern is essential for the embryo, as any perturbations in RA or FGF signalling will have severe consequences to the cardiovascular lineages. Furthermore, Hand1 function is essential for both proper heart tube development, and endothelial lineage specification. Since *hand1* is directly regulated by RA signalling, this may provide a link between the loss of RA and failed heart tube formation, and increased RA signalling and the posterior expansion of the vascular plexus.

## Chapter 6 References

Abu-Abed, S., Dolle, P., Metzger, D., Beckett, B., Chambon, P., Petkovich, M., 2001. The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. Genes Dev. 15**,** 226-40.

Adams, D. S., Robinson, K. R., Fukumoto, T., Yuan, S., Albertson, R. C., Yelick, P., Kuo, L., McSweeney, M., Levin, M., 2006. Early, H+-V-ATPase-dependent proton flux is necessary for consistent left-right patterning of non-mammalian vertebrates. Development. 133**,** 1657-71.

Afouda, B. A., Martin, J., Liu, F., Ciau-Uitz, A., Patient, R., Hoppler, S., 2008. GATA transcription factors integrate Wnt signalling during heart development. Development. 135**,** 3185-90.

Agarwal, C., Chandraratna, R. A., Johnson, A. T., Rorke, E. A., Eckert, R. L., 1996. AGN193109 is a highly effective antagonist of retinoid action in human ectocervical epithelial cells. J Biol Chem. 271**,** 12209-12.

Agathon, A., Thisse, C., Thisse, B., 2003. The molecular nature of the zebrafish tail organizer. Nature. 424**,** 448-52.

Agius, E., Oelgeschlager, M., Wessely, O., Kemp, C., De Robertis, E. M., 2000. Endodermal Nodal-related signals and mesoderm induction in Xenopus. Development. 127**,** 1173-83.

Albalat, R., 2009. The retinoic acid machinery in invertebrates: ancestral elements and vertebrate innovations. Mol Cell Endocrinol. 313**,** 23-35.

Alsan, B. H., Schultheiss, T. M., 2002. Regulation of avian cardiogenesis by Fgf8 signaling. Development. 129**,** 1935-43.

Amaya, E., Musci, T. J., Kirschner, M. W., 1991. Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. Cell. 66**,** 257-70.

Amaya, E., Stein, P. A., Musci, T. J., Kirschner, M. W., 1993. FGF signalling in the early specification of mesoderm in Xenopus. Development. 118**,** 477-87.

Arai, A., Yamamoto, K., Toyama, J., 1997. Murine cardiac progenitor cells require visceral embryonic endoderm and primitive streak for terminal differentiation. Dev Dyn. 210**,** 344-53.

Astorga, J., Carlsson, P., 2007. Hedgehog induction of murine vasculogenesis is mediated by Foxf1 and Bmp4. Development. 134**,** 3753-61.

Aw, S., Adams, D. S., Qiu, D., Levin, M., 2008. H,K-ATPase protein localization and Kir4.1 function reveal concordance of three axes during early determination of left-right asymmetry. Mech Dev. 125**,** 353-72.

Aw, S., Koster, J., Pearson, W., Nichols, C., Shi, N. Q., Carneiro, K., Levin, M., 2010. The ATP-sensitive K(+)-channel (K(ATP)) controls early left-right patterning in Xenopus and chick embryos. Dev Biol.

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

Barnes, R. M., Firulli, B. A., Conway, S. J., Vincentz, J. W., Firulli, A. B., 2010. Analysis of the Hand1 cell lineage reveals novel contributions to cardiovascular, neural crest, extra-embryonic, and lateral mesoderm derivatives. Dev Dyn.

Beck, C. W., Slack, J. M., 1998. Analysis of the developing Xenopus tail bud reveals separate phases of gene expression during determination and outgrowth. Mech Dev. 72**,** 41-52.

Begemann, G., Marx, M., Mebus, K., Meyer, A., Bastmeyer, M., 2004. Beyond the neckless phenotype: influence of reduced retinoic acid signaling on motor neuron development in the zebrafish hindbrain. Dev Biol. 271**,** 119-29.

Benazeraf, B., Francois, P., Baker, R. E., Denans, N., Little, C. D., Pourquie, O., 2010. A random cell motility gradient downstream of FGF controls elongation of an amniote embryo. Nature. 466**,** 248-52.

Bernard-Pierrot, I., Ricol, D., Cassidy, A., Graham, A., Elvin, P., Caillault, A., Lair, S., Broet, P., Thiery, J. P., Radvanyi, F., 2004. Inhibition of human bladder tumour cell growth by fibroblast growth factor receptor 2b is independent of its kinase activity. Involvement of the carboxy-terminal region of the receptor. Oncogene. 23**,** 9201-11.

Bertwistle, D., Walmsley, M. E., Read, E. M., Pizzey, J. A., Patient, R. K., 1996. GATA factors and the origins of adult and embryonic blood in Xenopus: responses to retinoic acid. Mech Dev. 57**,** 199-214.

Besser, D., Presta, M., Nagamine, Y., 1995. Elucidation of a signaling pathway induced by FGF-2 leading to uPA gene expression in NIH 3T3 fibroblasts. Cell Growth Differ. 6**,** 1009-17.

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

Birsoy, B., Kofron, M., Schaible, K., Wylie, C., Heasman, J., 2006. Vg 1 is an essential signaling molecule in Xenopus development. Development. 133**,** 15-20.

Bodmer, R., 1993. The gene tinman is required for specification of the heart and visceral muscles in Drosophila. Development. 118**,** 719-29.

Bodmer, R., Jan, L. Y., Jan, Y. N., 1990. A new homeobox-containing gene, msh-2, is transiently expressed early during mesoderm formation of Drosophila. Development. 110**,** 661-9.

Bohnsack, B. L., Lai, L., Dolle, P., Hirschi, K. K., 2004. Signaling hierarchy downstream of retinoic acid that independently regulates vascular remodeling and endothelial cell proliferation. Genes Dev. 18**,** 1345-58.

Bottcher, R. T., Niehrs, C., 2005. Fibroblast growth factor signaling during early vertebrate development. Endocr Rev. 26**,** 63-77.

Brade, T., Gessert, S., Kuhl, M., Pandur, P., 2007. The amphibian second heart field: Xenopus islet-1 is required for cardiovascular development. Dev Biol. 311**,** 297-310.

Brennan, R. G., 1993. The winged-helix DNA-binding motif: another helix-turn-helix takeoff. Cell. 74**,** 773-6.

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

Buchberger, A., Pabst, O., Brand, T., Seidl, K., Arnold, H. H., 1996. Chick NKx-2.3 represents a novel family member of vertebrate homologues to the Drosophila homeobox gene tinman: differential expression of cNKx-2.3 and cNKx-2.5 during heart and gut development. Mech Dev. 56**,** 151-63.

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

Campione, M., Steinbeisser, H., Schweickert, A., Deissler, K., van Bebber, F., Lowe, L. A., Nowotschin, S., Viebahn, C., Haffter, P., Kuehn, M. R., Blum, M., 1999. The homeobox gene Pitx2: mediator of asymmetric left-right signaling in vertebrate heart and gut looping. Development. 126**,** 1225-34.

Campo-Paysaa, F., Marletaz, F., Laudet, V., Schubert, M., 2008. Retinoic acid signaling in development: tissue-specific functions and evolutionary origins. Genesis. 46**,** 640-56.

Cartry, J., Nichane, M., Ribes, V., Colas, A., Riou, J. F., Pieler, T., Dolle, P., Bellefroid, E. J., Umbhauer, M., 2006. Retinoic acid signalling is required for specification of pronephric cell fate. Dev Biol. 299**,** 35-51.

Cascio, S., Gurdon, J. B., 1987. The initiation of new gene transcription during Xenopus gastrulation requires immediately preceding protein synthesis. Development. 100**,** 297-305.

Chambers, D., Medhurst, A. D., Walsh, F. S., Price, J., Mason, I., 2000. Differential display of genes expressed at the midbrain - hindbrain junction identifies

sprouty2: an FGF8-inducible member of a family of intracellular FGF antagonists. Mol Cell Neurosci. 15**,** 22-35.

Chambon, P., 1996. A decade of molecular biology of retinoic acid receptors. FASEB J. 10**,** 940-54.

Charron, F., Paradis, P., Bronchain, O., Nemer, G., Nemer, M., 1999. Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression. Mol Cell Biol. 19**,** 4355-65.

Chen, F., Cao, Y., Qian, J., Shao, F., Niederreither, K., Cardoso, W. V., 2010. A retinoic acid-dependent network in the foregut controls formation of the mouse lung primordium. J Clin Invest. 120**,** 2040-8.

Chen, Y., Jurgens, K., Hollemann, T., Claussen, M., Ramadori, G., Pieler, T., 2003. Cell-autonomous and signal-dependent expression of liver and intestine marker genes in pluripotent precursor cells from Xenopus embryos. Mech Dev. 120**,** 277-88.

Chen, Y., Pan, F. C., Brandes, N., Afelik, S., Solter, M., Pieler, T., 2004. Retinoic acid signaling is essential for pancreas development and promotes endocrine at the expense of exocrine cell differentiation in Xenopus. Dev Biol. 271**,** 144-60.

Chen, Y., Pollet, N., Niehrs, C., Pieler, T., 2001. Increased XRALDH2 activity has a posteriorizing effect on the central nervous system of Xenopus embryos. Mech Dev. 101**,** 91-103.

Christen, B., Beck, C. W., Lombardo, A., Slack, J. M., 2003. Regeneration-specific expression pattern of three posterior Hox genes. Dev Dyn. 226**,** 349-55.

Christen, B., Slack, J. M., 1997. FGF-8 is associated with anteroposterior patterning and limb regeneration in Xenopus. Dev Biol. 192**,** 455-66.

Christian, J. L., McMahon, J. A., McMahon, A. P., Moon, R. T., 1991. Xwnt-8, a Xenopus Wnt-1/int-1-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. Development. 111**,** 1045-55.

Chuang, C. Y., Lord, M. S., Melrose, J., Rees, M. D., Knox, S. M., Freeman, C., Iozzo, R. V., Whitelock, J. M., 2010. Heparan sulfate-dependent signaling of fibroblast growth factor 18 by chondrocyte-derived perlecan. Biochemistry. 49**,** 5524-32.

Ciruna, B., Rossant, J., 2001. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell. 1**,** 37-49.

Cleaver, O., Tonissen, K. F., Saha, M. S., Krieg, P. A., 1997. Neovascularization of the Xenopus embryo. Dev Dyn. 210**,** 66-77.

Cleaver, O. B., Patterson, K. D., Krieg, P. A., 1996. Overexpression of the tinman-related genes XNkx-2.5 and XNkx-2.3 in Xenopus embryos results in myocardial hyperplasia. Development. 122**,** 3549-56.

Clements, D., Friday, R. V., Woodland, H. R., 1999. Mode of action of VegT in mesoderm and endoderm formation. Development. 126**,** 4903-11.

Collop, A. H., Broomfield, J. A., Chandraratna, R. A., Yong, Z., Deimling, S. J., Kolker, S. J., Weeks, D. L., Drysdale, T. A., 2006. Retinoic acid signaling is essential for formation of the heart tube in Xenopus. Dev Biol. 291**,** 96-109.

Conlon, F. L., Sedgwick, S. G., Weston, K. M., Smith, J. C., 1996. Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. Development. 122**,** 2427-35.

Conlon, F. L., Smith, J. C., 1999. Interference with brachyury function inhibits convergent extension, causes apoptosis, and reveals separate requirements in the FGF and activin signalling pathways. Dev Biol. 213**,** 85-100.

Copenhaver, W. M., 1926. Experiments on the development of the heart of *Ambystoma punctatum*. J. Exp. Zool. 43**,** 321-371.

Cornell, R. A., Musci, T. J., Kimelman, D., 1995. FGF is a prospective competence factor for early activin-type signals in Xenopus mesoderm induction. Development. 121**,** 2429-37.

Costa, R. M., Soto, X., Chen, Y., Zorn, A. M., Amaya, E., 2008. spib is required for primitive myeloid development in Xenopus. Blood. 112**,** 2287-96.

Cox, C. M., D'Agostino, S. L., Miller, M. K., Heimark, R. L., Krieg, P. A., 2006. Apelin, the ligand for the endothelial G-protein-coupled receptor, APJ, is a potent angiogenic factor required for normal vascular development of the frog embryo. Dev Biol. 296**,** 177-89.

D'Souza, S. L., Elefanty, A. G., Keller, G., 2005. SCL/Tal-1 is essential for hematopoietic commitment of the hemangioblast but not for its development. Blood. 105**,** 3862-70.

Dalgin, G., Goldman, D. C., Donley, N., Ahmed, R., Eide, C. A., Christian, J. L., 2007. GATA-2 functions downstream of BMPs and CaM KIV in ectodermal cells during primitive hematopoiesis. Dev Biol. 310**,** 454-69.

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

Debiais, F., Lefevre, G., Lemonnier, J., Le Mee, S., Lasmoles, F., Mascarelli, F., Marie, P. J., 2004. Fibroblast growth factor-2 induces osteoblast survival through a phosphatidylinositol 3-kinase-dependent, -beta-catenin-independent signaling pathway. Exp Cell Res. 297**,** 235-46.

Dell'Era, P., Ronca, R., Coco, L., Nicoli, S., Metra, M., Presta, M., 2003. Fibroblast growth factor receptor-1 is essential for in vitro cardiomyocyte development. Circ Res. 93**,** 414-20.

Devic, E., Paquereau, L., Vernier, P., Knibiehler, B., Audigier, Y., 1996. Expression of a new G protein-coupled receptor X-msr is associated with an endothelial lineage in Xenopus laevis. Mech Dev. 59**,** 129-40.

Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M., Storey, K., 2003. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron. 40**,** 65-79.

Doherty, J. R., Johnson Hamlet, M. R., Kuliyev, E., Mead, P. E., 2007. A flk-1 promoter/enhancer reporter transgenic Xenopus laevis generated using the Sleeping Beauty transposon system: an in vivo model for vascular studies. Dev Dyn. 236**,** 2808-17.

Dosch, R., Gawantka, V., Delius, H., Blumenstock, C., Niehrs, C., 1997. Bmp-4 acts as a morphogen in dorsoventral mesoderm patterning in Xenopus. Development. 124, 2325-34.

Dostal, A., Nemeckova, J., Gaillyova, R., 2009. The 18q deletion syndrome and analysis of the critical region for orofacial cleft at 18q22.3. J Craniomaxillofac Surg. 37, 272-5.

Drysdale, T. A., Patterson, K. D., Saha, M., Krieg, P. A., 1997. Retinoic acid can block differentiation of the myocardium after heart specification. Dev Biol. 188, 205-15.

Drysdale, T. A., Tonissen, K. F., Patterson, K. D., Crawford, M. J., Krieg, P. A., 1994. Cardiac troponin I is a heart-specific marker in the Xenopus embryo: expression during abnormal heart morphogenesis. Dev Biol. 165, 432-41.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J., Nemer, M., 1997. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. EMBO J. 16, 5687-96.

Durston, A. J., Timmermans, J. P., Hage, W. J., Hendriks, H. F., de Vries, N. J., Heideveld, M., Nieuwkoop, P. D., 1989. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature. 340, 140-4.

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

Elinson, R. P., Rowning, B., 1988. A transient array of parallel microtubules in frog eggs: potential tracks for a cytoplasmic rotation that specifies the dorso-ventral axis. Dev Biol. 128**,** 185-97.

Ema, M., Takahashi, S., Rossant, J., 2006. Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors. Blood. 107**,** 111-7.

Endoh, M., Ogawa, M., Orkin, S., Nishikawa, S., 2002. SCL/tal-1-dependent process determines a competence to select the definitive hematopoietic lineage prior to endothelial differentiation. EMBO J. 21**,** 6700-8.

Escriva, H., Bertrand, S., Germain, P., Robinson-Rechavi, M., Umbhauer, M., Cartry, J., Duffraisse, M., Holland, L., Gronemeyer, H., Laudet, V., 2006. Neofunctionalization in vertebrates: the example of retinoic acid receptors. PLoS Genet. 2**,** e102.

Essner, J. J., Vogan, K. J., Wagner, M. K., Tabin, C. J., Yost, H. J., Brueckner, M., 2002. Conserved function for embryonic nodal cilia. Nature. 418**,** 37-8.

Eswarakumar, V. P., Lax, I., Schlessinger, J., 2005. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. 16**,** 139-49.

Fainsod, A., Deissler, K., Yelin, R., Marom, K., Epstein, M., Pillemer, G., Steinbeisser, H., Blum, M., 1997. The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. Mech Dev. 63**,** 39-50.

Fainsod, A., Steinbeisser, H., De Robertis, E. M., 1994. On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. EMBO J. 13**,** 5015-25.

Ferdous, A., Caprioli, A., Iacovino, M., Martin, C. M., Morris, J., Richardson, J. A., Latif, S., Hammer, R. E., Harvey, R. P., Olson, E. N., Kyba, M., Garry, D. J., 2009. Nkx2-5 transactivates the Ets-related protein 71 gene and specifies an endothelial/endocardial fate in the developing embryo. Proc Natl Acad Sci U S A. 106**,** 814-9.

Festag, M., Viertel, B., Steinberg, P., Sehner, C., 2007. An in vitro embryotoxicity assay based on the disturbance of the differentiation of murine embryonic stem cells into endothelial cells. II. Testing of compounds. Toxicol In Vitro. 21**,** 1631-40.

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

Fletcher, R. B., Harland, R. M., 2008. The role of FGF signaling in the establishment and maintenance of mesodermal gene expression in Xenopus. Dev Dyn. 237**,** 1243-54.

Frei, E., Schuh, R., Baumgartner, S., Burri, M., Noll, M., Jurgens, G., Seifert, E., Nauber, U., Jackle, H., 1988. Molecular characterization of spalt, a homeotic gene required for head and tail development in the Drosophila embryo. EMBO J. 7**,** 197-204.

Fu, Y., Yan, W., Mohun, T. J., Evans, S. M., 1998. Vertebrate tinman homologues XNkx2-3 and XNkx2-5 are required for heart formation in a functionally redundant manner. Development. 125**,** 4439-49.

Furthauer, M., Van Celst, J., Thisse, C., Thisse, B., 2004. Fgf signalling controls the dorsoventral patterning of the zebrafish embryo. Development. 131**,** 2853-64.

Garavito-Aguilar, Z. V., Riley, H. E., Yelon, D., 2010. Hand2 ensures an appropriate environment for cardiac fusion by limiting Fibronectin function. Development. 137**,** 3215-20.

Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C., Niehrs, C., 1995. Antagonizing the Spemann organizer: role of the homeobox gene Xvent-1. EMBO J. 14**,** 6268-79.

Gessert, S., Kuhl, M., 2009. Comparative gene expression analysis and fate mapping studies suggest an early segregation of cardiogenic lineages in Xenopus laevis. Dev Biol. 334**,** 395-408.

Ghosh, T. K., Song, F. F., Packham, E. A., Buxton, S., Robinson, T. E., Ronksley, J., Self, T., Bonser, A. J., Brook, J. D., 2009. Physical interaction between TBX5 and MEF2C is required for early heart development. Mol Cell Biol. 29**,** 2205-18.

Ghyselinck, N. B., Dupe, V., Dierich, A., Messaddeq, N., Garnier, J. M., Rochette-Egly, C., Chambon, P., Mark, M., 1997. Role of the retinoic acid receptor beta (RARbeta) during mouse development. Int J Dev Biol. 41**,** 425-47.

Gisselbrecht, S., Skeath, J. B., Doe, C. Q., Michelson, A. M., 1996. heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the Drosophila embryo. Genes Dev. 10**,** 3003-17.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., Niehrs, C., 1998. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature. 391**,** 357-62.

Goncalves, M. B., Agudo, M., Connor, S., McMahon, S., Minger, S. L., Maden, M., Corcoran, J. P., 2009. Sequential RARbeta and alpha signalling in vivo can induce adult forebrain neural progenitor cells to differentiate into neurons through Shh and FGF signalling pathways. Dev Biol. 326**,** 305-13.

Gont, L. K., Steinbeisser, H., Blumberg, B., de Robertis, E. M., 1993. Tail formation as a continuation of gastrulation: the multiple cell populations of the Xenopus tailbud derive from the late blastopore lip. Development. 119**,** 991-1004.

Grand, E. K., Chase, A. J., Heath, C., Rahemtulla, A., Cross, N. C., 2004. Targeting FGFR3 in multiple myeloma: inhibition of t(4;14)-positive cells by SU5402 and PD173074. Leukemia. 18**,** 962-6.

Green, J. B., New, H. V., Smith, J. C., 1992. Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. Cell. 71**,** 731-9.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., Krasnow, M. A., 1998. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell. 92**,** 253-63.

Hale, F., 1935. The relation of vitamin A to anophthalmos in pigs. Am. J. Ophth. 18**,** 1087-1092.

Harland, R. M., 1991. *In situ* hybridization: an improved whole-mount method for *Xenopus* embryos. Methods Cell Biol. 36**,** 685-695.

Haselbeck, R. J., Hoffmann, I., Duester, G., 1999. Distinct functions for Aldh1 and Raldh2 in the control of ligand production for embryonic retinoid signaling pathways. Dev Genet. 25**,** 353-64.

Haworth, K. E., Kotecha, S., Mohun, T. J., Latinkic, B. V., 2008. GATA4 and GATA5 are essential for heart and liver development in Xenopus embryos. BMC Dev Biol. 8**,** 74.

Heasman, J., Wessely, O., Langland, R., Craig, E. J., Kessler, D. S., 2001. Vegetal localization of maternal mRNAs is disrupted by VegT depletion. Dev Biol. 240**,** 377-86.

Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., Rosenfeld, M. G., 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature. 387**,** 43-8.

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

Hollemann, T., Chen, Y., Grunz, H., Pieler, T., 1998. Regionalized metabolic activity establishes boundaries of retinoic acid signalling. Embo J. 17**,** 7361-72.

Hollemann, T., Schuh, R., Pieler, T., Stick, R., 1996. Xenopus Xsal-1, a vertebrate homolog of the region specific homeotic gene spalt of Drosophila. Mech Dev. 55**,** 19-32.

Holtzinger, A., Evans, T., 2007. Gata5 and Gata6 are functionally redundant in zebrafish for specification of cardiomyocytes. Dev Biol. 312**,** 613-22.

Hoppler, S., Brown, J. D., Moon, R. T., 1996. Expression of a dominant-negative Wnt blocks induction of MyoD in Xenopus embryos. Genes Dev. 10**,** 2805-17.

Horb, M. E., Slack, J. M., 2001. Endoderm specification and differentiation in Xenopus embryos. Dev Biol. 236**,** 330-43.

Horb, M. E., Thomsen, G. H., 1999. Tbx5 is essential for heart development. Development. 126**,** 1739-51.

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

Inui, M., Fukui, A., Ito, Y., Asashima, M., 2006. Xapelin and Xmsr are required for cardiovascular development in Xenopus laevis. Dev Biol. 298**,** 188-200.

Iraha, F., Saito, Y., Yoshida, K., Kawakami, M., Izutsu, Y., Daar, I. O., Maeno, M., 2002. Common and distinct signals specify the distribution of blood and vascular cell lineages in Xenopus laevis embryos. Dev Growth Differ. 44**,** 395-407.

Isaacs, H. V., Deconinck, A. E., Pownall, M. E., 2007. FGF4 regulates blood and muscle specification in Xenopus laevis. Biol Cell. 99**,** 165-73.

Isaacs, H. V., Tannahill, D., Slack, J. M., 1992. Expression of a novel FGF in the Xenopus embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. Development. 114**,** 711-20.

Jiang, Y., Drysdale, T. A., Evans, T., 1999. A role for GATA-4/5/6 in the regulation of Nkx2.5 expression with implications for patterning of the precardiac field. Dev Biol. 216**,** 57-71.

Jiang, Y., Evans, T., 1996. The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. Dev Biol. 174**,** 258-70.

Jones, E. A., Woodland, H. R., 1987. The development of animal cap cells in Xenopus: a measure of the start of animal cap competence to form mesoderm. Development. 101**,** 557-563.

Jurgens, G., 1988. Head and tail development of the Drosophila embryo involves spalt, a novel homeotic gene. EMBO J. 7**,** 189-96.

Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., McKeehan, W. L., 1993. An essential heparin-binding domain in the fibroblast growth factor receptor kinase. Science. 259**,** 1918-21.

Kato, M., Wang, H., Kainulainen, V., Fitzgerald, M. L., Ledbetter, S., Ornitz, D. M., Bernfield, M., 1998. Physiological degradation converts the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2. Nat Med. 4**,** 691-7.

Kattman, S. J., Huber, T. L., Keller, G. M., 2006. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell. 11**,** 723-32.

Kawakami, Y., Raya, A., Raya, R. M., Rodriguez-Esteban, C., Belmonte, J. C., 2005. Retinoic acid signalling links left-right asymmetric patterning and bilaterally symmetric somitogenesis in the zebrafish embryo. Nature. 435**,** 165-71.

Keegan, B. R., Feldman, J. L., Begemann, G., Ingham, P. W., Yelon, D., 2005. Retinoic acid signaling restricts the cardiac progenitor pool. Science. 307**,** 247-9.

Keller, R., 1991. Early embryonic development of Xenopus laevis. Methods Cell Biol. 36**,** 61-113.

Kelley, C., Yee, K., Harland, R., Zon, L. I., 1994. Ventral expression of GATA-1 and GATA-2 in the Xenopus embryo defines induction of hematopoietic mesoderm. Dev Biol. 165**,** 193-205.

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

Kengaku, M., Okamoto, H., 1995. bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in Xenopus. Development. 121**,** 3121-30.

Keren-Politansky, A., Keren, A., Bengal, E., 2009. Neural ectoderm-secreted FGF initiates the expression of Nkx2.5 in cardiac progenitors via a p38 MAPK/CREB pathway. Dev Biol. 335**,** 374-84.

Khokha, M. K., Yeh, J., Grammer, T. C., Harland, R. M., 2005. Depletion of three BMP antagonists from Spemann's organizer leads to a catastrophic loss of dorsal structures. Dev Cell. 8**,** 401-11.

Kikkawa, M., Yamazaki, M., Izutsu, Y., Maeno, M., 2001. Two-step induction of primitive erythrocytes in Xenopus laevis embryos: signals from the vegetal endoderm and the overlying ectoderm. Int J Dev Biol. 45**,** 387-96.

Kinder, S. J., Loebel, D. A., Tam, P. P., 2001. Allocation and early differentiation of cardiovascular progenitors in the mouse embryo. Trends Cardiovasc Med. 11**,** 177-84.

Knochel, W., Beck, J., Meyerhof, W., 1987. Nucleotide sequence of the Xenopus tropicalis larval beta globin gene. Nucleic Acids Res. 15**,** 10062.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C., Heasman, J., 1999. Mesoderm induction in Xenopus is a zygotic event regulated by maternal VegT via TGFbeta growth factors. Development. 126**,** 5759-70.

Koga, M., Kudoh, T., Hamada, Y., Watanabe, M., Kageura, H., 2007. A new triple staining method for double in situ hybridization in combination with cell lineage tracing in whole-mount Xenopus embryos. Dev Growth Differ. 49**,** 635-45.

Kohlhase, J., Hausmann, S., Stojmenovic, G., Dixkens, C., Bink, K., Schulz-Schaeffer, W., Altmann, M., Engel, W., 1999. SALL3, a new member of the human spalt-like gene family, maps to 18q23. Genomics. 62**,** 216-22.

Koide, T., Downes, M., Chandraratna, R. A., Blumberg, B., Umesono, K., 2001. Active repression of RAR signaling is required for head formation. Genes Dev. 15**,** 2111-21.

Koster, M., Dillinger, K., Knochel, W., 1999. Genomic structure and embryonic expression of the Xenopus winged helix factors XFD-13/13'. Mech Dev. 88**,** 89-93.

Kumano, G., Ezal, C., Smith, W. C., 2006. ADMP2 is essential for primitive blood and heart development in Xenopus. Dev Biol. 299**,** 411-23.

Lai, L., Bohnsack, B. L., Niederreither, K., Hirschi, K. K., 2003. Retinoic acid regulates endothelial cell proliferation during vasculogenesis. Development. 130**,** 6465-74.

Lane, M. C., Sheets, M. D., 2000. Designation of the anterior/posterior axis in pregastrula Xenopus laevis. Dev Biol. 225**,** 37-58.

Lane, M. C., Smith, W. C., 1999. The origins of primitive blood in Xenopus: implications for axial patterning. Development. 126**,** 423-34.

Lanner, F., Lee, K. L., Sohl, M., Holmborn, K., Yang, H., Wilbertz, J., Poellinger, L., Rossant, J., Farnebo, F., 2010. Heparan sulfation-dependent fibroblast growth factor signaling maintains embryonic stem cells primed for differentiation in a heterogeneous state. Stem Cells. 28**,** 191-200.

Lea, R., Papalopulu, N., Amaya, E., Dorey, K., 2009. Temporal and spatial expression of FGF ligands and receptors during Xenopus development. Dev Dyn. 238**,** 1467-79.

Lee, D., Park, C., Lee, H., Lugus, J. J., Kim, S. H., Arentson, E., Chung, Y. S., Gomez, G., Kyba, M., Lin, S., Janknecht, R., Lim, D. S., Choi, K., 2008. ER71 acts downstream of BMP, Notch, and Wnt signaling in blood and vessel progenitor specification. Cell Stem Cell. 2**,** 497-507.

Lee, P. L., Johnson, D. E., Cousens, L. S., Fried, V. A., Williams, L. T., 1989. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science. 245**,** 57-60.

Lenhard, T., Schober, A., Suter-Crazzolara, C., Unsicker, K., 2002. Fibroblast growth factor-2 requires glial-cell-line-derived neurotrophic factor for exerting its neuroprotective actions on glutamate-lesioned hippocampal neurons. Mol Cell Neurosci. 20**,** 181-97.

Levin, M., Thorlin, T., Robinson, K. R., Nogi, T., Mercola, M., 2002. Asymmetries in H+/K+-ATPase and cell membrane potentials comprise a very early step in left-right patterning. Cell. 111**,** 77-89.

Leyns, L., Bouwmeester, T., Kim, S. H., Piccolo, S., De Robertis, E. M., 1997. Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. Cell. 88**,** 747-56.

Li, P., Pashmforoush, M., Sucov, H. M., 2010. Retinoic acid regulates differentiation of the secondary heart field and TGFbeta-mediated outflow tract septation. Dev Cell. 18**,** 480-5.

Liao, E. C., Paw, B. H., Oates, A. C., Pratt, S. J., Postlethwait, J. H., Zon, L. I., 1998. SCL/Tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. Genes Dev. 12**,** 621-6.

Liao, W., Bisgrove, B. W., Sawyer, H., Hug, B., Bell, B., Peters, K., Grunwald, D. J., Stainier, D. Y., 1997. The zebrafish gene cloche acts upstream of a flk-1 homologue to regulate endothelial cell differentiation. Development. 124**,** 381-9.

Lien, C. L., Wu, C., Mercer, B., Webb, R., Richardson, J. A., Olson, E. N., 1999. Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. Development. 126**,** 75-84.

Lin, S. C., Dolle, P., Ryckebusch, L., Noseda, M., Zaffran, S., Schneider, M. D., Niederreither, K., 2010. Endogenous retinoic acid regulates cardiac progenitor differentiation. Proc Natl Acad Sci U S A. 107**,** 9234-9.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., Harvey, R. P., 1993. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development. 119**,** 969.

Liu, F., Walmsley, M., Rodaway, A., Patient, R., 2008. Fli1 acts at the top of the transcriptional network driving blood and endothelial development. Curr Biol. 18**,** 1234-40.

Lloret-Vilaspasa, F., Jansen, H. J., de Roos, K., Chandraratna, R. A., Zile, M. H., Stern, C. D., Durston, A. J., 2010. Retinoid signalling is required for information transfer from mesoderm to neuroectoderm during gastrulation. Int J Dev Biol. 54**,** 599-608.

Logan, M., Mohun, T., 1993. Induction of cardiac muscle differentiation in isolated animal pole explants of Xenopus laevis embryos. Development. 118**,** 865-75.

Lohnes, D., Kastner, P., Dierich, A., Mark, M., LeMeur, M., Chambon, P., 1993. Function of retinoic acid receptor gamma in the mouse. Cell. 73**,** 643-58.

Lohnes, D., Mark, M., Mendelsohn, C., Dolle, P., Dierich, A., Gorry, P., Gansmuller, A., Chambon, P., 1994. Function of the retinoic acid receptors (RARs) during

development (I). Craniofacial and skeletal abnormalities in RAR double mutants. Development. 120**,** 2723-48.

Lohr, J. L., Danos, M. C., Yost, H. J., 1997. Left-right asymmetry of a nodal-related gene is regulated by dorsoanterior midline structures during Xenopus development. Development. 124**,** 1465-72.

Lufkin, T., Lohnes, D., Mark, M., Dierich, A., Gorry, P., Gaub, M. P., LeMeur, M., Chambon, P., 1993. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. Proc Natl Acad Sci U S A. 90**,** 7225-9.

Lustig, K. D., Kroll, K., Sun, E., Ramos, R., Elmendorf, H., Kirschner, M. W., 1996a. A Xenopus nodal-related gene that acts in synergy with noggin to induce complete secondary axis and notochord formation. Development. 122**,** 3275-82.

Lustig, K. D., Kroll, K. L., Sun, E. E., Kirschner, M. W., 1996b. Expression cloning of a Xenopus T-related gene (Xombi) involved in mesodermal patterning and blastopore lip formation. Development. 122**,** 4001-12.

Lutz, L. B., Cole, L. M., Gupta, M. K., Kwist, K. W., Auchus, R. J., Hammes, S. R., 2001. Evidence that androgens are the primary steroids produced by Xenopus laevis ovaries and may signal through the classical androgen receptor to promote oocyte maturation. Proc Natl Acad Sci U S A. 98**,** 13728-33.

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

Maciag, T., Cerundolo, J., Ilsley, S., Kelley, P. R., Forand, R., 1979. An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. Proc Natl Acad Sci U S A. 76**,** 5674-8.

Maden, M., 1999. Heads or tails? Retinoic acid will decide. Bioessays. 21**,** 809-12.

Maeno, M., Mead, P. E., Kelley, C., Xu, R. H., Kung, H. F., Suzuki, A., Ueno, N., Zon, L. I., 1996. The role of BMP-4 and GATA-2 in the induction and differentiation of hematopoietic mesoderm in Xenopus laevis. Blood. 88**,** 1965-72.

Magnusson, P., Rolny, C., Jakobsson, L., Wikner, C., Wu, Y., Hicklin, D. J., Claesson-Welsh, L., 2004. Deregulation of Flk-1/vascular endothelial growth factor receptor-2 in fibroblast growth factor receptor-1-deficient vascular stem cell development. J Cell Sci. 117**,** 1513-23.

Mahlapuu, M., Enerback, S., Carlsson, P., 2001a. Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations. Development. 128**,** 2397-406.

Mahlapuu, M., Ormestad, M., Enerback, S., Carlsson, P., 2001b. The forkhead transcription factor Foxf1 is required for differentiation of extra-embryonic and lateral plate mesoderm. Development. 128**,** 155-66.

Mandel, E. M., Kaltenbrun, E., Callis, T. E., Zeng, X. X., Marques, S. R., Yelon, D., Wang, D. Z., Conlon, F. L., 2010. The BMP pathway acts to directly regulate Tbx20 in the developing heart. Development. 137**,** 1919-29.

Marques, S. R., Lee, Y., Poss, K. D., Yelon, D., 2008. Reiterative roles for FGF signaling in the establishment of size and proportion of the zebrafish heart. Dev Biol. 321**,** 397-406.

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

Mead, P. E., Kelley, C. M., Hahn, P. S., Piedad, O., Zon, L. I., 1998. SCL specifies hematopoietic mesoderm in Xenopus embryos. Development. 125**,** 2611-20.

Meadows, S. M., Salanga, M. C., Krieg, P. A., 2009. Kruppel-like factor 2 cooperates with the ETS family protein ERG to activate Flk1 expression during vascular development. Development. 136**,** 1115-25.

Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P., Mark, M., 1994. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development. 120**,** 2749-71.

Mic, F. A., Molotkov, A., Benbrook, D. M., Duester, G., 2003. Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. Proc Natl Acad Sci U S A. 100**,** 7135-40.

Miller, J. R., Rowning, B. A., Larabell, C. A., Yang-Snyder, J. A., Bates, R. L., Moon, R. T., 1999. Establishment of the dorsal-ventral axis in Xenopus embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. J Cell Biol. 146**,** 427-37.

Mills, K. R., Kruep, D., Saha, M. S., 1999. Elucidating the origins of the vascular system: a fate map of the vascular endothelial and red blood cell lineages in Xenopus laevis. Dev Biol. 209**,** 352-68.

Minowada, G., Jarvis, L. A., Chi, C. L., Neubuser, A., Sun, X., Hacohen, N., Krasnow, M. A., Martin, G. R., 1999. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development. 126**,** 4465-75.

Misfeldt, A. M., Boyle, S. C., Tompkins, K. L., Bautch, V. L., Labosky, P. A., Baldwin, H. S., 2009. Endocardial cells are a distinct endothelial lineage derived from Flk1+ multipotent cardiovascular progenitors. Dev Biol. 333**,** 78-89.

Mjaatvedt, C. H., Nakaoka, T., Moreno-Rodriguez, R., Norris, R. A., Kern, M. J., Eisenberg, C. A., Turner, D., Markwald, R. R., 2001. The outflow tract of the heart is recruited from a novel heart-forming field. Dev Biol. 238**,** 97-109.

Moftah, M. Z., Downie, S. A., Bronstein, N. B., Mezentseva, N., Pu, J., Maher, P. A., Newman, S. A., 2002. Ectodermal FGFs induce perinodular inhibition of limb chondrogenesis in vitro and in vivo via FGF receptor 2. Dev Biol. 249**,** 270-82.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R., Schlessinger, J., 1997. Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science. 276**,** 955-60.

Mohun, T. J., Leong, L. M., Weninger, W. J., Sparrow, D. B., 2000. The morphology of heart development in Xenopus laevis. Dev Biol. 218**,** 74-88.

Moreno, T. A., Kintner, C., 2004. Regulation of segmental patterning by retinoic acid signaling during Xenopus somitogenesis. Dev Cell. 6**,** 205-18.

Moretti, A., Caron, L., Nakano, A., Lam, J. T., Bernshausen, A., Chen, Y., Qyang, Y., Bu, L., Sasaki, M., Martin-Puig, S., Sun, Y., Evans, S. M., Laugwitz, K. L., Chien, K. R.,

2006. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell. 127**,** 1151-65.

Morikawa, Y., Cserjesi, P., 2004. Extra-embryonic vasculature development is regulated by the transcription factor HAND1. Development. 131**,** 2195-204.

Mudumana, S. P., Hentschel, D., Liu, Y., Vasilyev, A., Drummond, I. A., 2008. odd skipped related1 reveals a novel role for endoderm in regulating kidney versus vascular cell fate. Development. 135**,** 3355-67.

Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., Evans, R. M., 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell. 89**,** 373-80.

Nakamura, K., Yamamoto, A., Kamishohara, M., Takahashi, K., Taguchi, E., Miura, T., Kubo, K., Shibuya, M., Isoe, T., 2004. KRN633: A selective inhibitor of vascular endothelial growth factor receptor-2 tyrosine kinase that suppresses tumor angiogenesis and growth. Mol Cancer Ther. 3**,** 1639-49.

Nascone, N., Mercola, M., 1995. An inductive role for the endoderm in Xenopus cardiogenesis. Development. 121**,** 515-23.

Neugebauer, J. M., Amack, J. D., Peterson, A. G., Bisgrove, B. W., Yost, H. J., 2009. FGF signalling during embryo development regulates cilia length in diverse epithelia. Nature. 458**,** 651-4.

Niederreither, K., Subbarayan, V., Dolle, P., Chambon, P., 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat Genet. 21**,** 444-8.

Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P., Dolle, P., 2001. Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development. 128**,** 1019-31.

Niehrs, C., 2004. Regionally specific induction by the Spemann-Mangold organizer. Nat Rev Genet. 5**,** 425-34.

Nieuwkoop, P. D., 1969. The formation of the mesoderm in urodelean amphibians I. The induction by the endoderm. W. Roux' Arch. Ent. Org. 162**,** 341-373.

Nieuwkoop, P. D., Faber, J., 1994. Normal table of Xenopus laevis (Daudin) : a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Garland Pub., New York.

Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M., Hirokawa, N., 1998. Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. Cell. 95**,** 829-37.

Nutt, S. L., Dingwell, K. S., Holt, C. E., Amaya, E., 2001. Xenopus Sprouty2 inhibits FGF-mediated gastrulation movements but does not affect mesoderm induction and patterning. Genes Dev. 15**,** 1152-66.

Okada, Y., Nonaka, S., Tanaka, Y., Saijoh, Y., Hamada, H., Hirokawa, N., 1999. Abnormal nodal flow precedes situs inversus in iv and inv mice. Mol Cell. 4**,** 459-68.

Ormestad, M., Astorga, J., Landgren, H., Wang, T., Johansson, B. R., Miura, N., Carlsson, P., 2006. Foxf1 and Foxf2 control murine gut development by limiting

mesenchymal Wnt signaling and promoting extracellular matrix production. Development. 133**,** 833-43.

Pabst, O., Schneider, A., Brand, T., Arnold, H. H., 1997. The mouse Nkx2-3 homeodomain gene is expressed in gut mesenchyme during pre- and postnatal mouse development. Dev Dyn. 209**,** 29-35.

Pandur, P., Lasche, M., Eisenberg, L. M., Kuhl, M., 2002. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature. 418**,** 636-41.

Papalopulu, N., Clarke, J. D., Bradley, L., Wilkinson, D., Krumlauf, R., Holder, N., 1991. Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in Xenopus embryos. Development. 113**,** 1145-58.

Park, E. J., Ogden, L. A., Talbot, A., Evans, S., Cai, C. L., Black, B. L., Frank, D. U., Moon, A. M., 2006. Required, tissue-specific roles for Fgf8 in outflow tract formation and remodeling. Development. 133**,** 2419-33.

Parrish, M., Ott, T., Lance-Jones, C., Schuetz, G., Schwaeger-Nickolenko, A., Monaghan, A. P., 2004. Loss of the Sall3 gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality. Mol Cell Biol. 24**,** 7102-12.

Pasquale, E. B., 1990. A distinctive family of embryonic protein-tyrosine kinase receptors. Proc Natl Acad Sci U S A. 87**,** 5812-6.

Peterkin, T., Gibson, A., Patient, R., 2003. GATA-6 maintains BMP-4 and Nkx2 expression during cardiomyocyte precursor maturation. EMBO J. 22**,** 4260-73.

Peterkin, T., Gibson, A., Patient, R., 2007. Redundancy and evolution of GATA factor requirements in development of the myocardium. Dev Biol. 311**,** 623-35.

Petkovich, M., Brand, N. J., Krust, A., Chambon, P., 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature. 330**,** 444-50.

Pham, V. N., Lawson, N. D., Mugford, J. W., Dye, L., Castranova, D., Lo, B., Weinstein, B. M., 2007. Combinatorial function of ETS transcription factors in the developing vasculature. Dev Biol. 303**,** 772-83.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., De Robertis, E. M., 1999. The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature. 397**,** 707-10.

Piccolo, S., Sasai, Y., Lu, B., De Robertis, E. M., 1996. Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell. 86**,** 589-98.

Poole, T. J., Finkelstein, E. B., Cox, C. M., 2001. The role of FGF and VEGF in angioblast induction and migration during vascular development. Dev Dyn. 220**,** 1-17.

Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. W., Orkin, S. H., 1996. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. Cell. 86**,** 47-57.

Reifers, F., Walsh, E. C., Leger, S., Stainier, D. Y., Brand, M., 2000. Induction and differentiation of the zebrafish heart requires fibroblast growth factor 8 (fgf8/acerebellar). Development. 127**,** 225-35.

Reiter, J. F., Verkade, H., Stainier, D. Y., 2001. Bmp2b and Oep promote early myocardial differentiation through their regulation of gata5. Dev Biol. 234**,** 330-8.

Ribes, V., Fraulob, V., Petkovich, M., Dolle, P., 2007. The oxidizing enzyme CYP26a1 tightly regulates the availability of retinoic acid in the gastrulating mouse embryo to ensure proper head development and vasculogenesis. Dev Dyn. 236**,** 644-53.

Ribes, V., Le Roux, I., Rhinn, M., Schuhbaur, B., Dolle, P., 2009. Early mouse caudal development relies on crosstalk between retinoic acid, Shh and Fgf signalling pathways. Development. 136**,** 665-76.

Riley, P., Anson-Cartwright, L., Cross, J. C., 1998. The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. Nat Genet. 18**,** 271-5.

Rones, M. S., McLaughlin, K. A., Raffin, M., Mercola, M., 2000. Serrate and Notch specify cell fates in the heart field by suppressing cardiomyogenesis. Development. 127**,** 3865-76.

Ryan, A. K., Blumberg, B., Rodriguez-Esteban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., de la Pena, J., Sabbagh, W., Greenwald, J., Choe, S., Norris, D. P., Robertson, E. J., Evans, R. M., Rosenfeld, M. G., Izpisua Belmonte, J. C., 1998. Pitx2 determines left-right asymmetry of internal organs in vertebrates. Nature. 394**,** 545-51.

Ryan, K., Garrett, N., Mitchell, A., Gurdon, J. B., 1996. Eomesodermin, a key early gene in Xenopus mesoderm differentiation. Cell. 87**,** 989-1000.

Sakuma, R., Ohnishi Yi, Y., Meno, C., Fujii, H., Juan, H., Takeuchi, J., Ogura, T., Li, E., Miyazono, K., Hamada, H., 2002. Inhibition of Nodal signalling by Lefty mediated through interaction with common receptors and efficient diffusion. Genes Cells. 7**,** 401-12.

Salanga, M. C., Meadows, S. M., Myers, C. T., Krieg, P. A., 2010. ETS family protein ETV2 is required for initiation of the endothelial lineage but not the hematopoietic lineage in the Xenopus embryo. Dev Dyn. 239**,** 1178-87.

Salic, A. N., Kroll, K. L., Evans, L. M., Kirschner, M. W., 1997. Sizzled: a secreted Xwnt8 antagonist expressed in the ventral marginal zone of Xenopus embryos. Development. 124**,** 4739-48.

Samuel, L. J., Latinkic, B. V., 2009. Early activation of FGF and nodal pathways mediates cardiac specification independently of Wnt/beta-catenin signaling. PLoS One. 4**,** e7650.

Sater, A. K., Jacobson, A. G., 1990. The restriction of the heart morphogenetic field in Xenopus laevis. Dev Biol. 140**,** 328-36.

Scharf, S. R., Gerhart, J. C., 1980. Determination of the dorsal-ventral axis in eggs of Xenopus laevis: complete rescue of uv-impaired eggs by oblique orientation before first cleavage. Dev Biol. 79**,** 181-98.

Schlange, T., Andree, B., Arnold, H. H., Brand, T., 2000. BMP2 is required for early heart development during a distinct time period. Mech Dev. 91**,** 259-70.

Schmerer, M., Evans, T., 2003. Primitive erythropoiesis is regulated by Smad-dependent signaling in postgastrulation mesoderm. Blood. 102**,** 3196-205.

Schoenebeck, J. J., Keegan, B. R., Yelon, D., 2007. Vessel and blood specification override cardiac potential in anterior mesoderm. Dev Cell. 13**,** 254-67.

Schultheiss, T. M., Lassar, A. B., 1997. Induction of chick cardiac myogenesis by bone morphogenetic proteins. Cold Spring Harb Symp Quant Biol. 62**,** 413-9.

Schultheiss, T. M., Xydas, S., Lassar, A. B., 1995. Induction of avian cardiac myogenesis by anterior endoderm. Development. 121**,** 4203-14.

Schweickert, A., Weber, T., Beyer, T., Vick, P., Bogusch, S., Feistel, K., Blum, M., 2007. Cilia-driven leftward flow determines laterality in Xenopus. Curr Biol. 17**,** 60-6.

Seghezzi, G., Patel, S., Ren, C. J., Gualandris, A., Pintucci, G., Robbins, E. S., Shapiro, R. L., Galloway, A. C., Rifkin, D. B., Mignatti, P., 1998. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. J Cell Biol. 141**,** 1659-73.

Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., Schwartz, R. J., 1998. GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. Mol Cell Biol. 18**,** 3405-15.

Shah, S. B., Skromne, I., Hume, C. R., Kessler, D. S., Lee, K. J., Stern, C. D., Dodd, J., 1997. Misexpression of chick Vg1 in the marginal zone induces primitive streak formation. Development. 124**,** 5127-38.

Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., Bernstein, A., Rossant, J., 1997. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell. 89**,** 981-90.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., Schuh, A. C., 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 376**,** 62-6.

Sharpe, C. R., 1991. Retinoic acid can mimic endogenous signals involved in transformation of the Xenopus nervous system. Neuron. 7**,** 239-47.

Sirbu, I. O., Duester, G., 2006. Retinoic-acid signalling in node ectoderm and posterior neural plate directs left-right patterning of somitic mesoderm. Nat Cell Biol. 8**,** 271-7.

Sirbu, I. O., Zhao, X., Duester, G., 2008. Retinoic acid controls heart anteroposterior patterning by down-regulating Isl1 through the Fgf8 pathway. Dev Dyn. 237**,** 1627-35.

Sive, H. L., Grainger, R. M., Harland, R. M. Eds.), 2000. Early Development of Xenopus Laevis: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Skromne, I., Stern, C. D., 2001. Interactions between Wnt and Vg1 signalling pathways initiate primitive streak formation in the chick embryo. Development. 128**,** 2915-27.

Smart, N., Hill, A. A., Cross, J. C., Riley, P. R., 2002. A differential screen for putative targets of the bHLH transcription factor Hand1 in cardiac morphogenesis. Mech Dev. 119 Suppl 1**,** S65-71.

Smith, J. C., Price, B. M., Green, J. B., Weigel, D., Herrmann, B. G., 1991. Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. Cell. 67**,** 79-87.

Smith, S. J., Kotecha, S., Towers, N., Latinkic, B. V., Mohun, T. J., 2002. XPOX2-peroxidase expression and the XLURP-1 promoter reveal the site of embryonic myeloid cell development in Xenopus. Mech Dev. 117**,** 173-86.

Smith, W. C., Harland, R. M., 1992. Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. Cell. 70**,** 829-40.

Smith, W. C., McKendry, R., Ribisi, S., Jr., Harland, R. M., 1995. A nodal-related gene defines a physical and functional domain within the Spemann organizer. Cell. 82**,** 37-46.

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

Spemann, H., 1938. Embryonic Development and Induction. New Haven, Yale University Press.

Spemann, H., Mangold, H., 1924. Über induktion von embryonalanlagen durch implantation artfremder organisatoren. Wilhelm Roux Arch. Entw. Mech. Org. 100**,** 599-638.

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

Stainier, D. Y., Weinstein, B. M., Detrich, H. W., 3rd, Zon, L. I., Fishman, M. C., 1995. Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. Development. 121**,** 3141-50.

Stavridis, M. P., Collins, B. J., Storey, K. G., 2010. Retinoic acid orchestrates fibroblast growth factor signalling to drive embryonic stem cell differentiation. Development. 137**,** 881-90.

Steinfeld, R., Van Den Berghe, H., David, G., 1996. Stimulation of fibroblast growth factor receptor-1 occupancy and signaling by cell surface-associated syndecans and glypican. J Cell Biol. 133**,** 405-16.

Stennard, F., Carnac, G., Gurdon, J. B., 1996. The Xenopus T-box gene, Antipodean, encodes a vegetally localised maternal mRNA and can trigger mesoderm formation. Development. 122**,** 4179-88.

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

Sudarwati, S., Nieuwkoop, P. D., 1971. Mesoderm formation in the anuran Xenopus-laevis. W. Roux' Arch. Ent. Org. 166**,** 189-204.

Sumanas, S., Gomez, G., Zhao, Y., Park, C., Choi, K., Lin, S., 2008. Interplay among Etsrp/ER71, Scl, and Alk8 signaling controls endothelial and myeloid cell formation. Blood. 111**,** 4500-10.

Sun, Y., Liang, X., Najafi, N., Cass, M., Lin, L., Cai, C. L., Chen, J., Evans, S. M., 2007. Islet 1 is expressed in distinct cardiovascular lineages, including pacemaker and coronary vascular cells. Dev Biol. 304**,** 286-96.

Sweetman, D., Smith, T. G., Farrell, E. R., Munsterberg, A., 2005. Expression of csal1 in pre limb-bud chick embryos. Int J Dev Biol. 49**,** 427-30.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C. C., Lin, X., Heasman, J., 2005. Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. Cell. 120**,** 857-71.

Tashiro, S., Sedohara, A., Asashima, M., Izutsu, Y., Maeno, M., 2006. Characterization of myeloid cells derived from the anterior ventral mesoderm in the Xenopus laevis embryo. Dev Growth Differ. 48**,** 499-512.

Teng, M., Duong, T. T., Johnson, A. T., Klein, E. S., Wang, L., Khalifa, B., Chandraratna, R. A., 1997. Identification of highly potent retinoic acid receptor alpha-selective antagonists. J Med Chem. 40**,** 2445-51.

Thompson, M. A., Ransom, D. G., Pratt, S. J., MacLennan, H., Kieran, M. W., Detrich, H. W., 3rd, Vail, B., Huber, T. L., Paw, B., Brownlie, A. J., Oates, A. C., Fritz, A., Gates, M. A., Amores, A., Bahary, N., Talbot, W. S., Her, H., Beier, D. R., Postlethwait, J. H., Zon, L. I., 1998. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. Dev Biol. 197**,** 248-69.

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

Tonissen, K. F., Drysdale, T. A., Lints, T. J., Harvey, R. P., Krieg, P. A., 1994. XNkx-2.5, a Xenopus gene related to Nkx-2.5 and tinman: evidence for a conserved role in cardiac development. Dev Biol. 162**,** 325-8.

Tran, H. T., Sekkali, B., Van Imschoot, G., Janssens, S., Vleminckx, K., 2010. Wnt/beta-catenin signaling is involved in the induction and maintenance of primitive hematopoiesis in the vertebrate embryo. Proc Natl Acad Sci U S A. 107**,** 16160-5.

Tseng, H. T., Shah, R., Jamrich, M., 2004. Function and regulation of FoxF1 during Xenopus gut development. Development. 131**,** 3637-47.

Ullrich, A., Schlessinger, J., 1990. Signal transduction by receptors with tyrosine kinase activity. Cell. 61**,** 203-12.

Van Wauwe, J. P., Coene, M. C., Goossens, J., Van Nijen, G., Cools, W., Lauwers, W., 1988. Ketoconazole inhibits the in vitro and in vivo metabolism of all-trans-retinoic acid. J Pharmacol Exp Ther. 245**,** 718-22.

van Wijk, B., van den Berg, G., Abu-Issa, R., Barnett, P., van der Velden, S., Schmidt, M., Ruijter, J. M., Kirby, M. L., Moorman, A. F., van den Hoff, M. J., 2009. Epicardium and myocardium separate from a common precursor pool by crosstalk between bone morphogenetic protein- and fibroblast growth factor-signaling pathways. Circ Res. 105**,** 431-41.

Vincent, J. P., Oster, G. F., Gerhart, J. C., 1986. Kinematics of gray crescent formation in Xenopus eggs: the displacement of subcortical cytoplasm relative to the egg surface. Dev Biol. 113**,** 484-500.

Vincentz, J. W., Barnes, R. M., Firulli, B. A., Conway, S. J., Firulli, A. B., 2008. Cooperative interaction of Nkx2.5 and Mef2c transcription factors during heart development. Dev Dyn. 237**,** 3809-19.

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

Walmsley, M., Cleaver, D., Patient, R., 2008. Fibroblast growth factor controls the timing of Scl, Lmo2, and Runx1 expression during embryonic blood development. Blood. 111**,** 1157-66.

Walters, M. J., Wayman, G. A., Christian, J. L., 2001. Bone morphogenetic protein function is required for terminal differentiation of the heart but not for early expression of cardiac marker genes. Mech Dev. 100**,** 263-73.

Warkany, J., Schraffenberger, E., 1946. Congenital malformations induced in rats by maternal vitamin A deficiency. I. Defects of the eye. Arch. Ophth. 35**,** 150-169.

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

Waxman, J. S., Keegan, B. R., Roberts, R. W., Poss, K. D., Yelon, D., 2008. Hoxb5b acts downstream of retinoic acid signaling in the forelimb field to restrict heart field potential in zebrafish. Dev Cell. 15**,** 923-34.

Weaver, C., Farr, G. H., 3rd, Pan, W., Rowning, B. A., Wang, J., Mao, J., Wu, D., Li, L., Larabell, C. A., Kimelman, D., 2003. GBP binds kinesin light chain and translocates during cortical rotation in Xenopus eggs. Development. 130**,** 5425-36.

Weaver, C., Kimelman, D., 2004. Move it or lose it: axis specification in Xenopus. Development. 131**,** 3491-9.

Weston, A. D., Blumberg, B., Underhill, T. M., 2003. Active repression by unliganded retinoid receptors in development: less is sometimes more. J Cell Biol. 161**,** 223-8.

White, J. A., Heasman, J., 2008. Maternal control of pattern formation in Xenopus laevis. J Exp Zool B Mol Dev Evol. 310**,** 73-84.

White, R. J., Nie, Q., Lander, A. D., Schilling, T. F., 2007. Complex regulation of cyp26a1 creates a robust retinoic acid gradient in the zebrafish embryo. PLoS Biol. 5**,** e304.

Williams, E. J., Furness, J., Walsh, F. S., Doherty, P., 1994a. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron. 13**,** 583-94.

Williams, E. J., Furness, J., Walsh, F. S., Doherty, P., 1994b. Characterisation of the second messenger pathway underlying neurite outgrowth stimulated by FGF. Development. 120**,** 1685-93.

Wills, A. E., Choi, V. M., Bennett, M. J., Khokha, M. K., Harland, R. M., 2010. BMP antagonists and FGF signaling contribute to different domains of the neural plate in Xenopus. Dev Biol. 337**,** 335-50.

Wilson, J. G., Roth, C. B., Warkany, J., 1953. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. Am J Anat. 92**,** 189-217.

Wilson, J. G., Warkany, J., 1947a. Anomalies of the genito-urinary tract induced by maternal vitamin A deficiency in fetal rats. Anat Rec. 97**,** 376.

Wilson, J. G., Warkany, J., 1947b. Epithelial keratinization as evidence of fetal vitamin A deficiency. Proc Soc Exp Biol Med. 64**,** 419-22.

Wilson, J. G., Warkany, J., 1948. Malformations in the genito-urinary tract induced by maternal vitamin A deficiency in the rat. Am J Anat. 83**,** 357-407.

Wilson, J. G., Warkany, J., 1949. Aortic-arch and cardiac anomalies in the offspring of vitamin A deficient rats. Am J Anat. 85**,** 113-55.

Wilson, J. G., Warkany, J., 1950. Congenital anomalies of heart and great vessels in offspring of vitamin A-deficient rats. Am J Dis Child. 79**,** 963.

Xu, K., Chong, D. C., Rankin, S. A., Zorn, A. M., Cleaver, O., 2009. Rasip1 is required for endothelial cell motility, angiogenesis and vessel formation. Dev Biol. 329**,** 269-79.

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

Yamauchi, H., Hotta, Y., Konishi, M., Miyake, A., Kawahara, A., Itoh, N., 2006. Fgf21 is essential for haematopoiesis in zebrafish. EMBO Rep. 7**,** 649-54.

Yarden, Y., Ullrich, A., 1988. Growth factor receptor tyrosine kinases. Annu Rev Biochem. 57**,** 443-78.

Yatskievych, T. A., Ladd, A. N., Antin, P. B., 1997. Induction of cardiac myogenesis in avian pregastrula epiblast: the role of the hypoblast and activin. Development. 124**,** 2561-70.

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

Zeynali, B., Dixon, K. E., 1998. Effects of retinoic acid on the endoderm in Xenopus embryos. Dev Genes Evol. 208**,** 318-26.

Zhang, J., Houston, D. W., King, M. L., Payne, C., Wylie, C., Heasman, J., 1998. The role of maternal VegT in establishing the primary germ layers in Xenopus embryos. Cell. 94**,** 515-24.

Zhang, J., King, M. L., 1996. Xenopus VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning. Development. 122**,** 4119-29.

Zhao, R., Watt, A. J., Battle, M. A., Li, J., Bondow, B. J., Duncan, S. A., 2008. Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. Dev Biol. 317**,** 614-9.

Zhao, R., Watt, A. J., Li, J., Luebke-Wheeler, J., Morrisey, E. E., Duncan, S. A., 2005. GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. Mol Cell Biol. 25**,** 2622-31.

Zhao, X., Duester, G., 2009. Effect of retinoic acid signaling on Wnt/beta-catenin and FGF signaling during body axis extension. Gene Expr Patterns. 9**,** 430-5.

Zhao, X., Sirbu, I. O., Mic, F. A., Molotkova, N., Molotkov, A., Kumar, S., Duester, G., 2009. Retinoic acid promotes limb induction through effects on body axis extension but is unnecessary for limb patterning. Curr Biol. 19**,** 1050-7.

Zimmerman, L. B., De Jesus-Escobar, J. M., Harland, R. M., 1996. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell. 86**,** 599-606.

# Vita

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Platform Presentations

**Deimling, S.J.,** Drysdale, T.A. (2010). "Patterning the *Xenopus* lateral plate mesoderm." 13th International *Xenopus* Conference; Lake Louse, Alberta, Canada.

**Deimling, S.J.**, Drysdale, T.A. (2009). "Retinoic acid and FGF signals pattern the LPM in the post-gastrula *Xenopus* embryo." Department of Paediatrics Research Day; University of Western Ontario, Canada.
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**Deimling, S.J.**, Drysdale, T.A. (2008). "Retinoic acid patterns the lateral plate mesoderm in the post-gastrula *Xenopus* embryo. 4th Canadian Developmental Biology Conference; Banff, Alberta, Canada.
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**Deimling, S.J.**, Drysdale, T.A. (2007). "Patterning of the lateral plate mesoderm by retinoic acid." Paediatrics Research Day; University of Western Ontario. .

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Poster Presentations

**Deimling, S.J.**, Drysdale, T.A. (2010) "Retinoic Acid and FGF Signals Pattern the LPM in the Post Gastrula *Xenopus* Embryo." 5th Canadian National Developmental Biology Meeting; Mont-Tremblant, Quebec, Canada.

**Deimling, S.J.**, and Drysdale, T.A. (2009). "Retinoic acid and FGF signals pattern the LPM in the post-gastrula *Xenopus* embryo." 16th International Society of Developmental Biologists Congress; Edinburgh, Scotland, UK.

**Deimling, S.J.**, Drysdale T.A. (2009). "Opposing Retinoic acid and FGF signals pattern the early *Xenopus* LPM." Great Lakes Mammalian Development Meeting; Toronto, Ontario, Canada.

**Deimling, S.J.**, Drysdale T.A. (2007). "Retinoic acid signaling in anterior-posterior patterning during regional specification of the *Xenopus* gut." Great Lakes Mammalian Development Meeting; Toronto, Ontario, Canada.

**Deimling, S.J.**, Yong, Z., Drysdale, T.A. (2006). "Patterning of the lateral plate mesoderm in *Xenopus* by retinoic acid." 3rd Canadian Developmental Biology Meeting; Mont-Tremblant, Quebec, Canada.

**Deimling, S.J.**, Yong, Z., Drysdale, T.A. (2006). "Retinoic acid influences anterior-posterior patterning in the lateral mesoderm." Great Lakes Mammalian Development Meeting; Toronto, Ontario, Canada.

Yong, Z., **Deimling, S.**, Kerkhof, J., Soeder, S., Wang, J.H., Andrews, J., Carter, D., Drysdale, T.A. (2005). "Identification of retinoic acid responsive genes in the early *Xenopus* embryo." Great Lakes Mammalian Development Meeting; Toronto, Ontario, Canada.

**Publications:**

**Deimling, SJ.** and Drysdale, T.A. (2011) FGF is required to regulate anterior-posterior patterning in the *Xenopus* lateral plate mesoderm. Mech. Dev. In revision: MOD-1111.

**Deimling, S.J.** and Drysdale, T.A. (2009) Retinoic acid regulates anterior-posterior patterning within the lateral plate mesoderm of *Xenopus*. Mech. Dev. **126**, 913-923.

Collop, A.H., Broomfield, J.A., Chandraratna, R.A., Yong, Z., **Deimling, S.J.**, Kolker, S.J., Weeks, D. L., Drysdale, T. A. (2006) Retinoic acid signalling is essential for formation of the heart tube in *Xenopus*. Dev. Biol**. 291**, 96-109.