

Notch signalling in *Xenopus laevis* haematopoietic stem cell programming

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**‘Our real teacher has been and still is the embryo, who is, incidentally, the only teacher
who is always right’**

- Viktor Hamburger

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Abbreviations

AGM	aorta-gonad-mesonephros
ATG	translation start site
BLAST	basic local alignment tool
Bp	base pair
CSL	CBF1/suppressor of hairless/Lag1
cDNA	complementary deoxyribonucleic acid
DA	dorsal aorta
DIG	digoxigenin
DLP	dorsal lateral plate mesoderm
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetate
EGF	epidermal growth factor
EST	expressed sequence tag
EtOH	ethanol
GFP	green fluorescent protein
HCG	human chorionic gonadotropin
HSC	haematopoietic stem cell
ISS	<i>in situ</i> hybridisation on sections
ISV	intersomitic vessels
LNR	lin-12-notch repeats
MeOH	methanol
MO	morpholino oligonucleotide
NCBI	National Centre for Biotechnology Information
NECD	notch extracellular domain

NICD	notch intracellular domain
NO	nitric oxide
NRR	negative regulatory region
PCR	polymerase chain reaction
PCV	posterior cardinal vein
P-Sp	paraaortic splanchnopleural mesoderm
RACE	rapid amplification of cDNA ends
St	Nieuwkoop Faber stage
VBI	ventral blood island
VEGF	vascular endothelial growth factor
VMZ	ventral marginal zone
WISH	whole mount <i>in situ</i> hybridisation
WT	wild type

Abstract

Notch signalling in *Xenopus laevis* haematopoietic stem cell programming

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Multipotent haematopoietic stem cells (HSCs) originate in the dorsal aorta (DA) during vertebrate embryogenesis, and after migrating to a permanent niche, give rise to a continuous supply of mature blood cells of all lineages throughout adult life. Previous cell tracing experiments have shown that the cells of the DA migrate here from an early collection of haemangioblasts (bipotent precursors of blood and endothelial cells) which reside in the dorsolateral plate (DLP) mesoderm. Development of HSCs is tightly regulated by a number of key signalling pathways in both the DLP and the DA. In particular, notch signalling is considered an important factor in vascular, arterial and HSC development. Here, the relatively slow development and the spatial separation of definitive haematopoiesis from primitive haematopoiesis in *Xenopus laevis* has been exploited to reveal the first defect of reduced notch signalling in the *Xenopus* DA.

Two notch inputs to HSC programming have been identified in *Xenopus*: *notch4* and its target genes, *esr7* and *esr10*, are expressed from stage 31, immediately after migrating haemangioblast cells reach the midline of the embryo to form the DA, whilst *notch1* is expressed slightly later, from stage 34, and controls expression of two further notch target genes, *esr1* and *hesr1*. Using both morpholino knockdown of these six genes, and chemical inhibition of notch signalling using a specific γ -secretase inhibitor, notch signalling has been demonstrated to be essential for HSC programming but dispensable for earlier haemangioblast and arterial programming. Furthermore, *esr1*, downstream of both *notch1* and *notch4*, is shown to be responsible for repression of endothelial genes in the DA. Taken together, this demonstrates that a cascade of notch and notch effector genes are essential for the programming of *Xenopus* HSCs.

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Chapter 1. Introduction

Development of an embryonic vascular system and a functional haematopoietic system is fundamentally important to the life of a higher vertebrate so nutrients and oxygen can be effectively delivered to all cells in the organism, and waste products removed, allowing them to develop fully. The cardiovascular systems, including primitive blood, the heart, the dorsal aorta and a vascular system connecting these to the rest of the body, are laid down from early mesodermal precursors and importantly are the first organ systems to fully develop in an embryo.

1.1. Haematopoiesis

The haematopoietic system comprises many different cell types which circulate around the body via the vasculature, performing varied, and essential, functions throughout life. These mature, functional, blood cells have limited lifespans so must be constantly replaced to maintain the life of an organism. As such, haematopoiesis continues throughout life.

It is understood that adult haematopoietic cells derive from a population of multipotent haematopoietic stem cells (HSCs). A complex system, haematopoiesis is characterised as the production of all mature blood lineages from HSCs via several different intermediate progenitors, whose potentials are progressively restricted, and the processes by which these lineages develop into fully functional cells. The mechanisms specifying the development of these mature haematopoietic cells from HSCs are relatively well documented. However, the development of HSCs in an embryo is less well defined, and is currently an area of intense research. The ability of an HSC to generate all blood lineages is of great clinical significance as understanding the processes by which HSCs are formed, expanded and differentiated could provide therapy for haematological diseases.

1.2. The HSC

High dose irradiation leads to death of an organism through various mechanisms, including the failure of bone marrow. Over 60 years ago, the pursuit of cells capable of protecting humans exposed to lethal doses of irradiation brought about the concept of the HSC. Shielding haematopoietic organs such as the spleen or the femur (containing bone marrow), or intravenous injection of healthy bone marrow post-irradiation could protect from the lethal effects of irradiation. This led to the notion of a humoral or cellular component in the spleen and bone marrow which could stimulate haematopoietic recovery (reviewed in Little and Storb, 2002). Further investigations demonstrated that intravenous injection of highly proliferative cells found in the bone marrow resulted in the generation of large colonies of haematopoietic cells in the spleen of irradiated hosts 8-12 days post-transplantation, including erythroid, myeloid and megakaryocytic lineages (Till and McCulloch, 1961). This suggested the haematopoietic potential was conferred by a cellular, rather than humoral, component. Nodules on the spleen of recipients, referred to as spleen colonies, were found to occur in proportion to the number of cells injected, and so were proposed to each originate from a single injected cell. These spleen colony forming units (CFU-S) were clonal in origin, and had the capacity to be re-transplanted into a subsequent irradiated host where they would again form CFU-S, and thus had the ability to self-renew (Becker et al., 1963; Curry and Trentin, 1967; Siminovitch et al., 1963; Wu et al., 1967). Together, the observation that these cells had the capacity to self-renew with the prior observation of erythroid, myeloid and megakaryocytic lineages arising, these CFU-S cells were believed to be exhibiting multipotency, and could therefore be HSCs. Current opinion is that whilst HSCs can give rise to CFU-S cells via an intermediate progenitor, the pre-CFU-S, they are too primitive to do so within the 12 days used in the original CFU-S assay (Hodgson and Bradley, 1979; Spangrude and Johnson, 1990). More recently, the ability of the CFU-S cells to give long-term reconstitution of haematopoietic cells in irradiated mice has been called into question,

reinforcing the idea that the CFU-S may represent a multipotent derivative of the HSC (reviewed in Bellantuono, 2004; Na Nakorn et al., 2002; Osawa et al., 1996).

Direct evidence for the existence of HSCs came about during the 1990s. Advancing techniques, such as fluorescence activated cell sorting (FACS) using cell surface markers, enabled the purification of HSCs (Baum et al., 1992; Greaves et al., 1981; Spangrude et al., 1988). HSCs are characterised as expressing Sca1 and c-Kit highly on the cell surface whilst having no cell surface markers normally present on differentiated haematopoietic lineages. These cells are known as the LSK population (Lin-/Sca-1+/c-Kit+) (Okada et al., 1992; Spangrude et al., 1988). In addition to FACS, several other factors have been used to identify LT-HSCs including size, morphology and immunohistochemistry (Junqueira, 1975) and uptake of rhodamine-123 (Spangrude and Johnson, 1990).

Using single retroviral labelled murine foetal liver cells, long-term reconstitution of the whole haematopoietic system in irradiated mice was demonstrated, providing the first functional evidence of the HSC (Harrison et al., 1993; Jordan and Lemischka, 1990; Jordan et al., 1990; Keller and Snodgrass, 1990; Lemischka, 1991). The HSC is now defined as a cell that has the capacity to self-renew whilst providing long-term, high-level repopulation of all haematopoietic lineages when transplanted into lethally irradiated hosts (Lemischka, 1991; Matsuzaki et al., 2004; Osawa et al., 1996; Spangrude et al., 1991). The current definitive test of HSC activity is the *in vivo* long-term repopulation (LTR) assay (Medvinsky and Dzierzak, 1998; Muller et al., 1994). Donor cells are intravenously injected into lethally irradiated murine hosts and haematopoietic reconstitution is analysed six months post-transplantation. Serial transplantation of these cells into a subsequent irradiated host assays the ability of the cells to self-renew. Two populations of HSCs have been identified by the LTR assay; the first population is capable of reconstituting the host for a

minimum of six months, and is therefore capable of long-term repopulation (long-term repopulating haematopoietic stem cells, or LT-HSC). The second population however are only capable of reconstituting the host for 3-4 months, and have a limited ability to reconstitute a subsequent host, and so are defined as short-term repopulating haematopoietic stem cells (ST-HSC) (Adolfsson et al., 2001; Adolfsson et al., 2005; Morrison and Weissman, 1994). LT-HSCs are considered extremely rare, comprising 1-10 in 100,000 cells in adult bone marrow (Harrison et al., 1988; Harrison et al., 1993). Measuring HSC activity using the LTR assay not only requires cells to have a multipotential capacity to repopulate the haematopoietic system whilst also self-renewing, it also depends on the ability of the cells to home to, and seed, the appropriate haematopoietic tissue. Thus, the ability of the HSC to locate the haematopoietic niche is crucial for function (Bellantuono, 2004).

A recent model for HSC lineage restriction has been proposed (Adolfsson et al., 2005, and references therein, and figure 1.1). This composite model proposes that HSCs follow stepwise lineage restrictions to more committed haematopoietic progenitors. An initial binary decision is made by HSCs during their lineage restriction to become either a myeloid restricted 'common myeloid progenitor' (CMP) or a 'lymphoid primed multipotent progenitor' (LMPP). This LMPP can follow any lymphoid lineage or can form a more restricted myeloid precursor known as the granulocyte/macrophage progenitor (GMP).

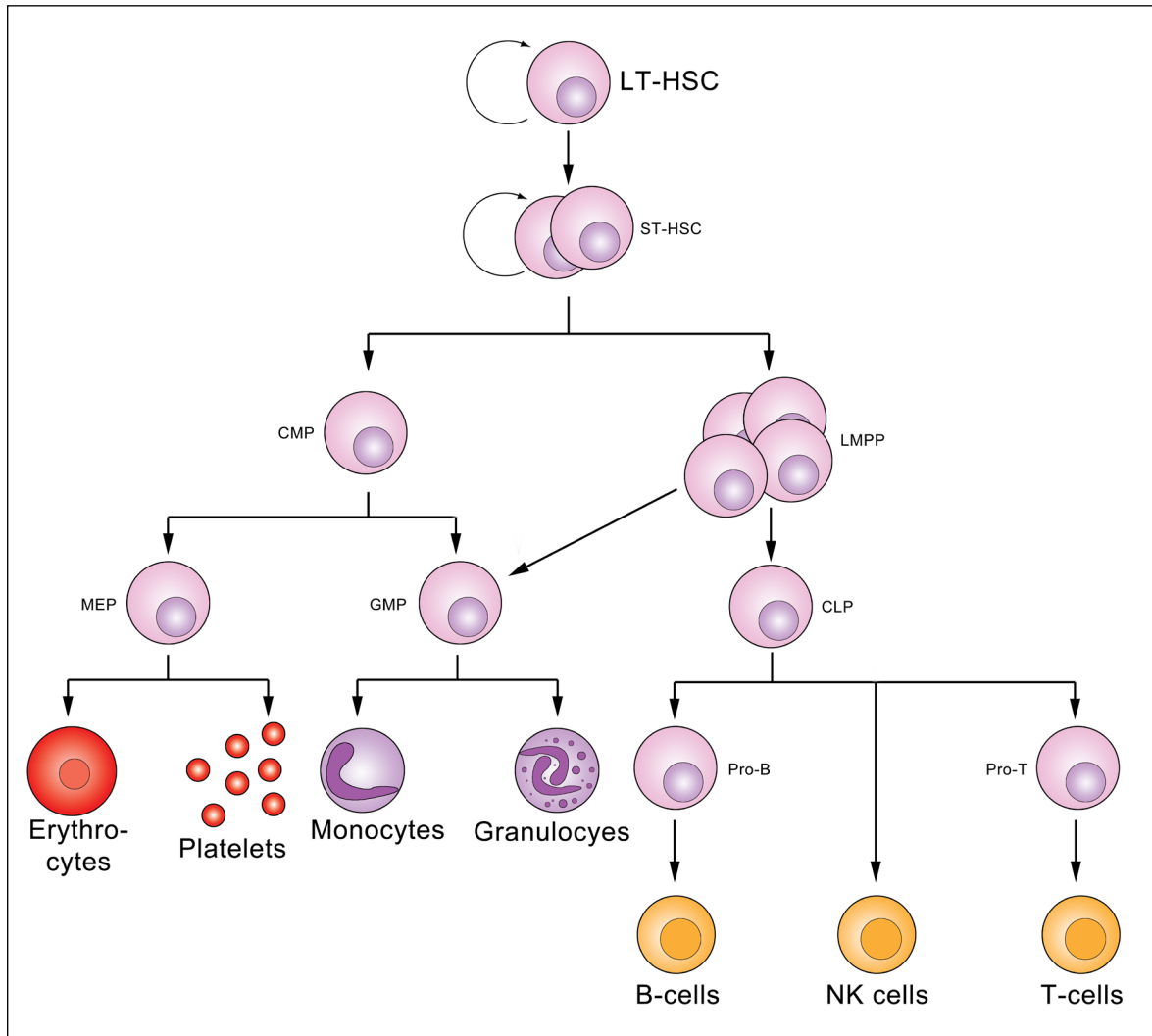


Figure 1.1 The haematopoietic lineage restriction pathways

A schematic diagram depicting the composite model of HSC lineage restriction. **Abbreviations:** LT-HSC – long-term repopulating haematopoietic stem cell; ST-HSC – short-term repopulating haematopoietic stem cell; CMP – common myeloid progenitor; LMPP – lymphoid-primed multipotent progenitor; MEP – myeloerythroid progenitor; GMP – granulocyte/macrophage progenitor; CLP – common lymphoid progenitor (Figured generated using data from Adolfsson et al., 2005).

1.3. The origins of haematopoiesis

Vertebrate haematopoiesis occurs in two distinct waves to facilitate the development of the embryo. The first transient, primitive, wave occurs in the murine extraembryonic yolk sac and establishes the development of primitive erythrocytes and macrophages needed to support the embryo during growth. The large nucleated erythrocytes circulate around the body facilitating oxygen delivery and carbon dioxide removal from cells around the embryo. A second wave of haematopoiesis, the definitive wave, occurs within the embryo and gives rise to HSCs. These HSCs enter circulation to seed the foetal liver before seeding the thymus, spleen and then bone marrow. HSCs will produce all blood lineages required to support the developing foetus and to sustain the adult throughout life.

1.3.1. Primitive haematopoiesis

In mouse, primitive haematopoiesis initiates in the presumptive blood islands within the extraembryonic yolk sac (YS) at embryonic day (E) 7-7.5, with the first primitive erythrocytes evident by E8 (Silver and Palis, 1997). In human embryos, primitive haematopoiesis initiates at E17 within the extraembryonic yolk-sac (YS) (reviewed in Godin and Cumano, 2002) and in chickens this happens in the avian yolk sac (aYS) (Dieterlen-Lievre, 1975). In lower vertebrates which do not have yolk sacs such as *Xenopus* and zebrafish, primitive haematopoiesis occurs in analogous structures called the ventral blood island (VBI) (Ciau-Uitz et al., 2000) and the intermediate cell mass (ICM) (Al-Adhami and Kunz, 1977), respectively.

The mammalian yolk sac is an extraembryonic bi-layer structure comprised of extraembryonic mesoderm and visceral endoderm. Fate mapping of primitive streak cells has shown that mesodermal cells of the yolk sac may be specified and allocated at different times, suggesting endothelial and blood lineages come from separate progenitor populations, rather than a

haemangioblast progenitor (reviewed in Ferkowicz and Yoder, 2005; Kinder et al., 1999). Furthermore, lineage labelling has shown that contribution by individual haemangioblast progenitors to both haematopoietic and endothelial lineages within a blood island is very rare (Ueno and Weissman, 2006). However, both populations have been shown to arise from *flk1*⁺ mesoderm (Lugus et al., 2009). Mesodermal cells responding to inductive signals from the neighbouring endoderm differentiate into endothelial precursor cells at the border, known as angioblasts. Inner mesodermal cells aggregate to form blood islands before differentiating into primitive nucleated erythrocytes (Bielinska et al., 1996; Cumano and Godin, 2007; Haar and Ackerman, 1971; Moore and Metcalf, 1970; Palis et al., 2010, and figure 1.2).

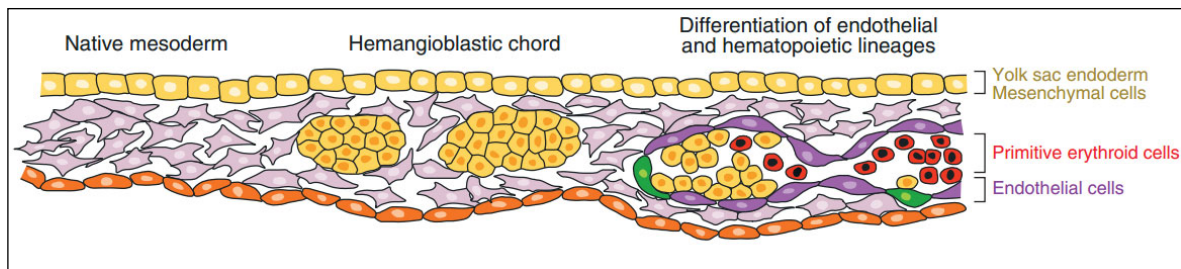


Figure 1.2 Formation of the yolk sac blood islands

Schematic diagram showing blood island formation within the yolk sac. Mesodermal cells can be seen developing into endothelial cells surrounding developing primitive erythrocytes (adapted from Cumano and Godin, 2007).

These large nucleated primitive erythrocytes express embryonic haemoglobin, a protein adapted to maximise the exchange of oxygen in the low-oxygen environment of a developing embryo (Palis et al., 1999). In addition to the erythrocytes, primitive macrophages are also developed in the blood islands to aid tissue remodelling. These macrophages are physiologically different from their definitive counterparts; bypassing the monocyte stage during differentiation and producing different enzymes (Cline and Moore, 1972; Naito et al., 1996). Some megakaryocytes have also been identified in the blood islands, exhibiting an accelerated production of platelets to prevent

haemorrhaging (Xu et al., 2001). In addition to the physiological differences between these primitive blood cells and their definitive counterparts, the primitive cells develop and mature more quickly in order to support the increasing metabolic requirements of the embryo.

1.3.2. Definitive haematopoiesis and the origin of the HSC

The second wave of haematopoiesis, definitive haematopoiesis, occurs within the embryo proper. In the murine embryo, the fetal liver is the major haematopoietic organ. In avian embryos, the bursa of Fabricius and thymus are the major haematopoietic organs whilst in *Xenopus* and zebrafish, definitive haematopoietic progenitors reside within the liver and kidney, respectively. Definitive haematopoiesis at these sites is initiated by haematopoietic stem cells which seed these haematopoietic organs. The origin of these HSCs has been intensely investigated over the last 40 years.

The mammalian YS was observed to be the primary site of haematopoiesis, and so proposals were put forward suggesting that the primary haematopoietic cells of the YS migrated via embryonic circulation to seed the fetal liver, and subsequently the bone marrow, where they would give rise to all adult blood lineages (Moore and Metcalf, 1970). These early experiments showed that the YS contained primitive erythroid progenitors, erythromyeloid progenitors, CFU-S cells and HSCs at different times in development, and thus a model was generated that the microenvironment of either the YS or fetal liver determined the potential of these haematopoietic progenitors. When transplanted directly into newborn mouse livers, E9.0 yolk sac cells could reconstitute primary recipients for at least 11 months, and secondary recipients for at least six months (Yoder et al., 1997). Whilst this suggests yolk sac derived cells have potential HSC activity, the inability of these cells to reconstitute lethally-irradiated adult mice questions the observation that these are HSCs. Lineage tracing has shown that *runx1*-expression yolk sac cells

can migrate and colonize the umbilical cord, the AGM region, and subsequently the embryonic liver (Samokhvalov et al., 2007), whilst temporal control of *runx1* expression suggests an exclusive extraembryonic mesodermal source of HSCs (Tanaka et al., 2012).

Experiments involving the grafting of a chicken yolk-sac onto a quail embryo revealed that definitive haematopoietic cells were of quail origin. This revealed that definitive haematopoiesis occurred at an intra-embryonic site, and thus primitive and definitive haematopoietic cells arose in distinct locations (Dieterlen-Lievre, 1975). Further avian grafting experiments confirmed an intra-embryonic site of definitive HSC production, and suggested the ventral wall of the DA as a likely source for the production of these definitive HSCs (Dieterlen-Lievre and Martin, 1981). Similar grafting experiments carried out using amphibian embryos also demonstrated the intra-embryonic origin of definitive haematopoietic cells, the dorsolateral plate mesoderm (DLP) (Maeno et al., 1985; Turpen et al., 1981). Later lineage labelling experiments in *Xenopus* finally confirmed this claim (Ciau-Uitz et al., 2000). Due to *in utero* development, it was not possible to do these types of grafting experiments using murine embryos.

As the dual origins of haematopoiesis in both avian and amphibian embryos was accepted, many studies began looking in depth at murine intra-embryonic haematopoietic sites, including the paraaortic splanchnopleural mesoderm (P-Sp) and its proposed derivative, the aorta-gonad-mesonephros (AGM). A subset of haematopoietic progenitors with B lymphoid potential was identified within the P-Sp, and subsequently in the YS (Cumano et al., 1993). The E9.0 aortic region was found to have B-lymphocyte potential following transplantation to irradiated hosts whilst YS precursors could not (Godin et al., 1993). CFU-S cells were also identified in the AGM at E9.0, at a much higher rate than those in the YS (Medvinsky et al., 1993). Later studies indicated that after E10.5 the AGM was capable of long-term reconstitution, again suggesting the AGM as

the origin of intra-embryonic HSCs (Medvinsky and Dzierzak, 1996; Muller et al., 1994). As murine circulation initiates at around E8.5 it was still possible that these intra-embryonic haematopoietic cells were seeded from the YS. As grafting studies similar to those undertaken in the amphibian were not possible for murine embryos, cultured pre and post-circulation AGM regions were studied and revealed that definitive HSCs autonomously develop in the AGM of the murine embryo (Cumano et al., 1996; Medvinsky and Dzierzak, 1996). Characterisation of the AGM region in human embryos, including the transplantation of human AGM cells to NOD-SCID immune deficient mouse thymuses, demonstrated the existence of intra-embryonic haematopoietic stem cells in humans (Tavian et al., 1996; Tavian et al., 1999; Tavian et al., 2001). In parallel to the AGM region, HSCs have been found to emerge in the head vasculature of E10.5-11.5 mouse embryos, suggesting the possibility of multiple intraembryonic origins of HSCs (Li et al., 2012).

Although the YS is currently not believed to be the site for definitive HSC emergence, it has been associated with a transient wave of definitive progenitors occurring after the emergence of the primitive haematopoietic cells but before the emergence of HSCs in the AGM (Palis et al., 2001; Palis et al., 1999; Yoshimoto et al., 2012). High proliferative potential colony forming cells (HPP-CFCs) have been identified using *in vitro* assays, demonstrating a YS multipotent progenitor able to generate erythroid and myeloid lineages (Palis et al., 2001; Palis et al., 1999). An analogous population of multipotent progenitors, termed erythromyeloid progenitors (EMPs) has also been identified in zebrafish, in the posterior blood island (PBI), arising independently of HSCs (Bertrand et al., 2007). Studies using circulation defective murine embryos also demonstrates that mice develop transient haematopoietic cell progenitors in the YS (Lux et al., 2008). It is possible that, although the YS does not give rise to definitive HSCs, these multipotential progenitors may develop into definitive HSCs once in a permissive microenvironment, such as the fetal liver or

AGM. However, early lethality of these embryos prevented analysis of AGM HSCs, and thus whether the precursors of HSCs developed in the YS or not remained to be demonstrated.

The description of haematopoietic clusters in the AGM was first described in the avian embryo (Dieterlen-Lievre and Martin, 1981), followed by mouse (Garcia-Porrero et al., 1995), human (Tavian et al., 1996), zebrafish (Thompson et al., 1998) and *Xenopus* (Ciau-Uitz et al., 2000). More in depth studies into the ventral wall of the DA and HSC cluster emergence in avian embryos demonstrated the presence of multipotent haematopoietic progenitors at this location (Cormier and Dieterlen-Lievre, 1988). Further analysis and functional studies have demonstrated that HSC activity is found within these clusters associated with the ventral wall of the DA and indicate that these haematopoietic clusters correspond to the translocation of newly formed HSCs into circulation (de Bruijn et al., 2002; Godin et al., 1999; North et al., 1999; North et al., 2002; Taoudi and Medvinsky, 2007). Following emergence from the ventral wall of the DA, these HSCs enter circulation to seed the appropriate haematopoietic tissues. Using limiting dilution experiments, it was estimated that only 3 HSCs were present in an E11 embryo but that this number massively increased over a 24 hour period to reach approximately 70 (Kumaravelu et al., 2002). Kumaravelu proposed that due to the average length of a cell cycle, this increase in HSCs could not be due to newly replicated cells, but instead proposed that this increase was due to the maturation of pre-HSCs to a definitive state. Subsequent studies have identified haematopoietic progenitor cells within the placenta, and revealed that the placental vasculature is capable of autonomously generating HSCs, or allowing the expansion of AGM derived HSCs (Alvarez-Silva et al., 2003; Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Rhodes et al., 2008; Zovein et al., 2008). As placental haematopoietic sites were not investigated during the HSC quantification study, it remains possible that the placenta may contribute more circulating HSCs than the AGM (reviewed in Medvinsky et al., 2011). Recent studies using three-dimensional visualisation of transparent

whole mouse embryos has identified the association of haematopoietic progenitors with the endothelial wall of the DA, the umbilical artery (UA) and the vitelline artery (VA) (Yokomizo and Dzierzak, 2010). Clusters were found to emerge first at E9 and continue until E14, with the cluster cell number peaking at E10.5, the same time that functional HSCs are produced (Yokomizo and Dzierzak, 2010). Interestingly, high numbers of c-Kit⁺ haematopoietic progenitor cells were identified, with an average of 578 identified in an E10.5 embryo and 445 in an E11.5 embryo, a number vastly higher than the predicted number of HSCs in the mouse at E11, as determined by limiting dilution assays, thus it has been estimated that as few as 0.2% of c-Kit⁺ haematopoietic progenitor cells would be functional HSCs (Kumaravelu et al., 2002; Yokomizo and Dzierzak, 2010).

Following HSC entry to circulation, the murine fetal liver is colonised and remains the major embryonic haematopoietic organ until the end of gestation. The thymus is colonised and becomes a site of lymphopoietic activity at E11, the spleen is active by E12, and haematopoietic cells begin to colonise the bone marrow at E15-16. As fetal liver haematopoiesis regresses at the end of gestation, the bone marrow takes over as the primary haematopoietic organ for the mouse throughout adult life (reviewed in Cumano and Godin, 2007, and figure 1.3).

1.4. The haemangioblast

Endothelial and haematopoietic genes are co-expressed in early haematopoietic cells including the DLP and yolk sac blood islands, highlighting the prospect of a bipotential progenitor cell with the ability to differentiate into both blood and endothelial lineages (Ciau-Uitz et al., 2010b; Ciau-Uitz et al., 2000; Murray, 1932).

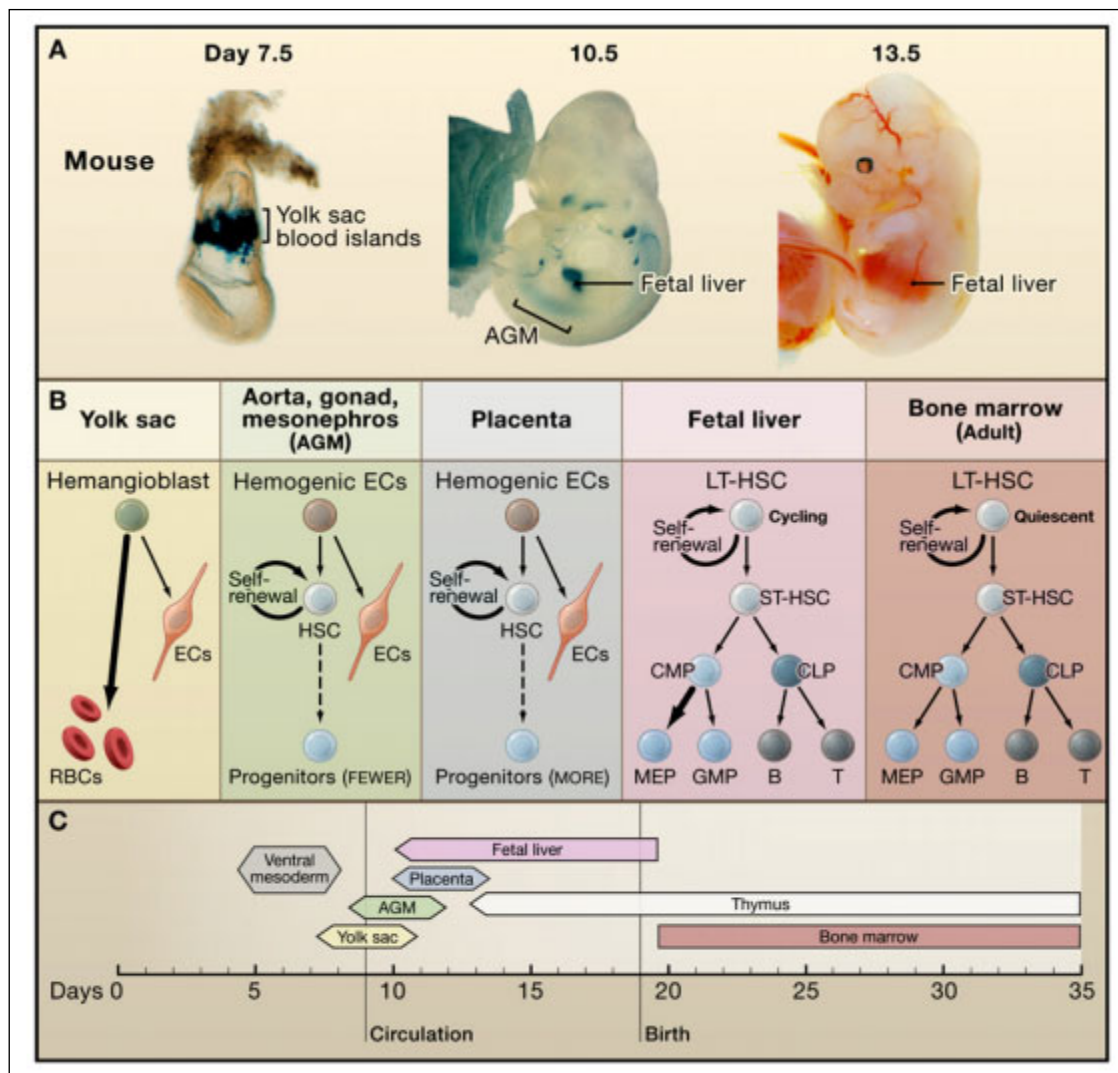


Figure 1.3 Developmental regulation of haematopoiesis in murine embryos

A. Haematopoiesis first occurs in the murine yolk sac, followed by the AGM, placenta and fetal liver. **B.** Haematopoiesis in specific haematopoietic organs favours the production of specific blood lineages. **C.** Developmental timing of haematopoietic sites. **Abbreviations:** ECs, endothelial cells; RBCs, red blood cells; LT-HSC, long-term haematopoietic stem cell; ST-HSC, short-term haematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor (figure taken from Orkin and Zon, 2008).

The first observation of the close temporal and spatial association between blood and endothelial cells in the YS was in the chick and it was proposed that these cells may have a common origin, a bi-potential progenitor called the haemangioblast (Murray, 1932, and references therein).

Molecular evidence for the existence of the haemangioblast shows that early endothelial and haematopoietic progenitors share the expression of several genes and cell surface markers, including *CD34*, *fli1*, *flk1*, *flt1*, *gata2*, *PECAM-1*, *scl* and *tie2* (reviewed in Godin and Cumano, 2002). In addition, several of these genes have been shown to be essential for both endothelial and blood lineage development (Godin and Cumano, 2002). For example, the *cloche* mutant in zebrafish disrupts both haematopoietic and endothelial cell development (Stainier et al., 1995), as does disruption of ETS-family member *ETV6* in *Xenopus* (Ciau-Uitz et al., 2010b), demonstrating that early progenitors in mouse, zebrafish and *Xenopus* are capable of specifying both endothelial and haematopoietic lineages.

The use of an *in vitro* mouse embryonic stem cell differentiation has greatly facilitated the investigation of the haemangioblast (Doetschman et al., 1985). As embryonic development progresses rapidly, and the tissues are difficult to access, the functional existence of the haemangioblast in embryos has proved difficult to demonstrate, and cell culture work has provided an invaluable tool for haemangioblast research. Embryonic stem cells (ES) can be induced to differentiate into embryoid bodies (EB) containing cells deriving from all three germ layers, and these cells have aided the study of the existence of the haemangioblast (reviewed in Keller, 2005 and; Murry and Keller, 2008). Development of haematopoietic and endothelial cells within EB cultures mirrors the observed development in the mouse YS, including vascular channels occupied by haematopoietic cells (Doetschman et al., 1985). Within 3.5 days of *in vitro* ES cell differentiation, blast colony forming cells (BL-CFC) present in the EB have been

demonstrated to represent cells equivalent to the haemangioblast. Individual cells of this early transient population can be stimulated to generate both blood and endothelial lineages after exposure to vascular endothelial growth factor (VEGF) signalling *in vitro*, as demonstrated by mixing studies (Choi et al., 1998; Nishikawa et al., 1998a). However, the cell fate in these cell differentiation cultures is heavily dependent on the culture conditions, and may not entirely reflect the conditions of these cells in the embryo. The EB *in vitro* culture system has been extremely useful in the identification of signals required for haematopoietic and endothelial cell programming (Figure 1.4). For example, BMP4 has been identified as a crucial regulator of the haemangioblast, inducing both mesoderm production and the production of endothelial cells (*flk1*⁺ cells) and blood cells (*scl*⁺ cells) from this mesoderm (Park et al., 2004).

More recently, embryonic studies have provided much evidence for the existence of the haemangioblast *in vivo*. Lineage tracing studies have identified BL-CFCs within the primitive streak of murine embryos labelled with brachyury-GFP (Huber et al., 2004). Following explant culture of individual cells, both brachyury⁺/*flk*⁺ and brachyury⁺/*flk*⁻ populations were able to give rise to colonies containing both haematopoietic and endothelial cells (Huber et al., 2004). Importantly, these haematopoietic and endothelial cells were found to be of clonal origin as demonstrated by cell mixing and limiting dilution analysis, demonstrating the *in vivo* presence of haemangioblast-like cells. Photo-activated fluorescein labelling of individual cells in zebrafish embryos has demonstrated the existence of rare cells at the ventral ridge of the gastrula stage embryo with the ability to give rise to both haematopoietic and endothelial cell populations *in vivo* (Vogeli et al., 2006).

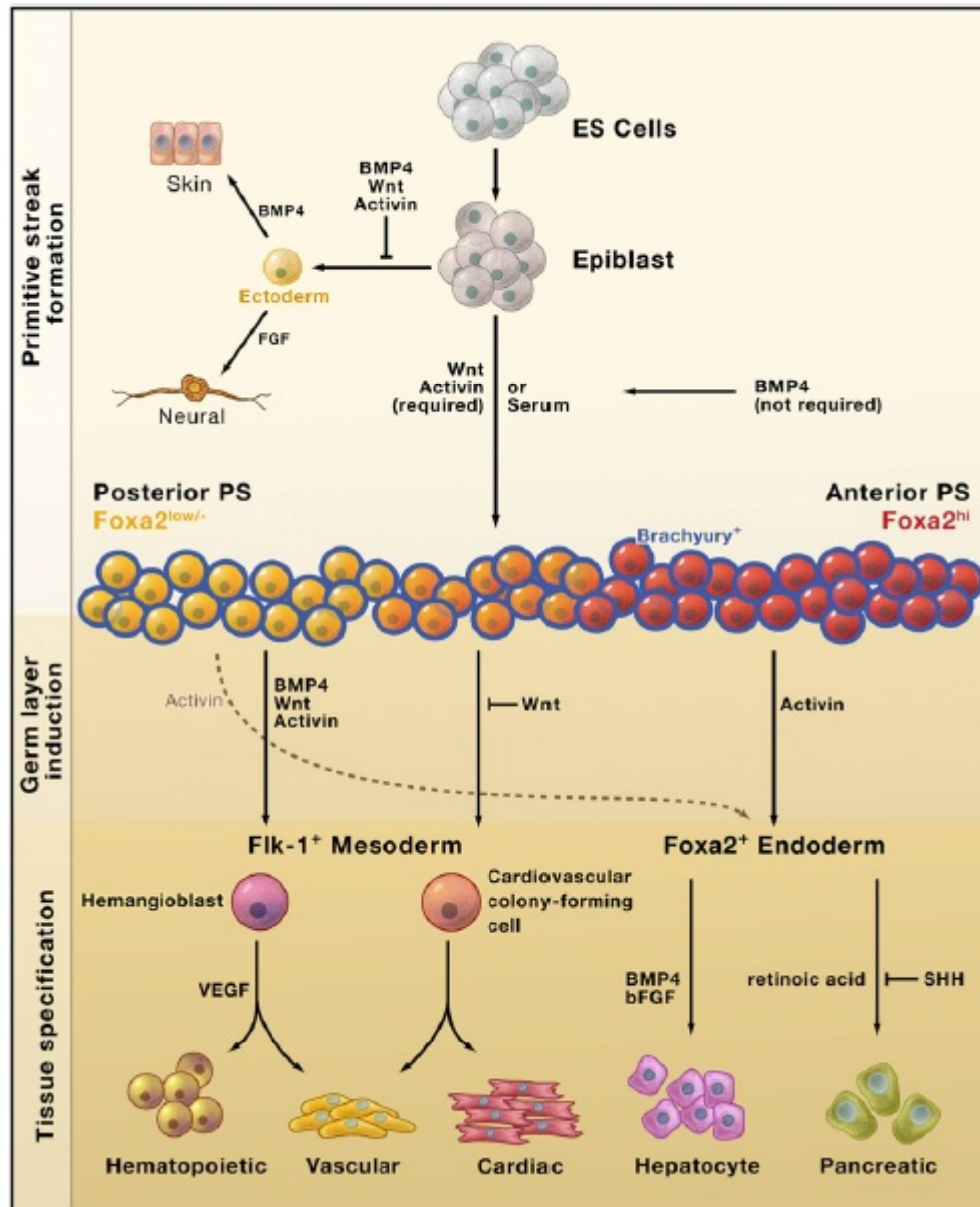


Figure 1.4 Embryonic stem cell (ES cell) differentiation in culture

Schematic model depicting the regulation of primitive streak formation, primary germ layer induction and tissue specification from mouse ES cells. Essential embryonic differentiation signals identified by ES cell culture are shown. After treatment with Wnt and Activin, or serum, cells resembling the epiblast of murine embryos generate primitive streak (PS)-like cells. After PS specification, posterior PS cells are specified to flk1⁺ mesoderm by continuing Wnt and Activin signals, along with BMP4 treatment. These flk1⁺ mesodermal cells go on to generate haematopoietic, vascular and cardiac cells (taken from Murry and Keller, 2008).

Furthermore, development of the zebrafish haemangioblast has been associated with development of cardiac lineages, also arising from mesodermal precursors (Peterkin et al., 2009; Simoes et al., 2011). A cross-antagonism between these adjoining tissues is responsible for the correct patterning of the haemangioblast and cardiac precursors (Simoes et al., 2011).

1.5. HSC emergence; haemogenic endothelium

How HSCs emerge within the AGM has been a topic of hot debate; numerous models have been proposed to explain the emergence of these cells within the lumen of the DA. Blood and endothelial lineages have a close relationship, with clusters of haematopoietic cells seen associated with the endothelial cells of ventral wall of the dorsal aorta (Ciau-Uitz et al., 2000; Dieterlen-Lievre and Martin, 1981; Garcia-Porrero et al., 1995; Gering and Patient, 2005; Godin et al., 1999). This has given rise to the suggestion that these haematopoietic cells arise via an endothelial intermediate with haematopoietic potential, known as haemogenic endothelium (Figure 1.5a, and reviewed in Bollerot et al., 2005; Zape and Zovein, 2011).

The first evidence for the existence of haemogenic endothelium was obtained by the specific labelling of avian aortic endothelial cells with an acetylated low density lipoprotein coupled with Dil (ac LDL-Dil) a label specifically taken up by endothelial cells (Jaffredo et al., 1998). These experiments showed that haematopoietic clusters in the lumen of the DA contained LDL-Dil, and thus were derived from cells with endothelial properties. LDL-Dil labelled cells were also observed ventral to the DA, within the underlying mesenchyme (Jaffredo et al., 1998). This indicated that a subset of endothelial cells from the endothelial wall of the aorta ingress into this underlying mesenchyme. Further long-term retroviral labelling of endothelial cells in the aorta again revealed endothelial cells ingressing ventral to the dorsal aorta (Jaffredo et al., 2000).

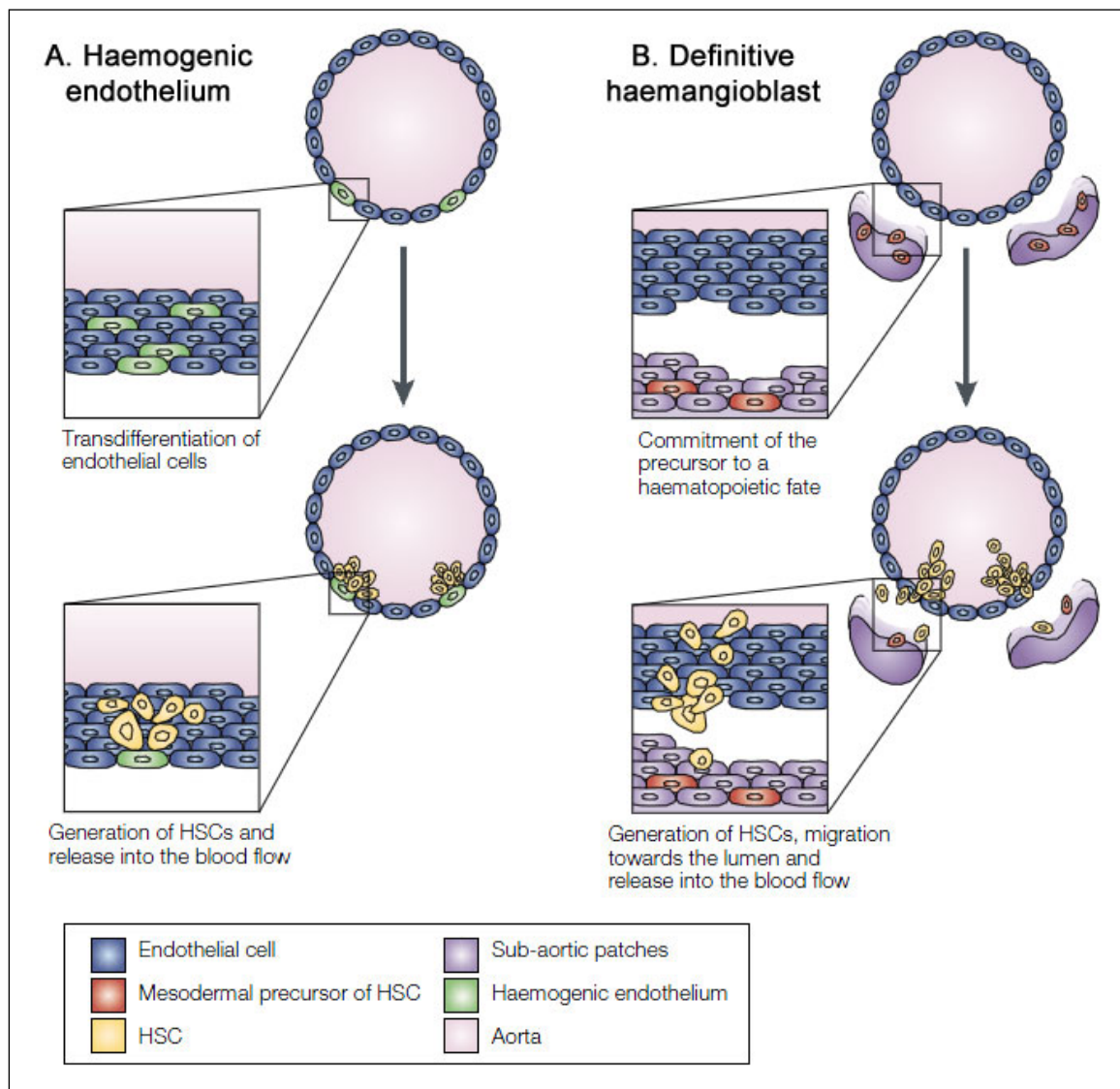


Figure 1.5 Models of intra-aortic haematopoietic cluster emergence

Schematic representation of models of intra-aortic haematopoietic cluster emergence.

A. Haemogenic endothelium. Haematopoietic clusters derive from endothelial cells within the ventral wall of the DA via transdifferentiation of endothelial cells. **B. Definitive haemangioblast.** HSCs derive from haemangioblasts in the mesenchyme underlying the DA. The HSCs migrate to the lumen of the DA to form haematopoietic clusters. (Adapted from Godin and Cumano, 2002).

These cells were identified as precursors to paraaortic foci, haematopoietic-like cells which develop within the mesenchyme (Jaffredo et al., 2000). Differentiation of embryo derived endothelial cells into haematopoietic cells provided further evidence for the existence of haemogenic endothelium (Nishikawa et al., 1998b).

Transcription factor Runx1 (Runt-related transcription factor 1) is fundamentally required for HSC formation and definitive haematopoiesis, and can be found expressed in intraluminal haematopoietic clusters, the ventral wall of the DA, and the mesenchyme underlying the DA (Cai et al., 2000; Gering and Patient, 2005; North et al., 1999; Okuda et al., 1996; Wang et al., 1996). Murine embryos haploinsufficient for *runx1* not only show HSCs present intraluminally, but also within the mesenchyme underlying the DA (North et al., 2002). HSCs in *runx1* haploinsufficient embryos are prematurely generated which may partially explain the differences between wild type and *runx1* haploinsufficient transgenic embryos. However, these observations suggest an HSC precursor, or haemangioblast, may be generated in the mesenchyme underneath the DA, which could migrate to the DA to form the haematopoietic clusters (Figure 1.5b). The identification of cells in the mesenchyme underlying the DA with the potential to form multilineage colonies *in vitro*, and some multilineage haematopoietic reconstitution *in vivo* has given support to this haemangioblast theory (Bertrand et al., 2005).

More recently, advancing imaging techniques has allowed the visualisation of HSC emergence in the DA, and together with advanced fate tracing techniques, has provided convincing evidence for the existence of haemogenic endothelium. In murine embryos, new sectioning techniques combined with time-lapse confocal microscopy has allowed the *in vivo* imaging of HSC emergence *de novo* from ventral aortic endothelial cells in the DA (Boisset et al., 2010). Zebrafish embryos have provided a useful model to study *in vivo* HSC emergence due to their transparent embryonic

body and abundance of transgenic lines (reviewed in Zhang and Liu, 2011). Time-lapse confocal microscopy on whole zebrafish embryos has again visualised the *de novo* emergence of HSCs from ventral aortic endothelial cells of the DA (Bertrand et al., 2010a; Kissa and Herbomel, 2010). Using *kdrl:Cre* transgenic zebrafish embryos to lineage label cells from haemogenic endothelium, HSCs were found to arise from this *kdrl* expressing endothelium (Bertrand et al., 2010a). Analysis of haematopoietic cells at 3 and 6 months old found that between 90% and 96% were of *kdrl*⁺ endothelium origin, thus demonstrating the endothelial origin of haematopoietic stem cells (Bertrand et al., 2010a). Similarly, using an advanced inducible Cre-Lox reporter under the control of the endothelial specific VE-cadherin promoter in murine embryos, lineage tracing of endothelial cells revealed the endothelial origin of murine HSCs (Zovein et al., 2008).

The mechanism by which HSCs emerge from the ventral aortic endothelium has been visualised in detail and termed an endothelial-to-haematopoietic transition (Kissa and Herbomel, 2010). In zebrafish, individual endothelial cells undergo contraction and bending toward the sub-aortic space before continuing contraction brings together the two lateral endothelial cells. These neighbouring lateral endothelial cells come into close contact and join, allowing the contracted central endothelial cell to round up and become motile as an HSC (Kissa and Herbomel, 2010). Transcription factor *runx1* is fundamentally required for this endothelial-to-haematopoietic transition. *Runx1*-depleted zebrafish embryos show a decreased rate of HSC emergence from ventral aortic endothelium, and those cells which do begin to emerge undergo rapid cell death before being able to round up into HSCs (Kissa and Herbomel, 2010). *Runx1* has also been shown as a critical factor for HSC emergence in murine embryos, whilst being dispensable for HSC survival after HSC emergence (Chen et al., 2009).

At the same time as haematopoietic clusters are emerging in the DA, blood is circulating throughout the vessels in the embryo, including the DA, exerting a sheer-stress force on the endothelium (Lehoux and Tedgui, 2003). This sheer-stress force has an impact on haematopoietic cells through nitric oxide (NO) signalling (Adamo et al., 2009; Krasnov et al., 2008; Michurina et al., 2004; North et al., 2009; Wang et al., 2011). Zebrafish embryos treated with small molecule agonists of NO signalling prior to circulation show an increased number of HSCs at 36hpf, while conversely embryos treated with NO signalling antagonists show a marked decrease in HSC number at the same stage (North et al., 2009). However, when using the same small molecule agonist or transgenic embryos with altered blood flow and looking earlier in development at the time when *runx1* expression initiates (24hpf), there is no effect on haematopoietic clusters (Wang et al., 2011). Thus, the suggestion is that NO signalling is crucial for the maintenance of haematopoietic clusters, but dispensable for emergence. In murine embryos, the absence of blood flow does not inhibit the emergence of haematopoietic clusters in the DA of embryonic sections (Boisset et al., 2010), supporting the zebrafish evidence for sheer stress and NO being dispensable for haematopoietic cluster emergence.

1.6. Development of the vasculature; vasculogenesis

Correct formation of the vasculature is essential for embryonic survival. The primary role of vasculature is to allow circulation of blood cells around the embryo, to deliver oxygen and nutrients, remove waste etc but in addition to this, the correct development of the vasculature is also fundamentally required for the production of definitive blood lineages, through a haemogenic endothelium stage (as discussed in section 1.5). The initial generation of vasculature in an embryo is through vasculogenesis whilst later remodelling and development of vessels to supply the ever changing needs of the organism occurs through angiogenesis (reviewed in Heinke et al., 2012).

After the initiation of gastrulation in the mammalian embryo, vasculogenesis initiates with the formation of endothelial cells within the YS and of angioblast precursors in the head mesenchyme. Several signalling pathways have been shown to be essential for vasculogenesis and lumen formation (reviewed in Rossant and Howard, 2002), including fibroblast growth factor (FGF) (Cox and Poole, 2000), hedgehog (Hh) (Vokes et al., 2004) and VEGF (Millauer et al., 1993). More recently, it has been shown that a *cis*-acting FOX:ETS enhancer motif is capable of specifically driving endothelial expression in the developing vasculature (De Val et al., 2008). This FOX:ETS motif is found in many known endothelial enhancers and is bound by Forkhead and Ets transcription factors. Combinatorial overexpression of FoxC2 and Etv2 has been shown to ectopically induce expression of vascular markers, whilst their depletion disrupts vascular development, highlighting their importance as regulators of vascular development (De Val et al., 2008).

The dorsal aorta derives from angioblasts in the lateral plate mesoderm (Chun et al., 2011; Wasteson et al., 2008; Zovein et al., 2010). Initially, bilateral paired dorsal aortae are formed either side of the midline in chick and mouse embryos. These paired aortae later fuse at the midline forming the DA (Garriock et al., 2010). In zebrafish and *Xenopus*, the lateral plate mesoderm angioblasts migrate to the midline where they form a single aorta.

1.7. Establishment of venous and arterial identity

It was believed for a long time that the structural and functional differences between arteries and veins was due to differences in the direction and pressure of blood flow (reviewed in Lawson and Weinstein, 2002). The identification of specific arterial and venous markers (Ephrin-B2; EfnB2, and Eph-B4; EphB4, respectively) led to the idea that arterial and venous identity is established during programming of the vascular system, prior to the onset of circulation (Wang et al., 1998).

Several other factors have since been implicated in pre-circulation venous/arterial specification (reviewed in Lin et al., 2007, and summarised in figure 1.6). Lineage tracing has revealed clusters of endothelial cells within the lateral mesoderm of zebrafish which are capable of contributing to either the DA or the posterior cardinal vein (PCV), but never both (Zhong et al., 2001). This suggests that the arterial and venous fates are determined much earlier in development, prior to angioblast migration. In support of this, migration of endothelial cells to the midline of the embryo has been observed to occur in two waves, with endothelial cells in the first wave contributing to the dorsal aorta, and the subsequent wave forming the vein (Williams et al., 2010). However, more recent investigations have found that the vein and artery are initially formed from one unspecified midline precursor vessel after migration of ECs in zebrafish, suggesting the ECs are specified, or at least segregated, at the midline (Herbert et al., 2009).

Notch signalling has been found to be a key determinant in the specification of arterial identity (reviewed in Lin et al., 2007), and this will be discussed comprehensively in chapter 1.8.3. Upstream of Notch signalling, Sonic Hedgehog (Shh) and VEGF signalling have also been demonstrated to be fundamental for correct arterial specification (Lawson et al., 2002). In zebrafish embryos lacking the midline patterning signal, Shh, circulation fails to initiate. Loss of arterial marker expression, including Notch family genes, combined with an inability to restrict venous-preferential marker, *flt4*, demonstrates an incorrectly programmed DA (Lawson et al., 2002). Furthermore, loss of Shh in zebrafish embryos prevents expression of *vegfa* in adjacent somitic tissue, whilst overexpression of *vegfa* mRNA can rescue arterial programming in zebrafish embryos deficient in Shh. Thus, Shh is placed upstream of VEGFA in the gene regulatory network governing arterial specification (Lawson et al., 2002). Morpholino knockdown and mRNA overexpression of *vegfa* in zebrafish reveals a specific requirement of VEGFA for arterial programming (Lawson et al., 2002). These studies indicate that VEGFA acts upstream of Notch,

but downstream of Shh to programme the DA. Studies using small molecule inhibitors of the Hh signalling pathway have revealed that Hh is also required to regulate the size of the avian dorsal aorta (Moran et al., 2011), whilst Hh has been shown to promote arterial specification at the expense of venous (Williams et al., 2010).

However, several differences between the effect of Hh and VEGFA on arterial specification have been noted, suggesting additional components downstream of Hh may be controlling arterial specification. Zebrafish embryos lacking Hh fail to form two distinct axial vessels, a phenotype more severe than that observed in VEGFA depleted embryos (Lawson et al., 2002). Consistently, avian vascular lumenisation can be ectopically induced by Shh, but not VEGF (Vokes et al., 2004) whilst mammalian studies have found VEGF-independent roles of Hh signalling in lumenisation (Coultas et al., 2010). Zebrafish mutants for patched1 (*ptc1*) and patched2 (*ptc2*), exhibiting a constitutively active Hh phenotype, have shown that not all Hh signalling controlling arterial programming is mediated by VEGF (Wilkinson et al., 2012). Normal and ectopic arterial expression is observed when upregulating Hh expression using *ptc1;ptc2* mutants in VEGFA depleted embryos, suggesting Hh has the capability to signal to the DA without VEGFA signalling. This Hh dependent role on arterial specification has been shown to be mediated through a calcitonin receptor-like receptor in zebrafish embryos (Wilkinson et al., 2012).

Venous fate is believed to be established by specific venous endothelium associated signalling pathways acting to repress arterial programming (reviewed in Lin et al., 2007). *COUP-TFII* is specifically expressed in venous endothelial cells and conditional knockout in endothelial cells results in venous cells exhibiting arterial characteristics, such as expression of arterial markers *efnb2* and notch signalling components (You et al., 2005). Furthermore, these arterialised venous vessels have the ability to form haematopoietic clusters intraluminally and to recruit smooth-

muscle cells, suggesting the venous endothelium has gained arterial functionality in addition to expression of arterial cell surface markers (You et al., 2005). Ectopic expression of *COUP-TFII* in mouse endothelium phenocopies defects found in *neuropilin1* (*NP-1*) or *notch1* deficient embryos, suggesting down regulation of notch signalling by COUP-TFII could account for venous characteristics (Huppert et al., 2000; Kawasaki et al., 1999; You et al., 2005). Similarly, the venous programme must be repressed in arterial endothelium for correct specification. It has been proposed that Hey2/Gridlock (*grl*), a member of the hairy and enhancer of split family downstream of notch signalling expressed specifically in the arterial endothelium, is responsible for repressing venous genes such as *ephb4* in arterial endothelium (Zhong et al., 2001; Zhong et al., 2000).

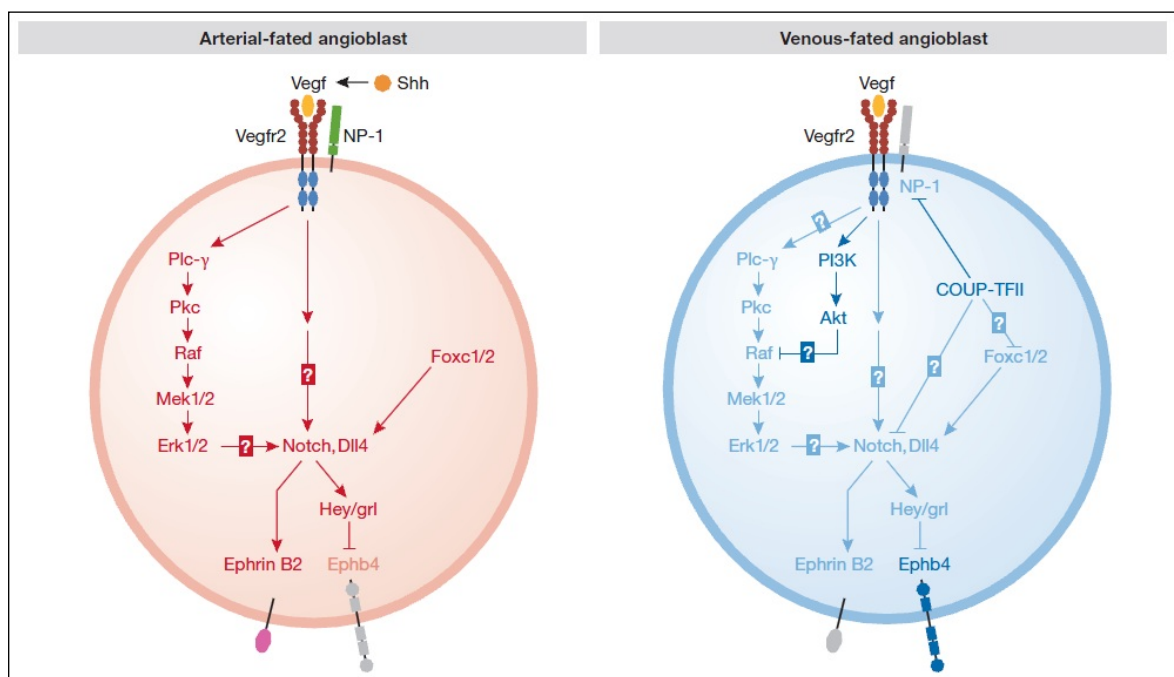


Figure 1.6 Signalling pathways regulating arterial and venous specification during embryonic development

A schematic diagram showing an overview of the current understanding of venous and arterial specification during embryogenesis (figure taken from Lin et al., 2007). However, recent experiments have also found Hey2/*grl* upstream of notch (Rowlinson and Gering, 2010, and see chapter 1.8.2).

1.8. Overview of the notch pathway

1.8.1. Notch signalling

Notch is a well conserved juxtacrine signalling pathway essential for cell fate determination. It was first discovered in *Drosophila melanogaster* mutants exhibiting notched/serrated wings in the early twentieth century (Morgan, 1917). Notch was initially cloned in *Drosophila melanogaster* (Artavanis-Tsakonas et al., 1983; Kidd et al., 1983), followed shortly by *Caenorhabditis elegans* (Yochem et al., 1988), *Xenopus laevis* (Coffman et al., 1990), human (Ellisen et al., 1991), rat (Weinmaster et al., 1991), mouse (Del Amo et al., 1992) and zebrafish (Bierkamp and Campos-Ortega, 1993). Whilst *Drosophila melanogaster* possesses only one notch receptor, *Caenorhabditis elegans* has two redundant notch receptors and mammals have four paralogues with some redundant and some non-redundant roles (reviewed in Kopan and Ilagan, 2009).

Notch receptors are large single-pass transmembrane receptor proteins (Figure 1.7, and reviewed in detail in Chillakuri et al., 2012). Whilst the notch receptor is transcribed and translated as one complete polypeptide, it is cleaved at the S1 cleavage site by furin-like convertase in the trans-Golgi (Logeat et al., 1998, and references therein). The two domains generate a non-covalently associated heterodimer and reside at the cell surface. The extracellular domain of the notch receptor (NECD) contains conserved tandem epidermal growth factor (EGF)-like repeats at the N-terminus. Although receptors from all species maintain the same molecular architecture, the NECD can vary in size and number of EGF-like repeats (reviewed in Kopan and Ilagan, 2009). A subset of these EGF-like repeats are calcium binding (cbEGF), essential for receptor-ligand binding (Fehon et al., 1990; Rebay et al., 1991). Two of the 36 EGF-like repeats in *Drosophila* NECD, found at positions 11 and 12, are both necessary and sufficient for receptor-ligand binding (reviewed in

Chillakuri et al., 2012; Rebay et al., 1991). After the EGF-like repeats are three Lin-12-Notch repeats (LNR) and a hydrophobic heterodimerisation domain, which together are termed the negative regulatory region (NRR). The NRR is responsible for preventing ligand-independent notch activation by concealing a cleavage site (S2 cleavage site) from metalloproteases, such as ADAM metalloprotease (Sanchez-Irizarry et al., 2004). The intracellular domain of the notch receptor (NICD) contains the transmembrane portion of the receptor, followed by a further cleavage site, the S3 site. The S3 site is cleaved by a γ -secretase complex to release the NICD after ligand binding and subsequent S2 cleavage (De Strooper et al., 1999). NICD also contains an RBP-Jk-associated module (RAM) domain and seven ankyrin repeats (ANK) to mediate interaction with CSL transcription factors and co-activator mastermind-like1 (MAML1) (Lubman et al., 2007; Nam et al., 2006; Roehl et al., 1996; Tamura et al., 1995). Following these is a transcription activation domain (TAD; absent in mammalian notch3 and notch4) and a C-terminal PEST domain to mediate proteolytic cleavage.

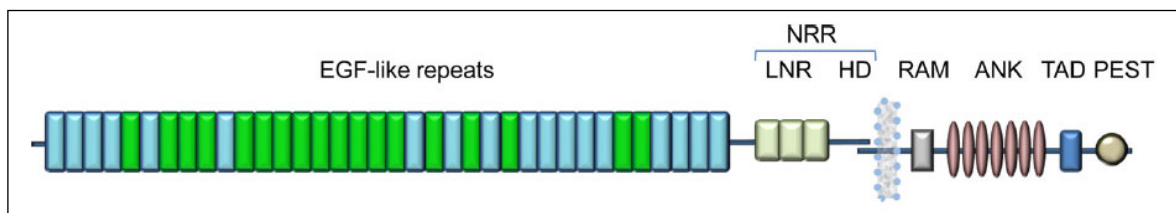


Figure 1.7 Schematic diagram showing the architecture of human Notch1 receptor

The NECD contains EGF-like repeats (Ca^{2+} binding in green, non Ca^{2+} binding in blue), Lin-12-notch repeats (LNR), and a heterodimerisation domain (HD). Together, the LNR and HR make up the negative regulatory region (NRR). The NICD has a transmembrane domain (not annotated), an RBP-Jk-associated module (RAM) domain, seven ankyrin repeats (ANK), a transcription activation domain (TAD) and a C-terminal PEST domain (adapted from Chillakuri et al., 2012).

Notch receptors bind to cell surface ligands of the Delta/Serrate/Lag-2 (DSL) family on neighbouring cells via its extracellular domain. Typically, these ligands fall into two sub-families;

Delta/Delta-like and Serrate/Jagged. *Drosophila* contains one each of Delta and Serrate, whilst mammals have three members of the Delta family (Dll1, Dll2, Dll4), and two members of the Jagged family (Jag-1 and Jag-2).

At the N-terminus of the ligands is a 'module at the N-terminus of Notch ligands' (MNNL) domain with unknown function, followed by a receptor binding DSL domain. Four highly conserved residues on the face of the DSL domain (human Jag-1 F¹⁹⁹, R²⁰¹, R²⁰³, F²⁰⁷) form the putative notch binding site (Cordle et al., 2008). After the DSL domain comes a region of EGF-like repeats, ranging in number between 1 and 16, depending on the ligand sub-family and the species (reviewed in Chillakuri et al., 2012). A cysteine rich domain (CRD) is found on Serrate/Jagged ligands, close to the transmembrane domain on the extracellular side whilst on some vertebrate ligands (Jag-1, Dll1 and Dll4), the intracellular portions of notch ligands contain a PDZL domain responsible for promoting adhesion of the ligand to the cytoskeleton (Mizuhara et al., 2005, and Figure 1.8). Ligand endocytosis, controlled by ubiquitin-ligases neutralized (neurl) and mindbomb (mib), is required for ligand maturation, producing a more active ligand which is translocated to the cell surface for interaction with notch receptors (reviewed in Weinmaster and Fischer, 2011).

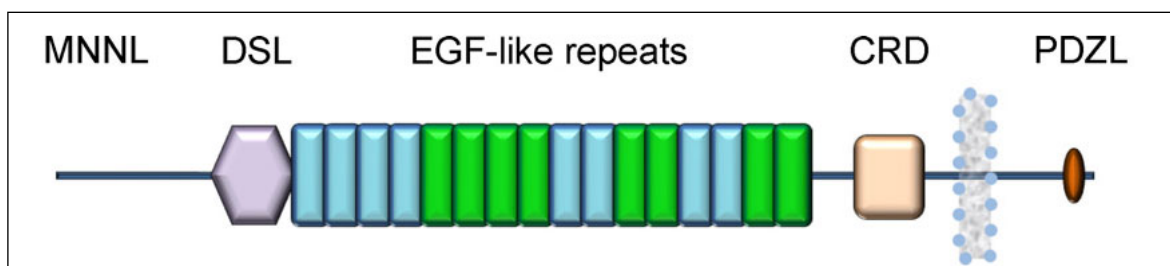


Figure 1.8 Schematic diagram showing the architecture of human Jagged-1 ligand

The extracellular portion of human Jag-1 consists of a 'module at the N-terminus of Notch ligands' (MNNL) domain with unknown structure, a receptor binding DSL domain, 16 EGF-like repeats and a cysteine rich domain (CRD). Intracellularly is a PDZL domain (adapted from Chillakuri et al., 2012).

Notch signalling is activated when Delta/Serrate/Lag2 (DSL) ligands on an adjoining cell bind to a notch receptor (reviewed in Chillakuri et al., 2012; and Kopan and Ilagan, 2009, and Figure 1.9). The ligand expressing cell attempts endocytosis of the ligand-receptor complex, leading to a conformational change in the receptor NRR region. Exposure of cleavage site S3 in the NRR region leads to ADAM metalloprotease mediated cleavage, removing the NECD. A second conformational change then occurs, allowing γ -secretase to cleave at S2 to release NICD from the transmembrane portion of the receptor. The NICD translocates to the nucleus where it will bind with transcription factor CBF1/suppressor of hairless/Lag1 (CSL) and co-activators (CoA) such as MAML1 to form a transcriptional activation complex; NICD is unable to bind DNA on its own. The transcriptional activation complex relieves repression of target genes, such as *Hes1*, which would otherwise be bound with CSL in a complex with co-repressors (CoR), such as histone deacetylases (HDACs), preventing transcriptional activation (reviewed in Kopan and Ilagan, 2009). Alternatively, if ligands are expressed on the same cell as the receptor, binding can still occur but instead of notch receptor activation, receptor-ligand interaction causes cis-inhibition, limiting notch activity (reviewed in del Alamo et al., 2011).

In addition to this ligand-induced cleavage of the notch receptor, the 'canonical' notch pathway, there have been other proposed mechanisms of notch activation, termed 'non-canonical' notch signalling (Rusconi and Corbin, 1998). Examples of non-canonical notch include, but are not limited to: interaction with Wnt signalling (reviewed in Andersen et al., 2012; Kwon et al., 2011), γ -secretase independent signalling (Berechid et al., 2002), furin independent signalling (Bush et al., 2001), CSL independent signalling (Shawber et al., 1996), and ligand independent signalling mediated via receptor endocytosis and degradation (reviewed in Baron, 2012; Hori et al., 2004; Romain et al., 2001). In addition, several non-DSL domain containing ligands have been proposed to bind the notch receptor to activate notch signalling (reviewed in Wang, 2011).

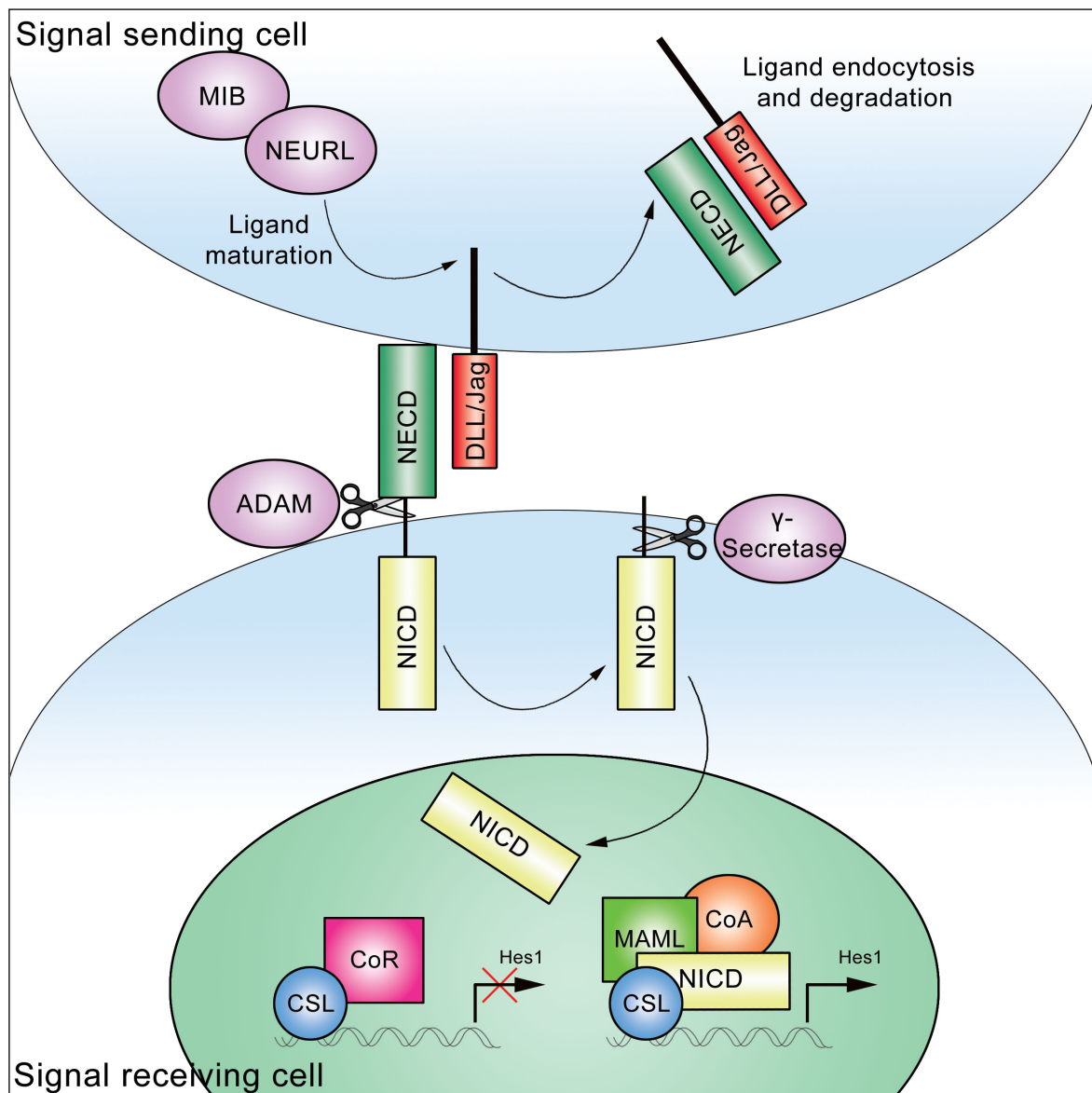


Figure 1.9 Schematic diagram showing notch signalling between neighbouring cells

Ligand-receptor binding initiates notch signalling. Once ligand-receptor binding has occurred, a conformational change allows ADAM metalloprotease to cleave extracellularly, at cleavage site S2. The NECD is released from the transmembrane domain of the receptor, causing a further conformational change which allows γ -secretase to cleave intracellularly, at cleavage site S3. NICD is then free to travel to the nucleus (green) where it relieves the repression on target genes, such as *hes1*, by transcription factor CSL.

Once NICD is cleaved at the S3 cleavage site, it is no-longer under the regulation of signalling from neighbouring cells. To ensure notch-mediated transcriptional activation is not sustained longer than required, NICD turnover is tightly controlled. The C-terminal PEST domain of NICD is ubiquitinated by Fbw7 E3 ubiquitin ligase, targeting the NICD for proteosomal degradation (Fryer et al., 2004; Tsunematsu et al., 2004). Mutations in the PEST domain have been associated with maintained notch signalling, including the observation that some T-cell acute lymphoblastic leukaemias (T-ALL) are caused by the inability to degrade NICD (Weng et al., 2004).

Post-translational modification of the NECD is known to regulate notch signalling through ligand specificity. Multiple sites for post-translational modification are found on the NECD, including an O-fucose site and an O-glucose site in EGF-like12, a cbEGF-like known to be required for receptor-ligand binding (Fehon et al., 1990; Moloney et al., 2000; Okajima et al., 2003; Rebay et al., 1991). O-fucosylation, occurring by O-fucosyltransferase activity, favours Notch-Serrate binding. Extending the fucose moiety with N-acetylglucosamine (GlnNAc) by the activity of Fringe glycosyltransferase favours Notch-Delta binding, at the expense of Notch-Serrate binding (reviewed in Chillakuri et al., 2012; and Kopan and Ilagan, 2009). NMR structural analysis of the addition of GlnNAc to Notch cbEGF12 shows a significant conformational change in the cbEGF12 structure (Hiruma-Shimizu et al., 2010). This analysis however was carried out on a single domain in the absence of Ca^{2+} . As cbEGF12 needs both N-terminal linkage to cbEGF11 and Ca^{2+} present to adopt a native structure, further analysis is needed on these domains in the presence of Ca^{2+} to understand how these post-translational modifications impact ligand binding *in vivo*.

Elegant experiments using laser ablation and cell tracking in *C.elegans* showed that Notch acts as a molecular determinant of cell fate, controlling binary cell decisions in adjacent cells (Seydoux and Greenwald, 1989). Studies in *Drosophila* built on these studies, introducing the theory of

lateral inhibition (Cabrera, 1990). Briefly, neighbouring cells initially showing no apparent distinction, both weakly expressing Delta and Notch, are patterned to form two different cell types through a disequibration of Notch and Delta expression. For example, during *Drosophila* neurogenesis, cells are initially indistinguishable through their expression of Delta and Notch. Once the disequibration occurs, cells expressing Delta at higher concentrations form neuroblasts whilst inhibiting their neighbouring cells from following the same fate. These neighbouring cells express higher levels of Notch and respond to the signals from the Delta-expressing cells to form epidermoblasts (Cabrera, 1990; reviewed in Katsube and Sakamoto, 2005). The disequibration of Notch and Delta expression in neighbouring cells is thought to arise through a small stochastic change in signalling between the two cells, followed by a transcriptional feedback mechanism to amplify the change in cell fate (reviewed in Fortini, 2009).

1.8.2. Transcriptional activation and notch targets

As NICD cannot bind directly to DNA it is unable to act as a transcriptional activator alone. Instead, NICD acts to mediate transcription through its DNA-binding partner, CSL (Furriols and Bray, 2001 and references therein). CSL was originally identified as a transcriptional repressor (Dou et al., 1994), binding to consensus sequence CGTGGGAA (Tun et al., 1994). However, it was soon discovered to be a molecular switch, acting as either a transcriptional repressor or an activator, depending on the recruitment of co-repressors or co-activators, respectively. In the absence of canonical notch signalling, CSL sits on the promoters of notch target genes, acting to repress them in a complex with co-repressors. When notch signalling is active, NICD translocates to the nucleus where it binds to CSL and recruits co-activators, including MAML, switching CSL from a repressive to an active complex (reviewed in Borggreffe and Oswald, 2009). Notch target promoters often contain more than one CSL binding site. Co-operative binding between two activating CSL/NICD/MAML complexes has been observed *in vitro*, with the two complexes

interacting through their ANK domains (Arnett et al., 2010; Liu et al., 2011; Nam et al., 2007). This dimerisation is necessary for transcriptional activation of some notch target genes, but not for others. In addition to notch activation complex dimerisation in the nucleus, the complex can also interact with other families of proteins, including: ASPP (Xin Lu, personal communication), BMP SMADs (Takizawa et al., 2003), TGF- β SMADs (Blokzijl et al., 2003), Wnt pathway transcription factor LEF-1 (Ross and Kadesch, 2001), Wnt pathway modulator β -catenin (Alves-Guerra et al., 2007), Mef2C (Shen et al., 2006), p53 (Zhao et al., 2007) and HIF1 (Zheng et al., 2008). Thus, notch signalling is more complex with many more molecular interactions than initially anticipated.

The hairy and enhancer of split genes (Hes) is a large family of highly conserved proteins that have been established as direct targets of notch signalling (reviewed in Borggreffe and Oswald, 2009; and Fischer and Gessler, 2007). *Hes1*, *hes5*, and *hes7* have all been identified as direct notch targets, whilst *hes2*, *hes3* and *hes6* are not (Bessho et al., 2001; Jarriault et al., 1995; Jarriault et al., 1998; Koyano-Nakagawa et al., 2000; Nishimura et al., 1998; Ohtsuka et al., 1999). *Hey1*, *hey2* and *heyL*, members of the Hes related with YRPW motif subfamily (Hey, also known as Hesr, Chf, Hrt, Herp and Gridlock), have also been shown to be direct notch targets (Iso et al., 2001a; Maier and Gessler, 2000). Hes and Hey proteins are classic basic helix-loop-helix transcription factors, commonly associated with transcriptional repression (Dawson et al., 1995). In addition to the DNA-binding basic domain (b) and the helix-loop-helix (HLH) serving as a dimerisation domain, there are an additional two α -helices forming an Orange domain which acts as a platform for protein-protein interactions (Dawson et al., 1995; Massari and Murre, 2000). Hes genes have a characteristic C-terminal tetrapeptide WRPW motif to recruit co-repressors TLE1-4 whilst Hey genes have a related YRPW or YQPW motif which is unable to recruit these TLE co-repressors (Dawson et al., 1995; Iso et al., 2001b). DNA binding of Hes proteins happens at N- and E-box sequences (CACNAG and CANNTG respectively) whilst Hey proteins preferentially bind

E-box motifs and cannot bind N-box sequences due to a small change in their basic domains (Iso et al., 2001b).

The mechanism for Hes and Hey proteins modulating transcription is currently unclear, although several models have been proposed (Fischer and Gessler, 2007). Through their bHLH and orange domains, Hes and Hey proteins are known to interact with each other, and a wide range of other proteins (reviewed in Fischer and Gessler, 2007). The formation of these homo- and heterodimers has been the focus of many studies aimed at understanding the mechanism behind transcriptional regulation by Hes and Hey proteins. The sequestering of activating transcription factors by Hes and Hey genes is one theory for the mode of repression. For example, Hey1 forms a heterodimer with muscle-specific MyoD, preventing the formation of a MyoD/E47 complex essential for myogenic differentiation (Sun et al., 2001). Another theory is that the Hes and Hey proteins may bind directly to transactivation domains of activating transcription factors. Binding of Hes or Hey genes to the transactivation domain of Scute in *Drosophila* compromises the transcriptional activation potential of Scute (Alifragis et al., 1997; Giagtzoglou et al., 2003; Giagtzoglou et al., 2005). Binding of the Hes WRPW motif to TLE generates a strong repressor complex, attracting further co-repressors such as histone deacetylases (reviewed in Fischer and Gessler, 2007). These Hes/TLE complexes can bind both Hes and TLE consensus sites in the genome, increasing the scope for transcriptional repression. Heterodimerisation of GATA and Hes/Hey proteins tightly regulates the activity of GATA factors; competition between co-activator p300 and co-repressor Hes1 for GATA1 interaction in haematopoietic progenitor cells has been proposed as a mechanism for regulating activity of GATA1 (Ishiko et al., 2005). Similarly, Hes1, Hey2 and Hey1 have all been found to interact with Runx proteins to regulate transcription (Garg et al., 2005; Zamurovic et al., 2004). In addition, interaction of Hey proteins with promoters lacking a TATA box but containing an initiator element, such as the *vegfr2/flk1* promoter, leads to

transcriptional repression in an E-box independent manner (Holderfield et al., 2006; reviewed in Siekmann et al., 2008). Artificial introduction of a TATA box to these promoters overcomes the ability of Hey proteins to repress transcription from these promoters, suggesting that they act to prevent transcription machinery binding at the promoter (Holderfield et al., 2006). Interaction of Hes and Hey proteins with several other proteins has been observed, highlighting the diversity of molecular interactions between Hes and Hey proteins to repress transcription (reviewed in Fischer and Gessler, 2007).

Although Hes and Hey proteins are commonly associated with transcriptional repression, Hes binding has been associated with transcriptional activation. Hes1 interaction with Runx1 or Runx2 can stimulate transcription; dimerisation of Hes1/Runx1 or Hes1/Runx2 inhibits the formation of the Hes1/TLE repressor complex, preventing Hes1 acting as a repressor and allowing transactivation of target genes (McLarren et al., 2000; McLaren et al., 2001; Shen and Christakos, 2005). In addition, whilst many *Hes* and *Hey* genes are transcriptional targets of Notch signalling, it also possible for Hey proteins to regulate Notch. In zebrafish embryos, Hey2 has been identified as a crucial factor for *Notch1b* expression in the DA (Rowlinson and Gering, 2010). Instead of responding to notch in a classical manner, *Hey2* expression is found downstream of *scl* and *cloche* in the posterior lateral mesoderm (PLM) and downstream of *etsrp* and *cloche* in the anterior lateral mesoderm (ALM), before being controlled by Hedgehog and VEGF signalling in the DA (Rowlinson and Gering, 2010).

Whilst Hes and Hes-related genes are the primary notch target genes, there have been several others identified (reviewed in Borggreffe and Oswald, 2009). CD25 and GATA3 have been identified as notch targets in T-cells (Amsen et al., 2007; Deftos et al., 2000; Fang et al., 2007), whilst NRARP and Deltex-1 have been shown to be direct targets of notch, and also negative

regulators of notch signalling, demonstrating a negative feedback mechanism (Deftos et al., 1998; Izon et al., 2002; Lamar et al., 2001). Recent Chip-seq analysis of Notch1-expressing embryonic stem cells has revealed that over 10,000 genes from many different families have a CSL binding site within 10kb of the transcription start site, highlighting the diversity of potential notch transcriptional targets (Hamidi et al., 2011).

1.8.3. Notch signalling in vasculogenesis, angiogenesis, arterial specification and haematopoiesis

Notch signalling regulates cell fate specification, differentiation and tissue patterning in metazoan embryos. Consistent with this observation, notch receptors and ligands are expressed in specific cells and tissues during embryonic development, including the cardiovascular system and haematopoietic cells. During specification of arterial identity in the DA, early expression of specific genes marks the genetic differentiation of arteries from veins (see section 1.7). *Notch1* and *Notch4* receptors have been identified as expressed in the developing artery, as have notch ligands *Dll1*, *Dll4*, *Jag-1* and *Jag-2*, suggesting they have a role in the development of the DA and/or haemogenic endothelium (Beckers et al., 1999; Del Amo et al., 1992; Reaume et al., 1992; Robert-Moreno et al., 2005; Shirayoshi et al., 1997; Shutter et al., 2000; Uyttendaele et al., 1996).

Using a zebrafish mutant of mindbomb (*mib*), a protein essential for notch ligand maturation, to disrupt notch signalling, notch has been implicated in arterial specification (Lawson et al., 2001). Together with vascular malformations, loss of notch signalling in zebrafish embryos results in the loss of artery-specific marker *ephrinB2* and the misexpression of venous-associated *EphB4* in arteries. Conversely, ectopic activation of notch signalling in veins prevents *EphB4* expression (Lawson et al., 2001). Similar phenotypes are found when mutating *Gridlock* (*Hey2*) in zebrafish embryos; vascular malformations are prominent and expression of *ephrinB2* is lost. However,

ectopic expression of *Gridlock* in veins does not lead to misexpression of arterial markers in the vein, instead it leads to a loss of vein markers, suggesting that the role of *Gridlock* in arteries is to suppress venous-associated genes (Rowlinson and Gering, 2010; Zhong et al., 2001; Zhong et al., 2000). COUP-TFII (encoded by *Nr2f2*) is thought to repress notch (and in turn, *Gridlock*) expression in the veins, in order to allow expression of venous genes (You et al., 2005). *Nr2f2* knockout mice show arterial characteristics in the vein whilst expression of *Nr2f2* in the artery causes a loss of arterial markers (You et al., 2005). Similarly, conditional overexpression of *Dll4* in mouse endothelial cells causes a gross enlargement of the DA, together with misexpression of arterial markers in the vein (Trindade et al., 2008). Mutation of *Rbpsuh* (encoding CSL) in mouse, or double knockout of *Notch1* and *Notch4*, leads to substantial vascular defects, including vascular remodelling defects and loss of *ephrinB2* in arterial cells (Krebs et al., 2004; Krebs et al., 2000). Similar results have been found in mice lacking *Dll4* (Duarte et al., 2004; Krebs et al., 2004), *Hey1* and *Hey2* (Fischer et al., 2004), ADAM metalloprotease (Hartmann et al., 2002), and γ -secretase (Herreman et al., 1999), showing that an intact notch pathway is essential for correct arterial and venous patterning. *Dll1*-null mutants exhibit normal arterial specification but show a requirement of *Dll1* in maintenance of the arterial programme, and thus different notch inputs are required throughout embryonic development to correctly specify and maintain arterial identity (Sorensen et al., 2009). This data is consistent with the expression of *Dll1* in arteries at E13-15, but absence of expression during arterial specification at E9.5-E10.5 (Beckers et al., 1999; Robert-Moreno et al., 2005). However, not all notch components are essential for arterial specification. *Jag-1*-null mouse embryos exhibit vascular remodelling defects and haemorrhaging resulting in embryonic lethality (Xue et al., 1999). However, analysis of arterial development in *Jag-1*-null embryos reveals normal arterial specification, including the correct expression of *ephrinB2* (Robert-Moreno et al., 2008). This discrepancy is thought to be due to a Jag-1 dependent endothelial role in the recruitment of smooth muscle cells (High et al., 2008).

Altered vascular development is not only controlled by loss of notch signalling, but also by ectopic notch signalling. Overexpression of *Notch4* in endothelial cells causes disorganised vascular networks and enlarged vessels, suggesting that the correct levels of notch signalling are critical for vascular development (Carlson et al., 2005). Similarly, overexpression of *Notch1* in *Tie2*-expressing endothelial cells causes vascular remodelling and smooth muscle recruitment defects along with cardiovascular and haematopoietic defects (Venkatesh et al., 2008). In agreement with these observations, it has been proposed that Notch does not act in an ON/OFF fashion; a dose-dependent model of notch signalling has also been described (Bigas and Espinosa, 2012).

A major limitation to studying the role of notch signalling in haematopoietic specification is that many notch pathway mutants die during embryonic development due to defects in vascular morphogenesis (Hrabe de Angelis et al., 1997; Krebs et al., 2004; Krebs et al., 2000; Limbourg et al., 2005; Xue et al., 1999). Mouse embryos homozygous for a null *Notch1* allele die at E9.5 (Krebs et al., 2000). Endothelial specific deletion of *Notch1* as controlled by *Tie2*-Cre-recombination also results in embryonic lethality at E9.5, suggesting that the primary defect causing lethality in *Notch1* deficient embryos is a vascular defect (Limbourg et al., 2005). In addition, as definitive haematopoietic clusters derive from ventral aortic endothelium during embryogenesis, it is not possible to study the direct effect of these above-mentioned mutations on haematopoietic clusters. Where aortic endothelium is incorrectly specified in these notch pathway mutants, it is predicted that subsequent programming of haemogenic endothelium is also compromised. Conditional knockouts are needed in order to study the direct effect of these notch pathway genes on haemogenic endothelium and haematopoietic cluster programming. To demonstrate the relationship between arterial programming and haemogenic endothelium, *Nr2f2* mutants showing ectopic notch activation in veins not only misexpress arterial markers in the vein, but also demonstrate ectopic cluster emergence, presumably through haemogenic endothelium (You

et al., 2005). Thus, arterial development may be a prerequisite for haematopoietic gene expression. Whilst arterial marker-independent ectopic expression of haematopoietic genes in the vein has also been demonstrated (Burns et al., 2005), proof that HSCs can be specified in the absence of an artery is still needed.

The first evidence for a role of notch signalling in definitive haematopoiesis came from *Notch1*-null murine embryos with a reduced ability to form transplantable repopulating LT-HSCs in the AGM (Kumano et al., 2003). Here, cells from *notch1*-null embryos were explanted and assayed for CFC-activity, and HSC activity. Although the number of endothelial cells were found to be the same in wild type and *notch1*-null embryos, HSC activity could not be identified in *notch1*-null explants (Kumano et al., 2003). In agreement with the proposed role for *Notch1* in haematopoietic development, *Notch1*-deficient stem cells are not capable of colonising the bone marrow in mice (Hadland et al., 2004). *Notch1*-null ES cells were generated and injected into wild-type host blastocysts for analysis throughout haematopoiesis, overcoming the lethality of *notch1*-null embryos. It was found that these *notch1*-null ES cells could proliferate, form EBs, differentiate into *flk1*⁺ mesodermal cells, and form CFCs normally, whilst they were unable to contribute to long-term haematopoietic cells, suggesting a requirement for notch1 in definitive haematopoiesis (Hadland et al., 2004). Expression of endothelial and haemogenic endothelium markers appeared normal in *Notch1*-null embryos by semiquantitative RT-PCR but there was a marked decrease in expression of haematopoietic markers (Kumano et al., 2003). However, detailed analysis of arterial specification was not carried out, so it still remains possible that the haematopoietic phenotype observed is due to incorrect arterial programming. In *mib* deficient zebrafish embryos, and *CSL* deficient mouse P-Sp culture, haematopoietic specification can be rescued by exogenous expression of *runx1* mRNA (Burns et al., 2005; Nakagawa et al., 2006). Hes1 was shown to bind to, and activate, *runx1*, and *runx1*-rescued *notch1*-null murine cells

demonstrated CFC activity, showing characteristics of haematopoietic cells. This suggests that the role of notch signalling in haematopoietic development is via a Notch-Hes1-Runx1 pathway (Burns et al., 2005; Nakagawa et al., 2006). Evidence for a direct notch signalling input into HSC development *in vivo*, in the absence of an arterial defect, came from *Jag-1* null murine embryos (Robert-Moreno et al., 2008). Whilst *Jag-1* null embryos show some vascular defects, arterial specification appeared normal, as demonstrated by the correct expression of arterial-specific *ephrinB2*, *CD44* and *SMA*. Importantly, *Jag-1* null embryos generated haemogenic endothelium and definitive haematopoietic cells in much smaller numbers than wild type littermates, as determined by Ly-6A-GFP transgene expression (specifically labelling haemogenic endothelium and HSCs) and CFC assays (Robert-Moreno et al., 2008). Notch is proposed to act by directly regulating *Gata2* and *Hes1* in haematopoietic cells, whilst notch targets *Hey1* and *Hey2* are also expressed in the DA (Robert-Moreno et al., 2005; Robert-Moreno et al., 2008).

Whilst it is becoming accepted that notch is essential for development of the haematopoietic system, it is known that notch is dispensable for the maintenance of HSCs (Duncan et al., 2005; Maillard et al., 2008). Instead notch is involved in haematopoietic lineage specification (reviewed in Gering and Patient, 2008).

Primitive haematopoiesis in *notch1*^{-/-} mice was found to be relatively normal, as analysed by colony forming cell (CFC) activity in the yolk sac (Kumano et al., 2003). Similarly, zebrafish embryos treated with notch inhibitor DAPT, a γ-secretase inhibitor which prevents cleavage of the notch receptor, or *mindbomb* mutant zebrafish, mutant for a protein essential for notch ligand maturation, showed no effect on primitive haematopoiesis (Burns et al., 2005; Gering and Patient, 2005). However, more recent studies show that decreased notch signalling causes a reduction in the number of erythrocytes without affecting apoptosis or cell proliferation (Lee et

al., 2009). This suggests a notch role in cell fate determination for primitive haematopoietic lineages, with notch signalling negatively regulating the number of endothelial cells and acting as a cell-fate switch between endothelial and haematopoietic lineages (Lee et al., 2009). This study is in direct conflict to the publication of notch signalling acting as a factor controlling apoptosis of primitive erythrocytes in the mouse yolk sac (Robert-Moreno et al., 2007). Thus, the exact role of notch signalling on primitive haematopoiesis is yet to be determined.

In addition to the above mentioned roles of notch signalling in vasculogenesis, arterial specification and primitive and definitive haematopoiesis, notch is a well characterised signal in the control of angiogenic sprouting of intersomitic vessels (reviewed in Siekmann et al., 2008). Notch is necessary to restrict angiogenic behaviour to tip cells, rather than stalk cells, in zebrafish embryos: loss of notch causes excessive ISV sprouting by promoting tip cell formation, whilst overexpression of notch causes a reduction of angiogenesis through promotion of stalk cell fate (Siekmann and Lawson, 2007). Angiogenesis of ISVs is mediated in cells not expressing notch through expression of *flt4* and subsequent detection of VEGF signalling, whilst stalk cells expressing notch do not express *flt4*, and are therefore not receptive to VEGF signalling (Siekmann and Lawson, 2007).

1.9. *Xenopus* as a model organism

Xenopus laevis (African clawed frog) is a species commonly used in developmental biology (reviewed in Beck and Slack, 2001). Large batches of eggs can be obtained throughout the year by inducing females with human chorionic gonadotropin (hCG). Fertilisation of the eggs occurs *in vitro*, giving rise to large batches of synchronously developing embryos. Alternatively, embryos can be collected after natural mating of females with males although embryos are generally less synchronous with this method. Embryos survive in simple salt-buffered solutions, surviving

initially on intraembryonic yolk reserves. Compared to murine development, *Xenopus* embryos develop rapidly; the body plan is laid down within the first 24 hours of development. *Xenopus* eggs are large; approximately 1 millimetre in size. This, together with their *ex utero* development, makes embryos ideal for fate mapping and grafting experiments.

Xenopus embryos have the ability to survive for a relatively long time without a fully functional cardiovascular system. Diffusion of oxygen through the epidermis to tissues in the embryo mean *Xenopus* is able to develop normally without blood flow where other species such as the mouse would not survive. This, together with the large number of molecular tools available for the analysis of gene function in development makes *Xenopus* embryos highly amenable for the study of blood and endothelial development.

1.9.1. Molecular tools of *Xenopus laevis*

Antisense morpholino oligonucleotides (MOs) provide a way to specifically knock down gene function (Heasman et al., 2000). This is an important development as traditional methods of gene targeting are not currently feasible in *Xenopus*. Morpholinos are nucleic acid analogues where the deoxyribose ring characteristic of DNA is replaced with a morpholine ring, and where phosphate linkage groups are replaced with non-ionic phosphorodiamidate to provide resistance to nucleases. They can be designed to bind anywhere in the 5' UTR of a gene, or up to 25 nucleotides downstream of the translation start site to prevent ribosome binding, thus preventing translation of the gene. Alternatively, morpholinos can be designed to target splice acceptor or donor sites, altering the splicing pattern of the gene to produce truncated or altered proteins (Eisen and Smith, 2008; Heasman, 2002). Small molecule chemical inhibitors have also become an established method of studying signalling pathways in *Xenopus*. Due to the *ex utero*

and *ex ovo* development of *Xenopus*, they are an ideal system in which to use chemical inhibitors (reviewed in Wheeler and Liu, 2012).

Some progress had been made in producing transgenic *Xenopus laevis* embryos using restriction enzyme-mediation integration (REMI) (Kroll and Amaya, 1996). This method uses plasmid DNA integrated into decondensed sperm nuclei to fertilise eggs. Alternatively, recombination using I-SceI meganuclease has been shown to produce transgenic *Xenopus* (Ogino et al., 2006; Pan et al., 2006). Transposase-mediated transgenic recombination has also been demonstrated in *Xenopus* using the Tol2 transposable element identified in medaka fish *Oryzias latipes* (Kawakami et al., 2004; Kawakami et al., 1998), and the sleeping beauty synthetic transposon system constructed from the genomes of multiple salmonid fish (Ivics et al., 1997; Sinzelle et al., 2006).

Xenopus laevis has a large expressed sequence tag (EST) database (reviewed in Gilchrist, 2012) and attempts are currently being made to sequence the genome. The *Xenopus* EST database provides a system to identify *Xenopus* homologues of known genes from other species. Using *in situ* hybridisation, both whole-mount and on sections, it is possible to study *Xenopus* gene expression throughout embryonic development.

1.9.2. Overview of *Xenopus* blood development

Early transplantation studies on amphibian embryos using diploid and triploid embryos revealed two temporally and spatially separated sites of haematopoiesis (Turpen et al., 1981). The ventral blood island (VBI), a yolk sac analogue located in the ventral mesoderm between the liver anlagen and the proctodeum, is the site of primitive haematopoiesis. Ventral to the somites and posterior to the pronephros is the dorsolateral plate (DLP) mesoderm containing precursors to the

amphibian equivalent of the AGM, the site of adult blood specification (Kau and Turpen, 1983; Maeno et al., 1985; Turpen et al., 1981).

It was originally thought that haematopoietic tissue was derived solely from the equatorial ventral mesoderm (ventral marginal zone; VMZ) of the blastula embryo. Early fate mapping experiments carried out by Dale and Slack detected, but disregarded, a dorsal contribution of mesoderm to haematopoietic tissues, and concluded that haematopoietic tissue derived solely from ventral tissue (Dale and Slack, 1987). Several studies have since re-investigated the contribution of dorsal mesoderm to blood, finding a significant contribution of the dorsal mesoderm to the VBI, in addition to the already known ventral contribution to both the VBI and DLP.

In one such study, it was demonstrated that the *Xenopus* homologue of *runx1* (originally named *Xaml1*) is expressed in the VBI at tailbud stages, and could therefore be used as a marker of primitive haematopoiesis. Cultured explants of dorsal mesoderm (dorsal marginal zone; DMZ) in isolation were shown to express *runx1*, and injection of a truncated dominant-negative form of *runx1* into the DMZ of the embryo affected primitive haematopoiesis in the anterior VBI, thus showing that the dorsal mesoderm contributed to the primitive haematopoietic tissues (Tracey et al., 1998). Lineage labelling experiments were carried out to clarify this observation. Injection of lineage tracer *lacZ* into individual blastomeres of a regularly-cleaving 32-cell embryo demonstrated that the VBI and DLP are spatially distinct and separately derived from the 32-cell stage; the anterior VBI is derived from dorsal blastomeres D1 and C1, the posterior VBI is derived from ventral blastomere D4 and the DLP from ventral blastomere C3 (Ciau-Uitz et al., 2000, and Figure 1.10). At later stages the lineage label from blastomere C3 which contributes to the DLP is found to migrate to the midline, labelling the dorsal aorta (DA) and the haematopoietic clusters

associated with the ventral wall of the DA (Ciau-Uitz et al., 2010a; Ciau-Uitz et al., 2000). These experiments confirm the findings of Turpen *et al*, that primitive and definitive haematopoiesis are spatially separate (Turpen et al., 1997). Although the origins of primitive and definitive haematopoiesis have been shown to be spatially separate from early stages, cells from the VBI and DLP are able to contribute to either primitive or definitive haematopoiesis when transplanted into the appropriate microenvironment at an early stage (stages 12-14). This suggests that haematopoietic cells show certain plasticity, to a point, and that local environmental cues have a crucial role in specifying mesoderm to form primitive and definitive haematopoietic cells. Despite this early plasticity, the migration of haematopoietic cells from the VBI to seed the DLP has been ruled out by lineage labelling, transplantation and explant studies which show that adult and embryonic blood have entirely separate origins in *Xenopus* (Bertwistle et al., 1996; Ciau-Uitz et al., 2000)

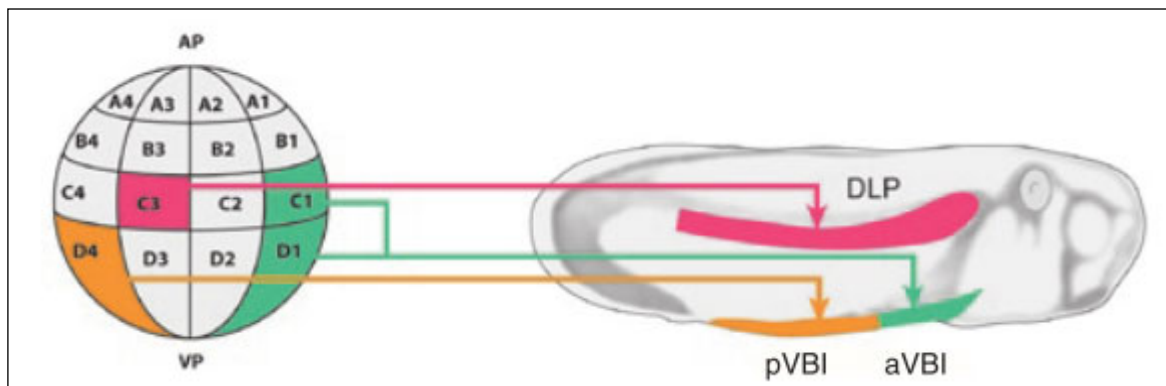


Figure 1.10 Separate origins of primitive and definitive blood in 32-cell stage *Xenopus laevis* embryos.

(Figure taken from Dzierzak and Speck, 2008).

1.9.2.1. Primitive haematopoiesis and the VBI

Lineage labelling and explant culture experiments have demonstrated that the VBI derives from both dorsal and ventral mesodermal tissue; the anterior VBI (aVBI) originating from the dorsal mesoderm (DMZ) and the posterior VBI (pVBI) originating from the ventral mesoderm (VMZ) (Ciau-Uitz et al., 2000; Tracey et al., 1998; Walmsley et al., 2002).

Cells of the aVBI travel to this site through the entire length of the embryo during gastrulation. Slightly anterior to the eventual site of the VBI these cells begin expressing both blood and endothelial genes, and through lineage labelling experiments have been shown to be a haemangioblast population. As development of the embryo progresses these haemangioblast cells migrate posteriorly to the site of the anterior VBI, and progressively become restricted to either blood or endothelial lineages (Ciau-Uitz et al., 2000; Walmsley et al., 2002; Walmsley et al., 2008). The peripheral cells of the aVBI express endothelial markers such as *fli1*, *hex* and *flk1* before forming the vitelline vessels of the VBI. The cells in the centre of the aVBI express early blood markers essential for the specification of primitive blood including *scl*, *lmo2*, *gata1* and *runx1*, and these cells will then give rise to primitive blood as determined by the expression of α -*globin* (Ciau-Uitz et al., 2000; Mead et al., 2001; Tracey et al., 1998; Walmsley et al., 2002).

Cells of the pVBI originating from ventral mesoderm do not have to travel as far during gastrulation as their aVBI counterparts. Analysis of gene expression and lineage labelling experiments suggest that cells of the pVBI do not form a haemangioblast population, or if such a population is generated it is done so very transiently as to not be detectable by currently available methods (Ciau-Uitz et al., 2000). Cells of the pVBI express *runx1*, *scl* and *lmo2*, followed by α -*globin*, and are thought to arise *de novo* at later stages of development, rather than via the migration of cells from the aVBI (Ciau-Uitz et al., 2000; Tracey et al., 1998; Walmsley et al., 2002;

Walmsley et al., 2008). It is however believed that signalling from the aVBI to the pVBI is required for the correct specification of primitive blood in the pVBI (Walmsley et al., 2002). Injection of RNA encoding a dominant-negative BMP receptor (tBR) into the DMZ of the 4-cell embryo, which will later give rise to the dorsal mesoderm, eliminates expression of blood and endothelial genes in the aVBI. In addition, expression of α -globin in the pVBI is also reduced, highlighting the need for signalling from the aVBI to the pVBI (Walmsley et al., 2002).

Myeloid cells are also being specified in the aVBI, as seen by the expression of the myeloid marker *pox/mpo* in the haemangioblast population. The myeloid cells will later migrate posteriorly through the blood island before dispersing throughout the embryo (Smith et al., 2002). Later in development, myeloid cells will be specified in the pVBI, independently of those in the aVBI, by independent mechanisms (Maeno et al., 2012).

Erythropoiesis begins in the VBI between stages 26 and 28, as detected by expression of the primitive erythroid marker α -globin. This production of erythrocytes continues until stage 40 when the VBI loses the ability to form primitive erythrocytes (Mangia et al., 1970; Ohinata and Enami, 1991). Following initial vascularisation of the embryo, erythrocytes enter circulation at stage 34, and precursors move from the VBI to the embryonic liver (Ohinata and Enami, 1991). In addition to the primitive erythroid and myeloid cells, the VBI will give rise to haematopoietic progenitors which go on to seed the liver, thymus and spleen, participating in haematopoiesis until late embryonic stages (Maeno et al., 1985). The precise origin of these cells in the VBI is currently unknown, as is the stage at which they are specified, but it is known that these cells can only contribute to haematopoiesis at larval stages, and do not contribute to haematopoiesis post-metamorphosis, into adult life (Hollyfield, 1966).

1.9.2.2. Definitive haematopoiesis, the DLP and the DA

A population of dorsolateral plate (DLP) mesodermal precursors contribute to the development of the DA and HSCs (reviewed in Ciau-Uitz et al., 2010a). Initial transplantation studies revealed the DLP as the primary source of adult haematopoietic cells in amphibia (Kau and Turpen, 1983; Maeno et al., 1985; Turpen et al., 1997; Turpen et al., 1981). These DLP cells expressing endothelial marker, *flk1*, migrate to the midline of the embryos following VEGF signalling from the hypochord, and go on to form the DA directly under the hypochord (Cleaver and Krieg, 1998). Later lineage labelling and gene expression studies have demonstrated that DLP cells can be observed migrating to the midline of the embryo, and are shown to contribute to the DA endothelium, the mesenchyme underlying the DA, and importantly to the intra-aortic clusters believed to be the source of definitive HSCs, suggesting the DLP contains precursors to the definitive HSCs (Ciau-Uitz et al., 2000; Walmsley et al., 2002). Cells of the DLP co-express blood and endothelial genes, including *fli1*, and cells ventral to the DLP co-express endothelial and pronephric markers (Ciau-Uitz et al., 2010b; Ciau-Uitz et al., 2000). That the DLP co-expresses blood and endothelial genes and later gives rise to major vessels and definitive HSCs signifies that the DLP may be classed as a definitive haemangioblast (Ciau-Uitz et al., 2000; Walmsley et al., 2002): this population of cells will be referred to as a haemangioblast population throughout this thesis. However, it is important to note that individual cell lineage analysis has not been carried out on these cells, therefore it can not be presumed that individual cells have the capacity to generate both endothelial and haematopoietic lineages - it is the population as a whole which is referred to as a haemangioblast..

It is known that both BMP and ETS transcription factors play an important role in programming the DLP, (Liu et al., 2008; Walmsley et al., 2002). A genetic hierarchy regulating the programming of the DLP has recently been uncovered (Figure 1.11, Ciau-Uitz, personal communication). In

addition to the genetic hierarchy within the DLP, signalling of *vegfa* from the somites to the DLP has been shown to be a requirement for correct DLP specification, including regulating the expression of haematopoietic marker *scl* (Ciau-Uitz et al., 2010b). A subset of *fli1* expressing DLP cells start to migrate to the DLP at stage 27, and finish migrating by stage 31, where they go on to develop a lumenised vessel (Ciau-Uitz et al., 2000, and Figure 1.12). This expression data fits with the known fate of *flk1* expressing cells in the DLP (Cleaver and Krieg, 1998). The *fli1* expressing cells remaining in the DLP go on to form the posterior cardinal veins and pronephric duct (Ciau-Uitz et al., 2000). At no point are cells which express haematopoietic markers such as *scl* and *gata2* seen migrating to the midline, however expression of *gata2* is re-initiated once the cells reach the midline, in the ventral wall of the DA, while *scl* expression can be seen in clusters associated with the ventral wall of the DA (Ciau-Uitz et al., 2000; Walmsley et al., 2002). Whilst *runx1* expression is not detected in the DLP during *Xenopus* development, expression is seen in the ventral wall of the DA and underlying mesenchyme from stage 35, and in the clusters associated with the ventral wall of the DA at stage 43 (Ciau-Uitz et al., 2000). This expression is consistent with the role of *runx1* in the endothelial to haematopoietic transition (Kissa and Herbomel, 2010; Li et al., 2006; North et al., 1999). Although there is currently no functional assay in *Xenopus* to test the intra-aortic cluster population for HSC activity, such as the LTR assay in mouse, the expression analysis data together with their temporal and spatial emergence strongly indicates that these are HSCs (Ciau-Uitz et al., 2000). Once these definitive HSCs are fully specified in the DA, they move on to seed the thymus, spleen and liver, where they will reside for the life of the adult. Although cells from both the VBI and DLP colonise these haematopoietic tissues, it is known that only the definitive progenitors initially specified in the DLP and DA remain post-metamorphosis to provide blood for the entire life of the adult (Maeno et al., 1985).

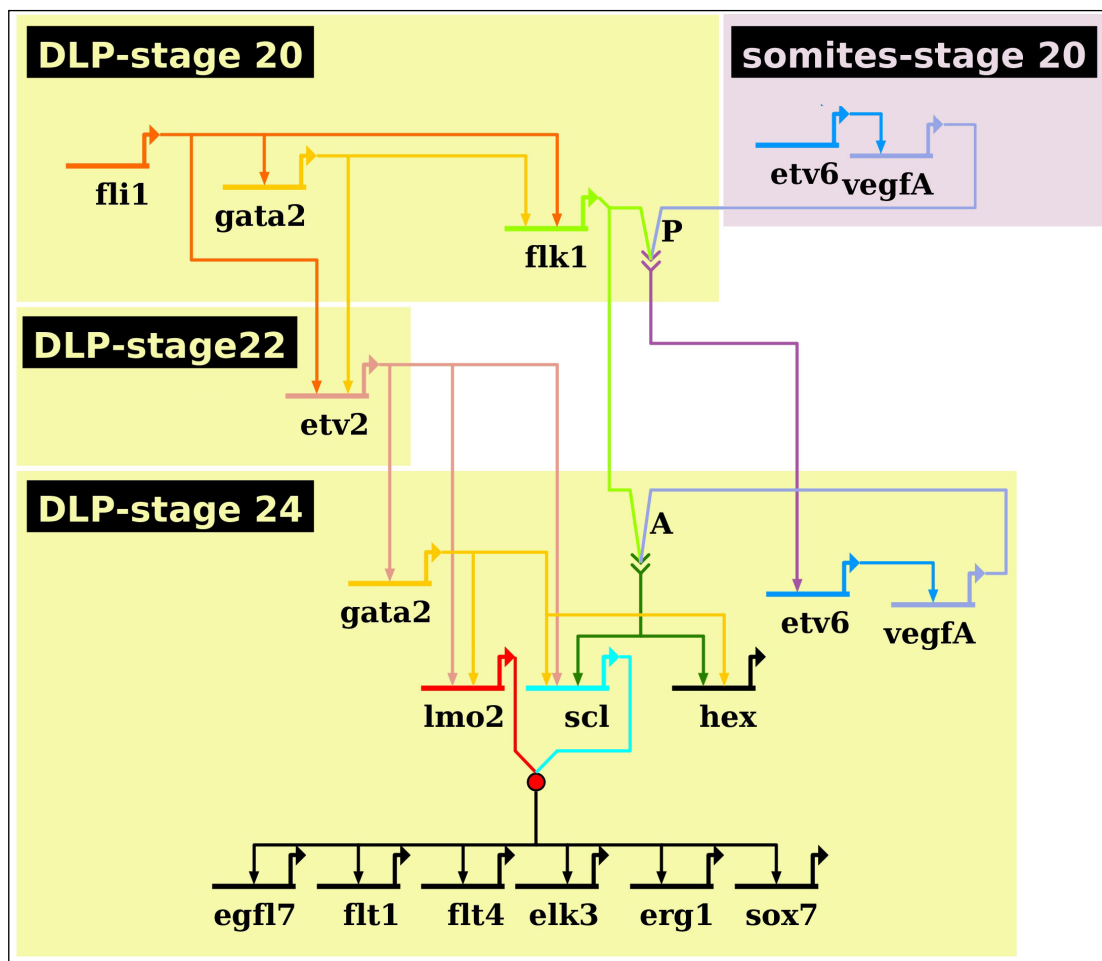


Figure 1.11 Gene regulatory network showing a hierarchy of genes required for DLP programming

A tightly controlled gene regulatory network is evident in the DLP for programming of the haemangioblast. Paracrine signalling from the somites via vegf is required in addition to the autocrine DLP programme. Thanks to Dr A. Cia-Uitz for this figure.

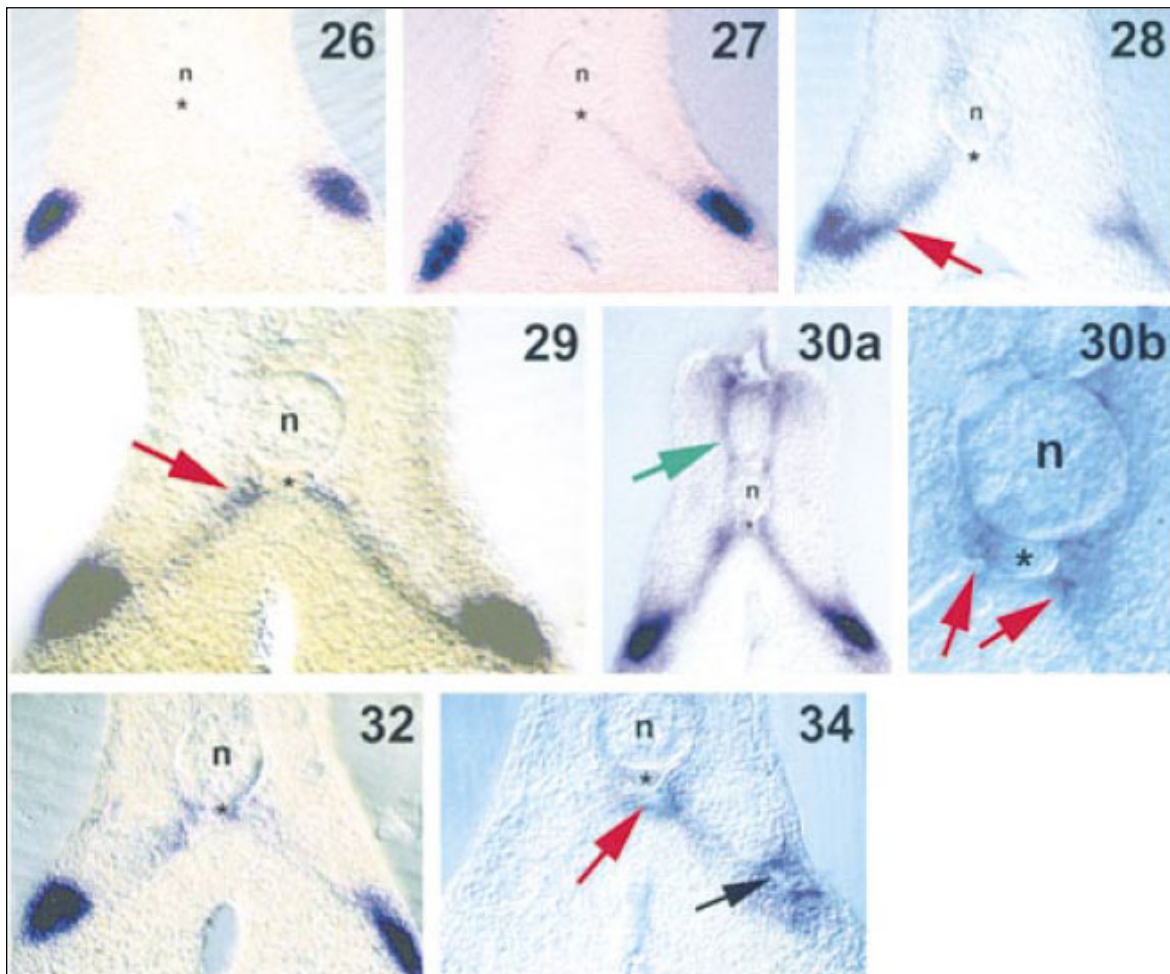


Figure 1.12 Migration of *fli1* expressing cells from the DLP to the midline

Time course showing *fli1* expressing endothelial cells migrating from the DLP to the midline. At stages 26 and 27, *fli1* expressing cells reside in the DLP. At stage 28 these cells begin migrating from the DLP to the midline (red arrow). At stage 30, *fli1* expressing cells can be seen migrating from the dorsal fin to the midline in response to VEGF secretion from the hypochord (green arrow). The expression of *fli1* staining between the DLP and the midline is absent at stage 32, indicating migration of the DLP cells has finished. At stage 34, *fli1* expressing cells can be seen at the midline, forming the DA (red arrow). Some cells remain in the DLP, going on to form the posterior cardinal vein (black arrow, stage 34). Stages are indicated by numbers in the top right corner of each image. Notochord (n) and hypochord (black asterisk, *) are indicated on each image. (Taken from Ciau-Uitz et al., 2000).

1.10. Overview and Objectives

Whilst several studies have revealed a requirement for notch signalling in vascular and arterial programming, a contribution of notch signalling specifically to HSC programming has been difficult to prove. Definitive haematopoietic clusters derive from ventral aortic endothelium during embryogenesis: where aortic endothelium is incorrectly specified in these notch pathway mutants it is predicted that subsequent programming of haemogenic endothelium will also be compromised. Thus, it is not possible to study the direct effect of these notch pathway mutations on haematopoietic cluster emergence. In addition, a major limitation to studying the role of notch signalling in haematopoietic specification is that many notch pathway mutants die during embryonic development due to defects in vascular morphogenesis (Hrabe de Angelis et al., 1997; Krebs et al., 2004; Krebs et al., 2000; Limbourg et al., 2005; Xue et al., 1999).

The aim of this thesis is to understand the role of notch signalling in haematopoietic stem cell development in *Xenopus laevis*. The *ex-utero* development of *X.laevis* embryos allows survival for a relatively long time without a fully functional cardiovascular system. Thus, where murine notch pathway mutants are embryonic lethal due to the defective cardiovascular system, *Xenopus* can overcome these problems due to diffusion of oxygen through the epidermis.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Maintenance of frogs

Mature male *Xenopus laevis* were purchased from the European Xenopus Research Centre (EXRC) or Nasco, and females were purchased from Nasco. They were maintained at 19°C on a 12-hour light/dark cycle and were fed two times a week – once on pellets and ox heart, and once on pellets alone.

2.1.2. Solutions

All solutions were prepared with RNA quality (Milli-Q®) water, and sterilised by autoclaving where appropriate.

AP Buffer:

0.1M Tris, pH 9.5; 50mM MgCl₂; 0.1M NaCl; 0.1% Tween

Bleaching Solution:

5% formamide (VWR); 0.5% SSC, 33% H₂O₂ (VWR)

50X Denhardt's solution:

2% BSA (Fraction V, Sigma); 2% Ficoll 400 (Sigma); 2% Polyvinylpyrrolidone (Sigma)

Hybridisation Mix for whole-mount *in situ* hybridisation (WISH):

50% formamide; 5X SSC; 1mg/ml yeast RNA; 100µg/ml Heparin (Sigma)

LB:

10g bactotryptone; 5g bactoyeast; 10g NaCl in 1 litre dH₂O

LB Agar:

LB containing 15g bactoagar per litre

Maleic Acid Buffer (MAB):

100mM maleic acid (Sigma); 150mM NaCl (Sigma); pH7.5.

MAB/Tw:

MAB with 0.1% Tween20 (Sigma)

MAB with block:

MAB with 2% boehringer block (Roche); dissolved at 67°C, stored at -20°C.

MEMFA:

0.1M MOPS (3-(N-morpholino) propanesulfonic acid), pH7.4; 2mM EGTA; 1mM MgSO₄. Add 3.7% formaldehyde (Sigma) immediately before use.

Modified Barth's Saline (MBS):

88mM NaCl; 1mM KCl; 2.4mM NaHCO₃; 15mM HEPES; 0.3mM CaNO₃.4H₂O; 0.41mM CaCl₂.6H₂O; 0.82mM MgSO₄.7H₂O; pH7.6. 10µg/ml penicillin/streptomycin (Sigma) added before use.

MS222:

5g MS222 (ethyl-3-amino benzoate methanesulfonate salt) in 1 litre dH₂O; pH7.0

PBS/Tw:

PBS (Invitrogen) containing 0.1% Tween20

20X SSC:

3M NaCl; 0.3M sodium citrate

25X TAE:

2M Tris-acetate; 50mM EDTA

TEA:

0.1M Triethanolamine (Fluka); pH7.9

2.1.3. *In situ* hybridisation probes

Probe	Restriction Enzyme	RNA Polymerase	Reference	Source
<i>aa4</i>	SmaI	T7	IMAGE: 5515354	A. Ciau-Uitz
<i>ami</i>	EcoRI	T7	IMAGE: 7204180	A. Ciau-Uitz
<i>cx37</i>	Sall	T7	IMAGE: 5512615	A. Ciau-Uitz
<i>dll1</i>	SmaI	T7	IMAGE: 6636221	A. Ciau-Uitz
<i>dll3</i>	NotI	T7	XL013d08	A. Ciau-Uitz
<i>dll4</i>	EcoRI	T7	IMAGE: 6875578	A. Ciau-Uitz
<i>egfl7</i>	SmaI	T7	IMAGE: 5542405	A. Ciau-Uitz
<i>ephB4</i>	NotI	T7	XL100e24	A. Ciau-Uitz
<i>ephrinB2</i>	NotI	T7	XL056a06	A. Ciau-Uitz
<i>esr1</i>	NotI	T7	XL064k09	A. Ciau-Uitz
<i>esr2</i>	NotI	T7	XL031a05	A. Ciau-Uitz
<i>esr4</i>	SmaI	T7	IMAGE: 6634542	A. Ciau-Uitz
<i>esr5</i>	SmaI	T7	IMAGE: 5047232	A. Ciau-Uitz
<i>esr6e</i>	Sall	T7	IMAGE: 5161871	A. Ciau-Uitz
<i>esr7</i>	Sall	T7	IMAGE: 4959168	A. Ciau-Uitz
<i>esr9</i>	NotI	T7	IMAGE: 4085131	A. Ciau-Uitz
<i>esr10</i>	SmaI	T7	IMAGE: 7976915	A. Ciau-Uitz
<i>etsrp/etv2</i>	NotI	T7	XL052p03	A. Ciau-Uitz
<i>flk1</i>	SmaI	T7	XL087o23	A. Ciau-Uitz
<i>flt1</i>	SmaI	T7	IMAGE: 4959298	A. Ciau-Uitz
<i>flt4</i>	Sall	T7	IMAGE: 4970772	A. Ciau-Uitz
<i>gata1</i>	XhoI	SP6	(Bertwistle et al., 1996)	M. Walmsley
<i>gata2</i>	XbaI	SP6	Walmsley <i>et al.</i> 1994	M. Walmsley
<i>gfi1a</i>	EcoRI	T7	IMAGE: 8547327	A. Ciau-Uitz
<i>αT4 globin</i>	EcoRI	SP6		M. Walmsley
<i>hairy1a</i>	SmaI	T7	IMAGE: 4030543	A. Ciau-Uitz
<i>hairy2b/hes4b</i>	NotI	T7	XL106p24	A. Ciau-Uitz
<i>hes2</i>	XhoI	T7	IMAGE: 495039	R. Stephenson
<i>hes3-like a</i>				Cloned by R. Stephenson
<i>hes6.1</i>			IMAGE: 8321242	R. Stephenson
<i>hes7.1</i>	NotI	T7	XL144j08	A. Ciau-Uitz
<i>hes8</i>				Cloned by R. Stephenson
<i>hesr1b</i>	NotI	T7	XL097h17	A. Ciau-Uitz
<i>hesr2</i>	EcoRV	T7	IMAGE: 8332260	A. Ciau-Uitz
<i>hes7.1</i>	NotI	T7	XL144j08	A. Ciau-Uitz
<i>jagged1</i>	SmaI	T7	IMAGE: 6634431	A. Ciau-Uitz
<i>jagged2</i>	NotI	T7	XL054p21	A. Ciau-Uitz

<i>mpo</i>	NotI	T7	XL038o23	A. Ciau-Uitz
<i>notch1</i>	NotI	T7	XL083p22	A. Ciau-Uitz
<i>notch2</i>	SmaI	T7	IMAGE: 4964146	A. Ciau-Uitz
<i>notch3</i>	RsrII	T7	IMAGE: 7641943	A. Ciau-Uitz
<i>notch4</i>	SmaI	T7	IMAGE: 4684242	A. Ciau-Uitz
<i>runx1</i>	SaII	T7	(Tracey et al., 1998)	A. Ciau-Uitz
<i>Scf</i>	XhoI	SP6		A. Ciau-Uitz
<i>spiB</i>	EcoRI	T7	IMAGE: 5537169	A. Ciau-Uitz
<i>tie2</i>	NotI	T7	XL064i22	A. Ciau-Uitz
<i>vegfa</i>	BamHI	T7	(Cleaver et al., 1997)	A. Ciau-Uitz

Table 2.1. Details of *in situ* hybridisation probes used

2.2. Methods

2.2.1. Preparation and manipulation of DNA

2.2.1.1. Bacterial transformation and culturing

DH5 α *Escherichia coli* cells (NEB) were incubated with plasmid DNA on ice for 30 minutes before being heat-shocked at 42°C for 60s and returned to ice for two minutes. Next, 500 μ l LB or SOC medium (NEB) was added and bacteria were cultured at 37°C for 60 minutes before being plated on LB agar containing 50 μ g/ml ampicillin or kanamycin, as required, and grown at 37°C overnight. Colonies were cultured in LB containing 50 μ g/ml ampicillin or kanamycin, as required, at 37°C overnight with moderate shaking.

2.2.1.2. Preparation of plasmid DNA

Small scale preparations of plasmid DNA (2ml) were carried out using QuickLyse Miniprep Kit (Qiagen), whilst large scale preparations (200ml) were carried out using GenElute HP Plasmid Kit (Sigma), according to the manufacturer's instructions.

2.2.1.3. Determination of DNA and RNA concentrations

The concentration of DNA and RNA in a solution was measured using a NanoDrop (Thermo Scientific), measuring the UV absorption at 260nm. Purity was measured as a ratio of absorption at 260:280nm.

2.2.1.4. DNA sequencing

DNA templates were sequenced by the MHU in-house sequencing facility using an ABI-3730 DNA analyser and reactions carried out using BigDye Terminator v3.1 (Applied Biosystems) as per manufacturer's instructions.

2.2.1.5. Preparation of DNA templates for *in vitro* reactions

DNA for *in vitro* transcription was linearised using the appropriate enzyme (section 2.2.1.6) and then purified using QIAquick PCR Purification Kit® (QIAGEN) according to the manufacturer's instructions.

2.2.1.6. Restriction enzyme digestion

DNA was digested using the appropriate restriction enzyme (NEB or Roche) and corresponding buffer supplied, according to manufacturer's instructions. Digestion of DNA was confirmed by visualising the digested DNA on a gel and comparing it to undigested DNA.

2.2.1.7. Gel electrophoresis

DNA and RNA were analysed on 1% agarose/0.5X TAE gel containing GelRed™ (Cambridge Bioscience) and run in 0.5X TAE at 50-130V. DNA and RNA intercalated with GelRed in a gel were viewed under UV light. DNA marker ladders (100bp or 1kb, NEB) were run and used to estimate length of DNA.

2.2.1.8. Concentration of DNA and RNA by ethanol precipitation

DNA and RNA were precipitated from solution by adding sodium acetate (pH5.2) to a final concentration of 0.3M followed by 2.5 volumes of ethanol. The solution was mixed and put on dry ice for 10 minutes, or at -20°C for at least 30 minutes. Samples were centrifuged at 4°C, 12,000g, for 30 minutes. Following centrifugation, the supernatant was removed and the pellet washed with 70% ethanol. The sample was centrifuged for five minutes at room temperature at 12,000g before removing the supernatant and drying the pellet in air and resuspending in autoclaved Milli-Q water.

2.2.1.9. Ligation of DNA fragments

Insert and vector were mixed together at a ratio of 3:1. Rapid DNA ligase and buffer (Roche) were added and left at RT for 5 minutes, as per the manufacturer's instructions. Cloning of PCR fragments was carried out directly using pGEM®-T Easy Vector Systems (Promega), as per the manufacturer's instructions.

2.2.1.10. Amplification of DNA by Polymerase Chain Reaction (PCR)

DNA polymerases were obtained from Roche, Sigma or Thermo Scientific. Template DNA was mixed with PCR amplification buffer, 0.2mM dNTPs, 10pmol of each forward and reverse primer, and DNA polymerase, in a total volume of 20 or 50µl, according to manufacturer's instructions. In a typical programme, the reaction had heated to 94°C for 4 minutes (step 1) for initial denaturation of the DNA, and to activate hot-start enzymes. Following this, up to 35 cycles were performed at 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 1 minute. A final extension at 72°C for 10 minutes was then performed. Products were analysed by running 5-10µl of the reaction on a gel. Primers used for PCR can be found in Table 2.2.

2.2.1.11. Construction of Gateway® clones

attB-flanked PCR products that contain morpholino binding sites were generated for each gene, as outlined in the manufacturer's instructions (Invitrogen) so that in-frame C- and N-terminal fusion proteins could be easily generated using the Gateway® system. BP recombination reactions with PCR products and an attP donor vector (pDONOR221) were carried out to create an attL Gateway® entry clone as specified in the manufacturer's instructions (Invitrogen). LR reactions between Gateway® entry clones and an attR *gfp* destination vector were carried out to make C-terminal *gfp* expression clones for morpholino knockdown assays. Constructs were sequenced using gene specific forward primers and GFP-CS2 reverse primer. The GFP destination vector was

constructed by Laura Mitchell and *esr1*, *esr7*, *esr10*, *hesr1*, *hesr2* and *notch1* entry clones were designed and constructed by myself.

Primer Name	F/R	Purpose	Sequence (5'-3')
esr1 gateway F	Forward	Gateway	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCGT GCACTCAGCTCTTACACA
esr1 gateway R	Reverse	Gateway	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCGGAAGAGA AAGTATCCAGAGA
esr3 F	Forward	Cloning/ 5' RACE	ATGGCACCCAGCACTGCTTTT
esr3 R	Reverse	Cloning/ 5' RACE	CCCCTTACTAGAGGTACAG
esr3 R3	Reverse	5' RACE	CATTGGGCTCCTGTGGTGGA
esr3 R2	Reverse	5' RACE	TGGCTTCTGCTTCTGGCTGCT
esr7 gateway F	Forward	Gateway	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCGA TTGTTACTCCAGAACCTC
esr7 gateway R	Reverse	Gateway	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACAAC TGGCTTTCGTAG
esr10 gateway F	Forward	Gateway	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCTC CCAGCTCCATCATATCCA
esr10 gateway R	Reverse	Gateway	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCCTTGTG ACAGTGTTCACT
hesr1 gateway F	Forward	Gateway	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCCA CACACAGCAGCCATGAAG
hesr1 gateway R	Reverse	Gateway	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCCCATT CTCATCCGCGCT
hesr2 gateway F	Forward	Gateway	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCCG GAGGATTGATATACTACTCGG
hesr2 gateway R	Reverse	Gateway	GGGGACCACTTTGTACAAGAAAGCTGGGTCCATGTCACT GTCAGAGCTGGT
hesr2F	Forward	5' RACE	TGCTGCAGGCAACCGGAGGCAAA
hesr2R1	Reverse	5' RACE	AGCTAAGGTGGGAGACAAGCCGT
hesr2R2	Reverse	5' RACE	GGGTGCAGGGAATGCTGGTGGGGT
hesr2R3	Reverse	5' RACE	TGCCAGGGCGTAGGGCCTGCT
notch1 gateway F	Forward	Gateway	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCTG GGACAGGGACCTTCATCA
notch1 gateway R	Reverse	Gateway	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTGGGGA ACTGGCACCTTTC
notch4 F	Forward	5' RACE	GTCAGAGCTCTCCATGTCAGAA
notch4 R1	Reverse	5' RACE	GTCTCACACACTGAACCTTGGA

notch4 R2	Reverse	5' RACE	CAGGGATTGGAGAGACACTCAT
notch4 Ex2Acc MO f1	Forward	Analysis of splice mo	GTACAACCCCAGATGGCTGTA
notch4 Ex2Acc MO f2	Forward	Analysis of splice mo	GACCATGTTTCCAGCTAATTC
notch4 Ex2Acc MO r1	Reverse	Analysis of splice mo	TGTCCCAGCAGATCCAAGCAGGT
notch4 Ex2Acc MO r2	Reverse	Analysis of splice mo	TGCAGCTTCCCCATTCTGACA

Table 2.2. Sequences of primers used

2.2.1.12. 5' Rapid Amplification of cDNA Ends

5' RACE was carried out using the 5'/3' RACE Kit, 2nd Generation (Roche) or First Choice® RLM-RACE Kit (Invitrogen) according to the manufacturer's protocol. Primers for 5' RACE can be found in Table 2.2. Cleanup of intermediary reactions was carried out using the High Pure PCR Product Purification Kit (Roche) as recommended in the manufacturer's protocol, and PCR was carried out using Expand Long Range dNTPack (Roche). PCR products were cloned into pGEM®-T Easy (Promega) according to the manufacturer's protocol, transformed into high efficiency DH5α *E. coli* (see section 2.2.1.1) and sent for sequencing.

2.2.2. DNA cleanup

2.2.2.1. Phenol/Chloroform protein removal

To remove protein from RNA or DNA solutions, one volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and the tubes vortexed thoroughly before spinning for 5 minutes at 12,000g. The upper, aqueous, phase of the solution containing the DNA or RNA was collected and

re-extracted as above using one volume of chloroform/isoamyl alcohol (24:1). An ethanol precipitation of the aqueous phase was then carried out (see section 2.2.1.8).

2.2.2.2. Plasmid DNA purification

Plasmid DNA was cleaned up using QIAGEN QIAquick PCR Purification Kit®, according to the manufacturer's instructions.

2.2.2.3. Gel extraction

DNA was extracted from agarose gels after electrophoresis using QIAquick Gel Extraction Kit® according to the manufacturer's instructions. To visualise the DNA on the gel in order cut out specific bands of DNA, the gel was illuminated on a UV box set to 70% illumination.

2.2.3. *In vitro* synthesis reactions

2.2.3.1. *In vitro* transcription of RNA probes for *in situ* hybridisation

Fluorescein and digoxigenin (DIG) labelled antisense probes for *in situ* hybridisation were transcribed from linearised template using the appropriate RNA polymerase (Roche) in the presence of buffer and fluorescein or DIG labelled nucleotides (Roche) at 37 °C for at least two hours. Linearised plasmid was removed by digestion with RNase-free DNase I (NEB) at 37°C for 30 minutes. An aliquot of sample was removed prior to DNase I addition and run on a gel against a sample of post-DNase I treated probe to assay for complete DNA template removal before stopping the reaction with EDTA at a final concentration of 10mM. The probe was precipitated with 0.3M NH₄OAc and ethanol on dry ice for 30 minutes, or at -20°C for at least 2 hours. Samples were centrifuged at 4°C, 12,000g, for 30 minutes. Following centrifugation, the supernatant was removed and the pellet washed with 70% ethanol. The sample was centrifuged for five minutes at room temperature at 12,000g before removing the supernatant and drying the pellet in air and resuspending in autoclaved Milli-Q water or hybridisation mix.

2.2.3.2. *In vitro* transcription of mRNA for embryo injection

Plasmids for gateway and overexpression constructs were digested with the appropriate restriction enzyme (see section 2.2.1.6) to produce DNA template, and cleaned up using QIAGEN QIAquick PCR Purification Kit (see section 2.2.2.2). Sense RNA for injection was transcribed using the appropriate RNA polymerase mMessage mMachine Kit® (Ambion), as per manufacturer's instructions. The RNA was then extracted using QIAGEN RNeasy Micro Kit® (see section 2.2.4.2). Quality and quantity of RNA was analysed using both gel electrophoresis (see section 2.2.1.7) and measuring the UV absorption at 260nm using a NanoDrop (see section 2.2.1.3).

2.2.3.3. cDNA synthesis

Reverse transcription of cDNA was performed using Superscript® III One-Step RT-PCR System (Invitrogen) according to the manufacturer's instructions. Template in the form of total RNA was used, produced via TRI Reagent® extraction (Sigma) (see section 2.2.4.1) and subsequently RNeasy micro kit extraction (see section 2.2.4.2).

2.2.4. RNA extraction

2.2.4.1. TRI Reagent® extraction

Three to five *Xenopus* embryos were homogenised in 1ml TRI Reagent® (Sigma) using a mechanical homogeniser. Samples were incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes before adding 0.1ml of chloroform and vortexing briefly. Samples were subsequently incubated at room temperature for 2-15 minutes before being spun at 12,000g for 15 minutes at 4°C. The aqueous phase, containing RNA, was transferred to a new tube before adding 0.5ml isopropanol and vortexing briefly. Samples were subsequently incubated at room temperature for 5-10 minutes before centrifuging at 12,000g for

10 minutes at 4°C. The supernatant was removed and the RNA pellet washed with 75% ethanol before centrifuging at 7,500g for 5 minutes at 4°C. The pellet was air dried and resuspended in autoclaved Milli-Q® water.

2.2.4.2. QIAGEN RNeasy Micro Kit®

RNA was additionally extracted using QIAGEN RNeasy Micro Kit®, according to the manufacturer's instructions.

2.2.5. Obtaining and manipulating embryos

2.2.5.1. Obtaining embryos

To obtain embryos, female *Xenopus laevis* were injected with 500U human chorionic gonadotropin (Intervet) into the dorsal lymph sac approximately 14 hours before desired egg collection, and kept at 19°C overnight. Testes were removed from a Schedule 1 male *Xenopus laevis* and stored in 70% Liebowitz (L-15) growth medium (Invitrogen) supplemented with penicillin/streptomycin (10µg/ml) for up to two weeks at 4°C. Eggs were squeezed from females and fertilised immediately *in vitro* with testes macerated in 0.1X MBS. Once the embryos had turned so that the animal pole was dorsal, after approximately 40 minutes, the embryos were de-jellied in 2% cysteine/1X MBS (pH8.0), washed three times in 0.1X MBS and twice in 1X MBS before being stored at 14-23°C until required.

2.2.5.2. Injection of morpholino oligonucleotides or mRNA into embryos

Injection apparatus (PL1-100 Medical Systems Corps) was calibrated by counting the number of injections needed to empty 1µl of H₂O from a needle at a given injection time, and from this the correct injection time for a 4nl injection was calculated. Embryos were transferred to 3% ficoll (Sigma)/1X MBS on injection grids and orientated appropriately for injection. Embryos were

injected with 4nl of RNA or morpholino oligonucleotides (Gene Tools) in a minimal volume of 3% ficoll/1X MBS. Embryos were transferred to 0.1X MBS at stage 6-8 and left to develop at 14-23°C until the desired stage. Morpholinos used can be found in Table 2.3.

Morpholino	Sequence (5'-3')	Reference	Effective concentration
<i>esr1</i> ATG mo	GCTGGTAGGAGCCATTATCCTAAGT	(Sakano et al., 2010)	40ng
<i>esr7</i> ATG mo	TGCCATTTTGAGGTTCTGGAGTAAC	-	30ng
<i>esr10</i> ATG mo	CTGTTTAGTAAGTGGATATGATGGA	(Taelman et al., 2006)	35ng
<i>hesr1</i> ATG mo	TAGTCGTGTCCCCGCTTCATGGCTG	(Taelman et al., 2006) referred to as <i>XHRT1</i>	20ng
<i>hesr2</i> ATG mo	TGATGAGGTTCCGAGTAGTATATCA	-	-
<i>notch1</i> ATG mo	TGCAGTCTCACTATAGGAACATAGA	-	50ng
<i>notch4</i> splice mo	GGGAGGAACCTGATTGAAAAACATA	-	30ng

Table 2.3. Details of morpholinos used

2.2.5.3. Small molecule treatment of embryos

Small molecules were dissolved in dimethyl sulfoxide (DMSO) (Sigma) and stored in small aliquots at -20°C. Embryos were incubated from the appropriate stage in small molecules in 0.1X MBS. Control treatments consisted of treating sibling embryos with the corresponding amount of DMSO from the same stage of development. DAPM was obtained from Calbiochem and used at 100µM.

2.2.5.4. Fixing and preparation of embryos for whole mount *in situ* hybridisation

Embryos were manually removed from their vitelline membranes with forceps, if necessary, and placed in eppendorf tubes, 10-20 per tube. They were fixed in 1ml of MEMFA for at least one hour at room temperature and washed overnight in 100% methanol, followed by two further

methanol washes for 5-10 minutes each. Embryos were stored at -20°C in methanol at least overnight but can be stored for several months until needed for *in situ* hybridisation.

2.2.5.5. Extraction of RNA from embryos

RNA was extracted from embryos using TRI Reagent® extraction (see section 2.2.4.1), followed by RNeasy Micro Kit® RNA extraction (see section 2.2.4.2).

2.2.6. Analysis of embryos

2.2.6.1. Whole-mount *in situ* hybridisation (WISH)

Embryos were fixed and stored as described in section 2.2.5.4. All washes were carried out in a volume of 1ml if done manually in 1.5ml eppendorf tubes, or when done in an *in situ* machine (Intavis) washes were done in a total volume of 40ml (24-well, small plates) or 80ml (48-well, large plates), unless otherwise stated. Embryos were rehydrated in a graded series of methanol washes with PBS/Tw (75%, 50% and 25%) before being washed three times in PBS/Tw, 5 minutes each wash. Embryos were bleached in bleaching solution on a light box to remove pigment before being washed three times for 5 minutes each in PBS/Tw to completely remove bleaching solution. Embryos at stage 34 or later were permeabilized with Proteinase K (8µg/ml PBS/Tw) for 1 minute before being washed three times for 5 minutes each in 2mg/ml glycine (Sigma) PBS/Tw and then a further three washes for 5 minutes in PBS/Tw to completely remove Proteinase K solution. Embryos were acetylated by washing three times with 0.1M TEA for 5 minutes each. To the second wash, 2.5µl/ml acetic anhydride (VWR) was added and to the third wash 5µl/ml was added. TEA and acetic anhydride were removed by washing three times, 5 minutes each, in PBS/Tw. Embryos were equilibrated in 0.5ml pre-heated (67°C) hybridisation buffer for 5 minutes before replacing with fresh hybridisation buffer (0.5ml) and incubating at 67°C for at least 4

hours. The hybridisation buffer was replaced with fresh hybridisation buffer containing DIG or fluorescein labelled probe and embryos were left at 67°C overnight to allow probe hybridisation. Following incubation of the probes, the probe containing hybridisation buffer was removed and stored for further use (probes can be used at least three times). Excess probe was removed by washing 10 minutes each in preheated 50% formamide/5X SSC, 25% formamide/2X SSC, 12.5% formamide/2X SSC, 2X SSC/0.1% Tween whilst gradually reducing temperature to 61°C, before a 30 minute wash in 0.2X SSC/0.1% Tween at 60°C. Following this, embryos were washed three times in PBS/Tw at room temperature, 5 minutes each, and then three times in MAB/Tw for 10 minutes each. Embryos were then blocked by incubating in MAB/block for 4 hours before replacing with fresh MAB/block containing 1/2000 dilution of anti-digoxigenin or anti-fluorescein Fab antibody fragments conjugated to alkaline phosphatase (Roche) and incubated overnight at 4°C for antibody detection of probes. Excess antibody was removed by washing 5 times in MAB, 90 minutes each, before washing in MAB at 4°C overnight. Embryos were washed in AP buffer 3 times, 5 minutes each, before developing *in situ* staining using BM purple (Roche) or BCIP (Roche). Embryos being developed were stored in the dark at room temperature until the colour was fully developed, and were then fixed in MEMFA for at least one hour. Excess staining was removed by washing embryos extensively in methanol at room temperature, before storing in methanol at -20°C.

2.2.6.2. Clearing WISH embryos

In order to visualise midline expression of genes detected by WISH, embryos were cleared by adding a 2:1 solution of benzyl benzoate/benzyl alcohol (sigma). As much methanol as possible was removed from embryos in storage before equilibrating embryos in 200-300µl benzyl benzoate/benzyl alcohol before replacing with fresh benzyl benzoate/benzyl alcohol and

observing under a light microscope. After photography embryos were stored in methanol at -20°C.

2.2.6.3. Imaging of embryos

Embryos were photographed using a light microscope and either Q-Imaging Micropublisher 5.0 RTV camera with Q-Capture Pro 7 software, or Nikon DXM1200 camera with ACT-1 Software. Embryos were photographed in either benzyl benzoate/benzyl alcohol (see section 2.2.6.2) or in PBS/Tw. Image manipulation was carried out using Adobe Photoshop CS4 or Adobe Illustrator CS4.

2.2.7. Creating gene regulatory network diagrams

Gene regulatory networks were compiled using BioTapestry, a programme designed and made available by the Davidson lab, California Institute of Technology.

Chapter 3. Expression analysis of notch pathway and target genes in the *Xenopus* dorsal aorta

3.1. Introduction

Notch signalling components are dynamically expressed during development in order to control specific cell fates. During development of the dorsal aorta (DA) and haematopoietic stem cells (HSCs), expression of notch signalling components has been observed in many species, including mouse. *Notch1* and *notch4* are expressed in the developing murine artery at E9.5 and E10.5, whilst the artery and haemogenic endothelium are being specified (Del Amo et al., 1992; Reaume et al., 1992; Robert-Moreno et al., 2005; Uyttendaele et al., 1996). However, *notch2* and *notch3* expression has not been observed in the DA during these stages suggesting they do not play a role in arterial specification or HSC programming (Robert-Moreno et al., 2005). *Dll1* expression has been observed in endothelial cells at E13-15 although it is not expressed in the developing DA at E9.5 or E10.5, during HSC emergence (Beckers et al., 1999; Robert-Moreno et al., 2005). This expression data is consistent with the role of *dll1* in arterial maintenance whilst being dispensable for initial arterial specification (Sorensen et al., 2009). *Dll4* expression can be observed in the developing artery at E9.5 and E10.5 (Robert-Moreno et al., 2005; Shutter et al., 2000) whilst *dll3* expression has not been observed in arterial endothelium (Dunwoodie et al., 1997; Robert-Moreno et al., 2005). Expression of both jagged ligands, *jag-1* and *jag-2*, has been observed in the DA at E9.5 and E10.5 also (Robert-Moreno et al., 2005). Notch target genes *hesr1/hey1* and *hesr2/hey2* are expressed in the DA at E9.5 and E10.5, and *hes1* is expressed from E10.5, suggesting notch signalling is active in the mouse DA at this time (Robert-Moreno et al., 2005).

In this chapter I investigate the expression of notch receptors and ligands, and notch target genes from the hairy and enhancer of split family, in the *Xenopus* DA. Consistent with mouse data, two notch receptors, *notch1* and *notch4* are expressed in the DA during arterial and haemogenic

endothelium specification, along with three notch ligands, *dll4*, *jag-1* and *jag-2*. *Notch1* and *notch4* are expressed in a temporally restricted manner in the DA; *notch4* expression initiates at stage 31, as the haemangioblast derivatives reach the midline of the embryo after migration, whilst *notch1* expression initiates at stage 34, immediately prior to expression of haemogenic endothelium marker *runx1*. Expression of *notch3* is initiated later in the DA, at stage 39, after arterial specification and initial haemogenic endothelium programming. The three notch ligands, *dll4*, *jag-1* and *jag-2*, are all expressed in midline endothelial cells from stage 31. Thus, all three ligands have the potential to activate either *notch4*, *notch1* or *notch3* in the DA. Analysis of hairy and enhancer of split related gene expression in the *Xenopus* DA reveals two temporally distinct waves of gene activation in response to the expression of the two earliest notch receptors. At stage 31, as *notch4* expression is initiated in midline endothelial cells, expression of three hairy and enhancer of split genes is observed. *Esr7* and *esr10* (paralogues of mouse *hes5*) and *hesr2/hey2* (an orthologue of mouse *hesr2/hey2* and zebrafish *gridlock*) are all expressed in the *Xenopus* DA from stage 31. Following expression of *notch1* in the DA at stage 34, two hairy and enhancer of split genes are expressed; *esr1*, a further paralogue of mouse *hes5*, and *hesr1/hey1*, an orthologue of mouse *hesr1/hey1*, are expressed from stage 35. This suggests that *notch1* and *notch4* receptor expression is linked with active notch signalling in the *Xenopus* DA.

Here, for the first time, I show that notch signalling components are expressed in the *Xenopus* DA in a temporally dynamic fashion, suggesting three independent notch inputs to DA and HSC programming and/or maintenance.

3.2. *Notch1*, *notch3* and *notch4* are expressed in the DA during arterial and haemogenic endothelium specification and/or maintenance

As previously discussed, *notch1* and *notch4* expression has been observed in the mouse DA during arterial specification. *Notch1*-null mice exhibit vascular and haematopoietic defects, whilst *notch1/notch4*-null mice have a more severe vascular phenotype. To understand whether or not notch signalling has a role in *Xenopus* arterial and haemogenic endothelium programming it is first essential to see if any of the four receptors are expressed in the *Xenopus* DA at this time.

In *X.laevis*, two notch receptor sequences had previously been identified: *notch1* (Coffman et al., 1990) and *notch2* (Ogino et al., 2008). A gene encoding *notch3* had been identified for *X.tropicalis*, a close relative of *X.laevis*, along with several other vertebrates (Theodosiou et al., 2009). *Notch3* had thus been presumed to be present in *X.laevis* but not identified due to the unsequenced *X.laevis* genome (Theodosiou et al., 2009). Expressed sequence tags (ESTs) for *X.tropicalis* have successfully been used to detect gene expression by *in situ* hybridisation in *X.laevis* on many occasions (Aldo Ciau-Uitz, personal communication). An EST for *X.tropicalis Notch3* was identified through searching a translated nucleotide *X.tropicalis* EST library using the mouse *notch3* translated mRNA sequence to blast the database (tblastx: 45% coverage, 7e-104). *Notch4* is the most divergent of the four notch receptors and having only been identified in mammals thus far is believed to be unique to mammals (Theodosiou et al., 2009). Using the murine *notch4* sequence for a tblastx search of the *X.laevis* EST database revealed an EST with 56% sequence similarity (48% coverage, 4e-63), presumed to be *X.laevis notch4*. More recently, an *X.tropicalis* orthologue of *notch4*, previously labelled as *notch3-like*, has been reassigned as *notch4* in the Ensemble genome database, giving credibility to the existence of *notch4* in *X.laevis*.

ESTs containing a fragment of the coding sequence for each notch receptor were obtained (see Table 2.1 for details) from which DIG-labelled RNA probes were generated to detect gene expression by *in situ* hybridisation. Analysis of gene expression at the midline of cleared embryos reveals the expression of *notch1* in the DA from stage 34, continuing through stage 39 (Figure 3.1, c-f, red arrowheads). *Notch1* expression can also be seen from stage 31 in the somites (Figure 3.1a-f, yellow arrowheads), neural tube (blue arrowheads) and notochord (orange arrowheads). The notochord is a common site for background probe trapping in *Xenopus*. Due to the vacuolous nature of the notochord, dig-labelled *in situ* probes become trapped here in a non-specific manner. Thus, it is not always the case that the gene is expressed in the notochord, and it should be noted that it could instead be non-specific probe trapping.

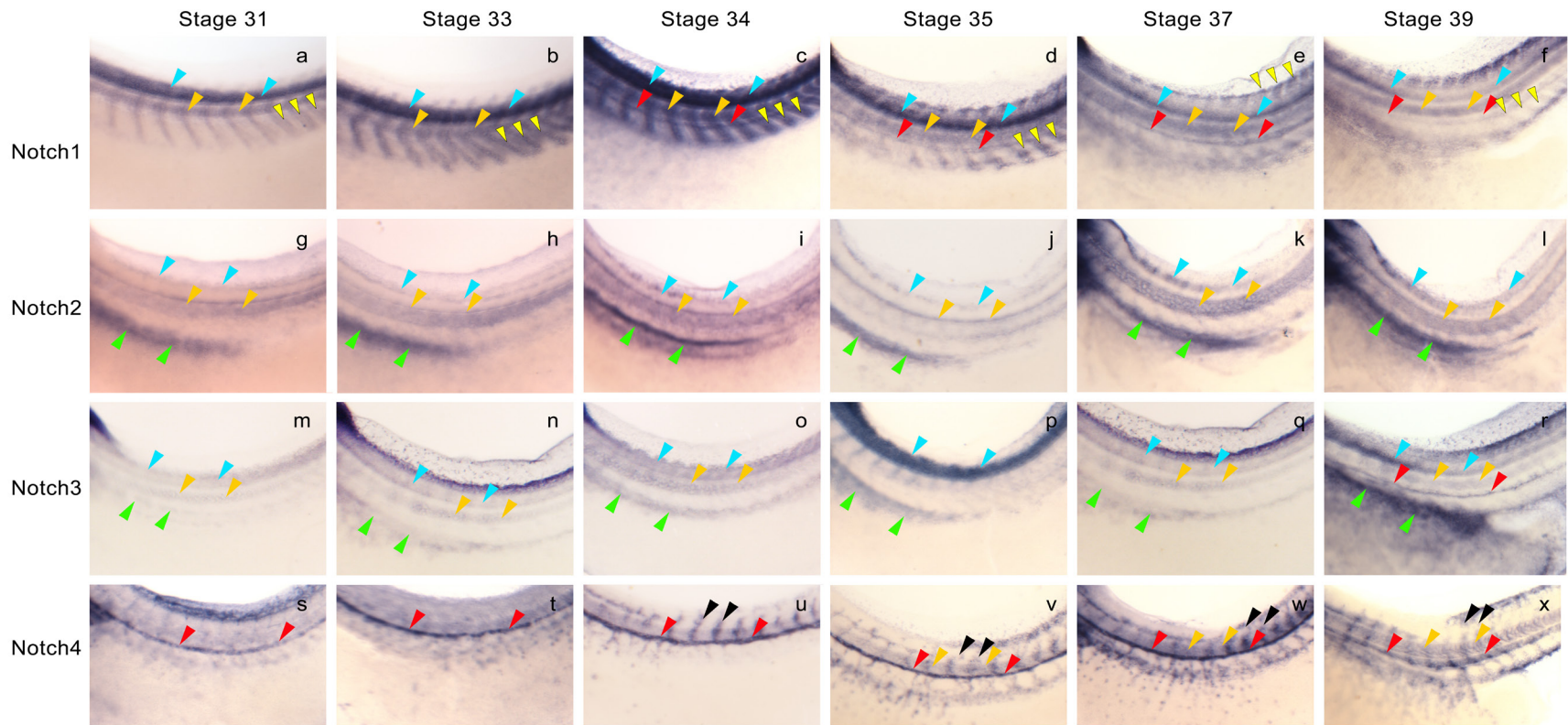
Notch2 and *notch3* have very similar expression patterns; expression can be seen in the neural tube (Figure 3.1g-l, blue arrowheads), notochord (orange arrowheads), and lateral mesoderm (green arrowheads). Whilst *notch2* is not expressed in the DA, *notch3* expression is initiated in the DA at stage 39 (Figure 3.1r, red arrowheads). Expression of *notch4* is observed in the DA from stage 31, immediately after the migration of cells from the DLP to the midline, continuing through stage 39 (Figure 3.1 s-x, red arrowheads). From stage 34 expression can also be seen in intersomitic vessels (black arrowheads), and in the notochord from stage 35. Thus, three of the four notch receptors are expressed in the *Xenopus* DA; *notch4* expression initiates as haemangioblast cells reach the midline, *notch1* expression initiates slightly later, at stage 35, whilst *notch3* is expressed much later.

In order to analyse the role of *notch1* and *notch4* in *Xenopus* arterial and HSC development, morpholino knockdown and chemical treatment approaches were undertaken (See Chapter 4, Chapter 5 and Chapter 6 for more information).

Figure 3.1 Expression of notch receptors in the *Xenopus* DA.

Notch1 expression is seen in the somites, neural tube and notochord (panels a-f). Expression in the DA is seen from stage 34 (panels c-f). *Notch2* is expressed in the neural tube, the notochord and lateral mesoderm (panels g-l). *Notch3* is expressed in the neural tube, the notochord and lateral mesoderm (panels m-r), and in the DA from stage 39 (panel r). *Notch4* expression is seen in the DA from stage 31 (panels s-x), the intersomitic vessels from stage 34 (panels u-x) and in the notochord from stage 35 (panels v-x).

Embryos were cleared using 2:1 BB:BA, and are oriented with anterior to the left and dorsal to the top. Red arrowheads mark expression in the DA, yellow arrowheads mark the somites, blue arrowheads mark the neural tube, orange arrowheads mark the notochord, black arrowheads mark intersomitic vessels and green arrowheads mark lateral mesoderm.



Treatment with a chemical inhibitor of notch signalling (DAPM) from the onset of DLP specification (stage 20) will inhibit the activity of all notch receptors, thus it is also essential to know if any notch receptors are expressed in haemangioblast cells of the DLP, and thus predict any early effects of reduced notch signalling in the DLP. Similarly, as injection of morpholinos occurs at the 2 cell stage, it is essential to understand where these genes are expressed during early embryonic development, and thus whether any early effects can be predicted.

Expression in the DLP is not seen for any of the four notch receptors (Figure 3.2). *Notch1* is expressed in the somites (Figure 3.2a-c, yellow arrowheads), hindbrain and neural tube (blue arrowheads), pronephros (orange arrowheads), presomitic mesoderm (white asterisk) and eye (green asterisk). *Notch2* expression is seen in the head ectoderm (Figure 3.2d-f, red arrowheads), pronephric duct (green arrowheads), neural tube (blue arrowheads) and somites (yellow arrowheads). *Notch3* expression is seen in the eye (Figure 3.2g-i, green asterisks), pronephros (orange arrowheads), neural tube (blue arrowheads) and somites (yellow arrowheads), whilst *notch4* expression is seen only in the midbrain-hindbrain boundary (Figure 3.2j-l, black arrowheads). Overstaining of *in situ* hybridisations for *notch4* to reveal the midbrain-hindbrain boundary expression has resulted in some background expression in the ectoderm.

Expression analysis of *notch1* and *notch4* during early embryonic development, prior to DLP specification, reveals expression in similar patterns for both genes. *Notch1* and *notch4* are expressed in anterior mesoderm and ectoderm (Figure 3.3, green arrowheads), the neural tube (blue arrowheads), somites (yellow arrowheads) and presomitic mesoderm (pink arrowheads). Haemangioblast cells are believed to lie in the lateral mesoderm from stage 13 (Aldo Ciau-Uitz, personal communication) and no expression of *notch1* or *notch4* is seen in the lateral mesoderm population.

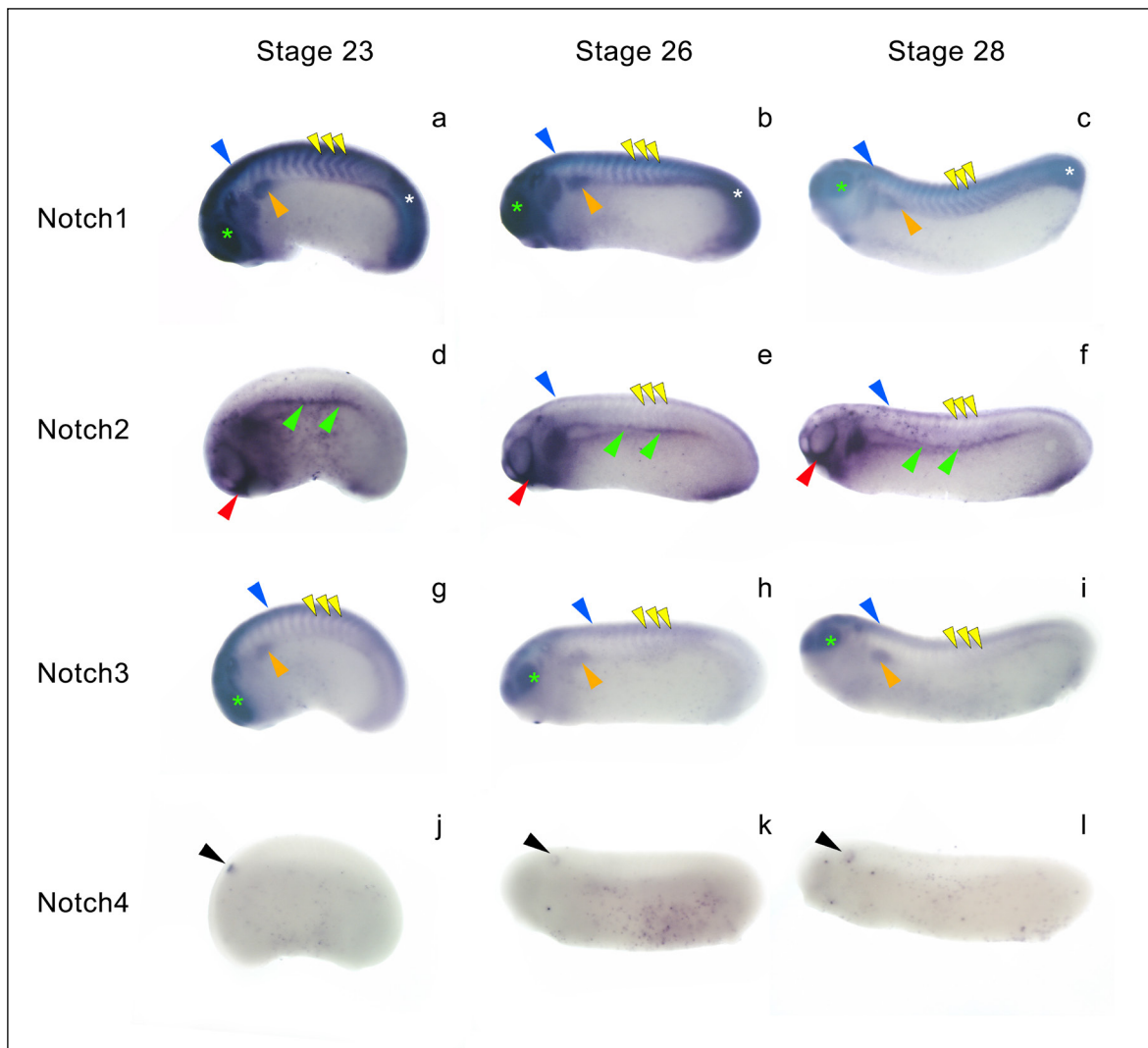


Figure 3.2 Expression analysis of notch receptors in the *Xenopus* DLP

Notch receptors are not expressed in the *Xenopus* DLP. Embryos are oriented with anterior to the left, dorsal to the top, and were not cleared before photography. Red arrowheads mark expression in head ectoderm, yellow arrowheads mark somitic expression, black arrowheads mark midbrain-hindbrain boundary, green arrowheads mark pronephric duct, blue arrowheads mark hindbrain and neural tube expression, orange arrowheads mark the pronephros, white asterisks mark presomitic mesoderm and green asterisks mark the eye.

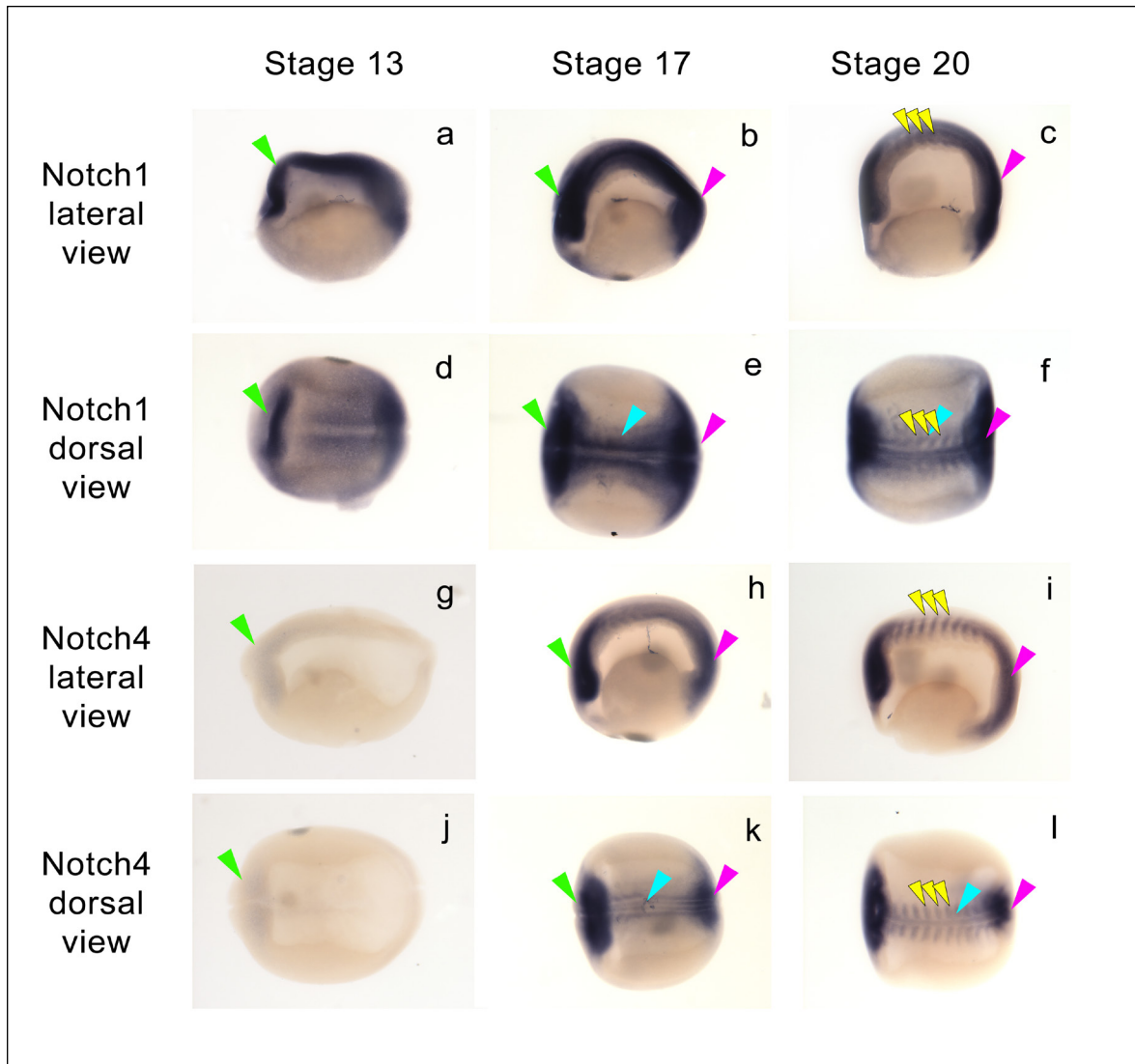


Figure 3.3 Expression analysis of *notch1* and *notch4* at early developmental stages

Notch1 and *notch4* are expressed in presomitic mesoderm, somites, anterior ectoderm and mesoderm, and in the neural tube. Embryos are oriented with anterior to the left. Panels a-c and g-i are visualised from the lateral view, with dorsal to the top. Panels d-f and j-l are visualised from the dorsal view. Embryos were cleared using 2:1 BB:BA. Green arrowheads mark anterior expression, blue arrowheads mark neural tube expression, yellow arrowheads mark somitic expression and pink arrowheads mark presomitic mesoderm expression.

Thus, as expression of notch receptors is not seen in the DLP or in haematopoietic precursors during early development, manipulation of notch signalling by either chemical inhibition or morpholinos should not have any effect on the endothelial and haematopoietic precursors before the onset of expression in the DA.

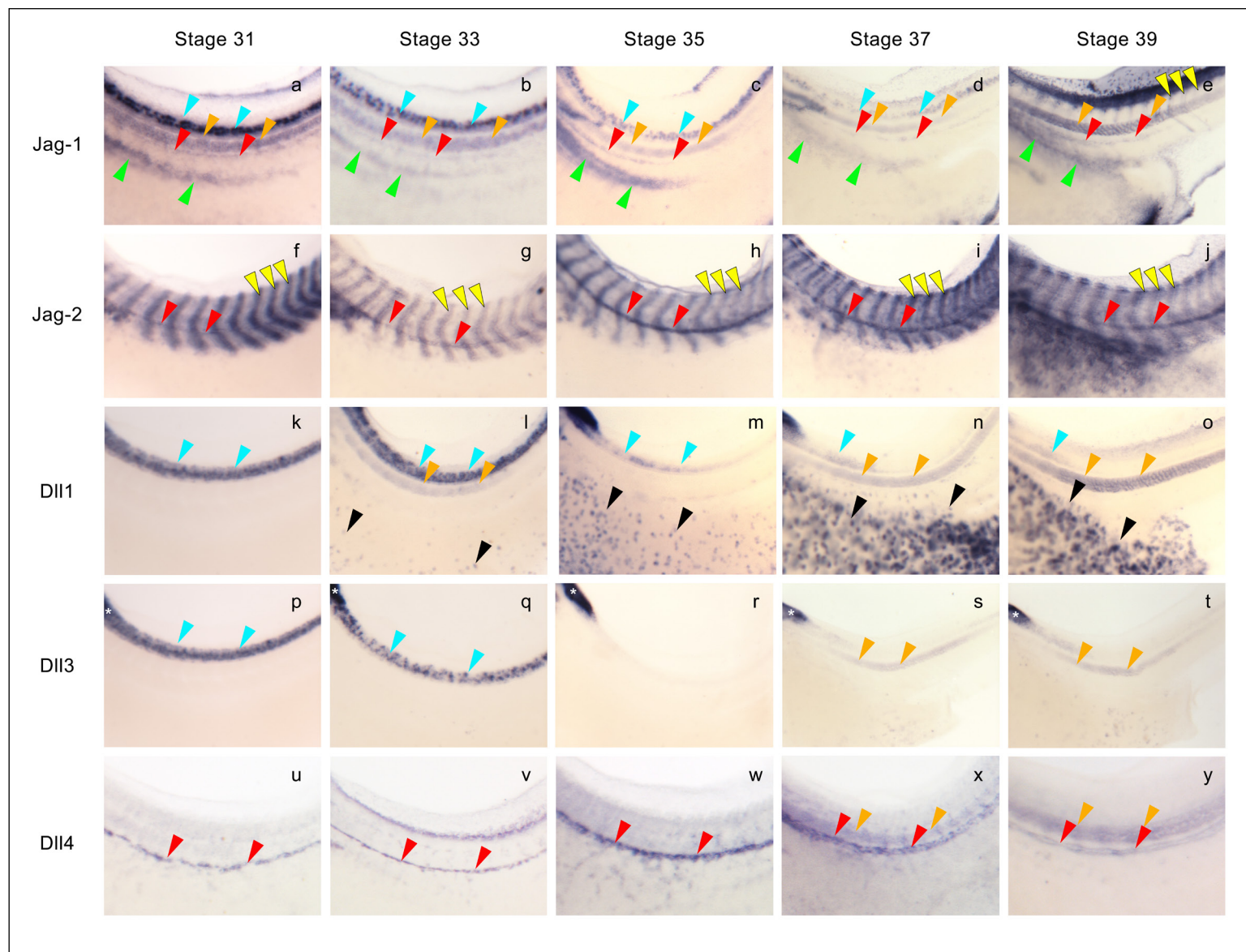
3.3. Three notch ligands are expressed in the *Xenopus* DA; *jag-1*, *jag-2* and *dll4* expression initiates as haemangioblast cells reach the midline to form the DA

Expression of notch receptors has been observed in the *Xenopus* DA (Figure 3.1). In order to activate canonical notch signalling in the DA, ligands are needed in the DA or surrounding tissues. To analyse notch ligand expression in the DA, expression analysis of each canonical notch ligand in the midline of cleared embryos was carried out using *in situ* hybridisation (see Table 2.1 for probe details). *Jag-1* is expressed in the DA (Figure 3.4a-e, red arrowheads), lateral mesoderm (green arrowheads), neural tube (orange arrowhead) and notochord (blue arrowhead) from stage 31-39. At stage 39, some expression in the somites is also seen (yellow arrowheads), as is some background probe trapping in the dorsal fin. *Jag-2* is expressed in the somites (Figure 3.4f-j, yellow arrowheads) and DA (red arrowheads). *Dll1* is expressed in the neural tube (Figure 3.4k-o, blue arrowheads) with expression peaking at stage 33 before weakening at stages 37-39. From stage 33, *Dll1* is seen in the notochord (orange arrowheads) and in endoderm (black arrowheads). *Dll3* is expressed in the neural tube at stage 31 and 33 (Figure 3.4p-q, blue arrowheads), the hindbrain from stage 31-39 (Figure 3.4p-t, white asterisks) and notochord from stage 37-39 (Figure 3.4s-t, orange arrowheads). *Dll4* is expressed in an endothelial specific manner with expression initially seen in the DA only (Figure 3.4u-y, red arrowheads). Some probe trapping is seen in the dorsal fin at stage 33, whilst expression can be seen in the notochord at stage 37-39. Thus, consistent with mouse data, three notch ligands are expressed in the DA during arterial and haemogenic endothelium specification; *jag-1*, *jag-2* and *dll4*.

Figure 3.4 Expression analysis of notch ligands in the Xenopus DA

Three notch ligands are expressed in the Xenopus DA; *jag-1*, *jag2* and *dll4* are all expressed from stage 31 through to stage 39.

Embryos were cleared with 2:1 BB:BA and are oriented with anterior to the left, dorsal to the top. Red arrowheads mark expression in the DA, green arrowheads mark lateral mesoderm, yellow arrowheads mark somites, black arrowheads mark endoderm, blue arrowheads mark neural tube, orange arrowheads mark notochord and white asterisks mark hindbrain.



3.4. Expression analysis of notch target genes in the *Xenopus* DA

As notch receptor and ligand expression has been identified in the *Xenopus* DA, it is presumed that notch signalling is active in these tissues. In order to better understand notch signalling in the *Xenopus* DA, expression analysis of notch target genes from the hairy and enhancer of split families was undertaken.

3.4.1. Hairy and enhancer of split related genes

Genes belonging to the hairy and enhancer of split related family (*hesr/hey/hrt/chf/grl*; herein referred to as *hesr*) have been identified as expressed in the mouse and zebrafish DA (Robert-Moreno et al., 2005; Zhong et al., 2000). In addition, a functional requirement for *gridlock* in arterial specification has been identified in zebrafish (Rowlinson and Gering, 2010; Zhong et al., 2001; Zhong et al., 2000), thus these are good candidates for *Xenopus* notch target genes in the DA.

Xenopus laevis is a pseudotetraploid organism: a genome duplication occurring approximately 30 million years ago has resulted in *X.laevis* often having two non-allelic forms of the same gene (Bisbee et al., 1977). Two paralogues of *hesr1* have been identified in *Xenopus laevis*; *hesr1a* and *hesr1b* (Taelman et al., 2006; referred to as XHRT1a and XHRT1b). These two genes have a sequence similarity of 99% (1884/1895 identical nucleotides, E-value 0.0). Differentiating between the two genes using *in situ* hybridisation would not be possible due to the high sequence similarity. Expression analysis of *hesr1b* (herein referred to as *hesr1*) was carried out to analyse expression in the DA (Figure 3.5k-p). Expression of *hesr1* can be seen in the somites (yellow arrowheads) and neural tube (blue arrowheads) from stage 31. From stage 35, expression can be seen in the DA (Figure 3.5n-p, red arrowheads), and from stage 37 can be seen in migrating hypaxial muscle populations (Figure 3.5o-p, black arrowheads). *Hesr2* is expressed in a

similar pattern to *hesr1* (Figure 3.5q-v); expression can be seen in the somites (yellow arrowheads) and neural tube (blue arrowheads). However, expression in the DA is seen earlier, from stage 31 (red arrowheads). In addition, from stage 33, expression can be seen in the endoderm ventrally in the embryo (not marked) and in the notochord (orange arrowheads). *Hesr2* expression in the somites gradually weakens from stage 31 to stage 39.

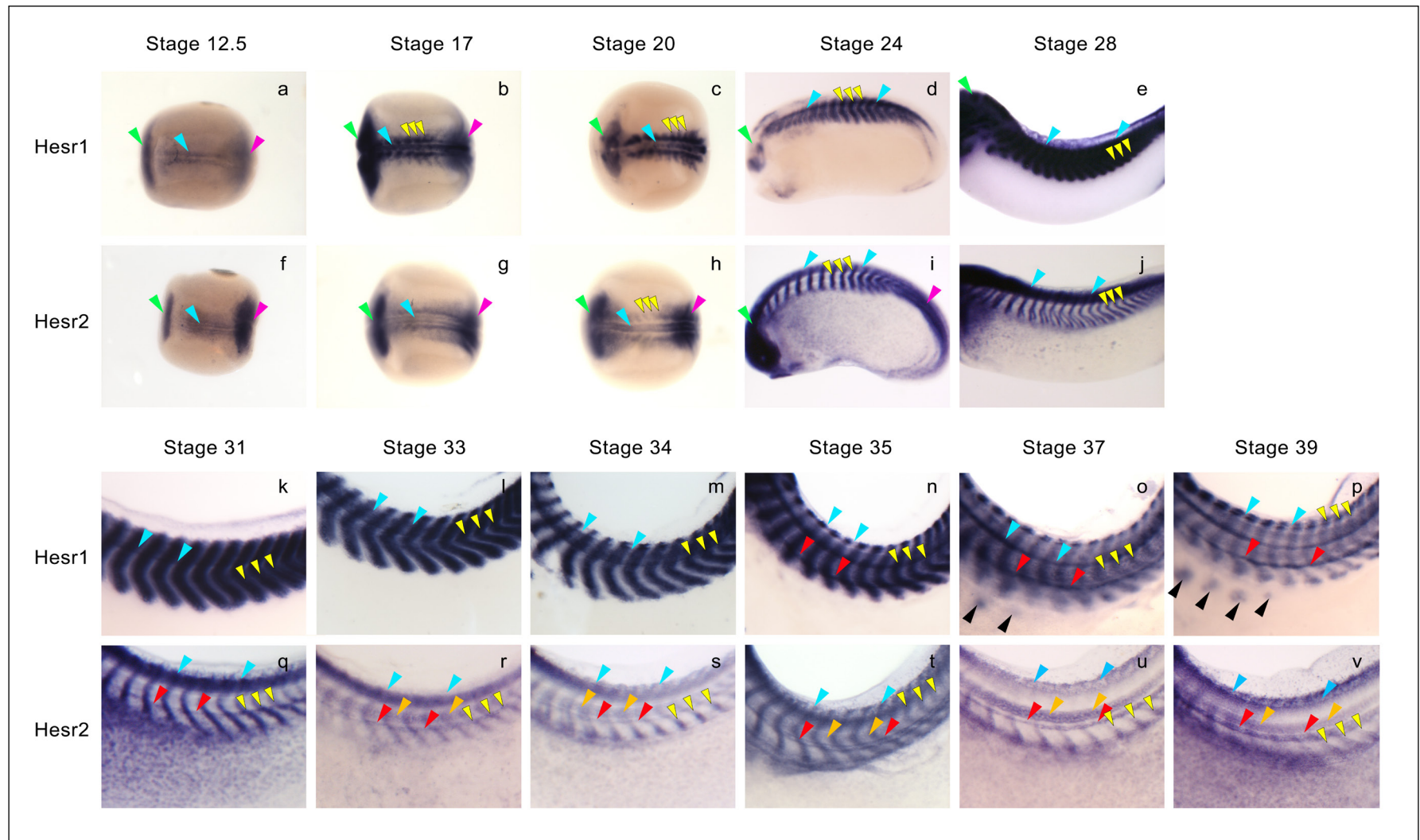
Therefore, consistent with mouse data, both *hesr1* and *hesr2* are expressed in the *Xenopus* DA. *Hesr2* is expressed from stage 31, after migration of cells from the DLP to the midline, and at the same time as *notch4* expression initiates. *Hesr1* expression initiates at stage 35, one stage after the onset of *notch1* expression in the DA.

As both *hesr1* and *hesr2* are expressed in the DA they are good candidates for notch effector genes in arterial and haemogenic endothelium specification. As such, knocking down these genes using morpholinos may aid the understanding of notch signalling in the DA (see Chapter 4 and Chapter 5). Early expression patterns of both *hesr1* and *hesr2* reveal that there is no expression in presumptive DA cells during early development (Figure 3.5a-j). Expression is seen in the somites (yellow arrowheads) and presomitic mesoderm (pink arrowheads), in anterior ectoderm and mesoderm (green arrowheads) and in the neural tube (blue arrowheads). Importantly, expression is absent from lateral mesoderm, thus, injection of morpholinos from the 2-cell stage should not have any direct early effect on the presumptive DA/HSC cells until the cells reach the midline and *hesr1/hesr2* expression initiates.

Figure 3.5 Expression analysis of hairy and enhancer of split related (*hesr*) genes in the *Xenopus* DA

Both *hesr1* and *hesr2* are expressed in the *Xenopus* DA; *hesr1* expression initiates in at stage 35 and is maintained through stage 39 whilst *hesr2* expression initiates earlier, at stage 31.

Embryos were cleared using 2:1 BB:BA. Panels d-e and i-v are imaged from the lateral view, with anterior to the left and dorsal to the top. Panels a-c and f-h are imaged from the dorsal view, with anterior to the left. Red arrowheads mark expression in the DA, blue arrowheads mark the neural tube, yellow arrowheads mark the somites, black arrowheads mark migrating hypaxial muscle populations, green arrowheads mark anterior ectoderm and mesoderm, pink arrowheads mark presomitic mesoderm and orange arrowheads mark the notochord.



3.4.2. Enhancer of split genes

Eight enhancer of split (*esr*) genes have been identified in *Xenopus laevis*; *esr1*, *esr2*, *esr4*, *esr5*, *esr6e*, *esr7*, *esr8* and *esr10*. Many of these genes also have secondary aliases, being referred to as *hes* genes: there is no physical distinction between *esr* and *hes* genes, it is a difference only of gene nomenclature. Similar to *hesr1*, some of these *esr* genes have two closely related genes, most likely pseudoalleles. *Esr1* and *esr1b* share sequence similarity of 448/493 (91%, E-value 0.0), and are paralogues of mouse *hes5*. Expression analysis of *esr1* reveals expression in the neural tube (Figure 3.6a-e, blue arrowheads) and notochord (orange arrowheads). From stage 35, expression is also seen in the DA (red arrowheads). *Esr2* (*Hes3.3*) is also seen expressed in the neural tube (Figure 3.6f-j, blue arrowheads) and notochord (orange arrowheads), but not in the DA. *Esr4* (*hes7.2*) and *esr5* have very similar expression patterns; expression, or background probe trapping, is seen in the notochord (Figure 3.6k-t, orange arrowheads), and strong expression can be seen in the presomitic mesoderm and the first developing somite, or somitomere (inset pictures). *Esr6e* (*hes3.1*) expression (or background probe trapping) is restricted to the notochord (Figure 3.7a-e, orange arrowheads). *Esr7* (*hes5.2a*), a second paralogue of mouse *hes5*, has a pseudoallele *esr7b/esr3* (*hes5.2b*) with 99% sequence identity (1377/1385, E-value 0.0). Expression analysis of *esr7* reveals expression in the DA (Figure 3.7f-h, red arrowheads) from stage 31-35. Expression is also seen in the neural tube (Figure 3.7f-j, blue arrowheads), the hindbrain (white asterisks) and from stage 39 in the notochord (orange arrowheads). *Esr9* (*hes9.1*) and *esr9b* share sequence similarity of 99% (994/996, E-value 0.0), and *esr9* is strongly expressed in the somites (Figure 3.7k-o, yellow arrowheads) and the ventral ectoderm (not marked). *Esr10*, another paralogue of mouse *hes5*, is expressed in a very similar pattern to *esr7*; expression is seen in the neural tube (Figure 3.7p-t, blue arrowheads) and hindbrain (white asterisks), and from stage 39 in the notochord (orange arrowheads). In addition, expression is seen in the DA (red arrowheads).

Figure 3.6 Expression analysis of *esr1-5* in the *Xenopus* DA

Esr1 is expressed in the DA from stage 35, continuing through stage 39, whilst *esr2*, *esr4* and *esr5* are not expressed in the DA at this time.

Embryos were cleared with 2:1 BB:BA and visualised laterally, with anterior to the left and dorsal to the top. Blue arrowheads mark expression in the neural tube, orange arrowheads mark the notochord, and red arrowheads mark the DA. *Esr4* and *esr5* inset (k-t): expression in presomitic mesoderm and somitomeres.

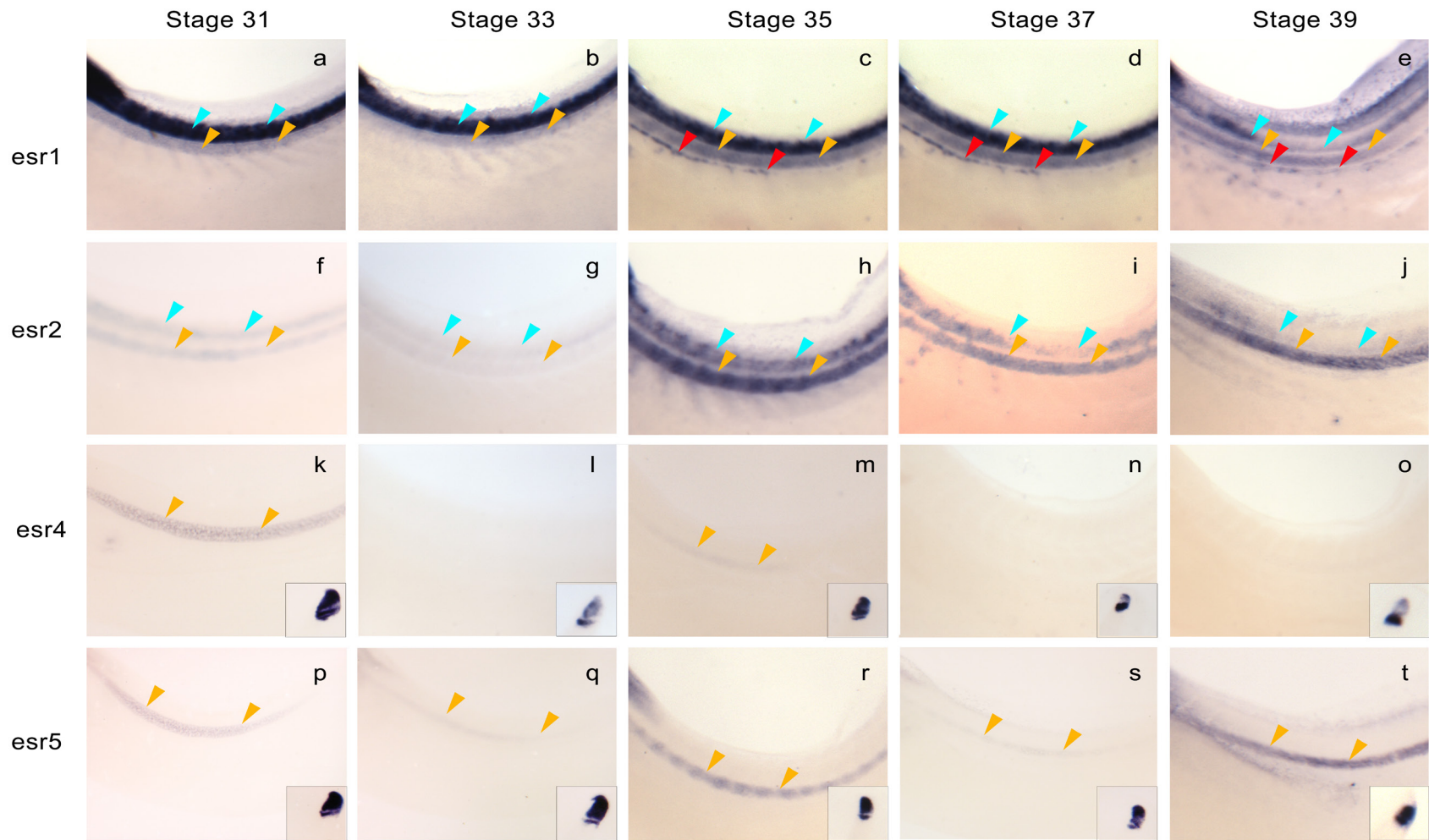
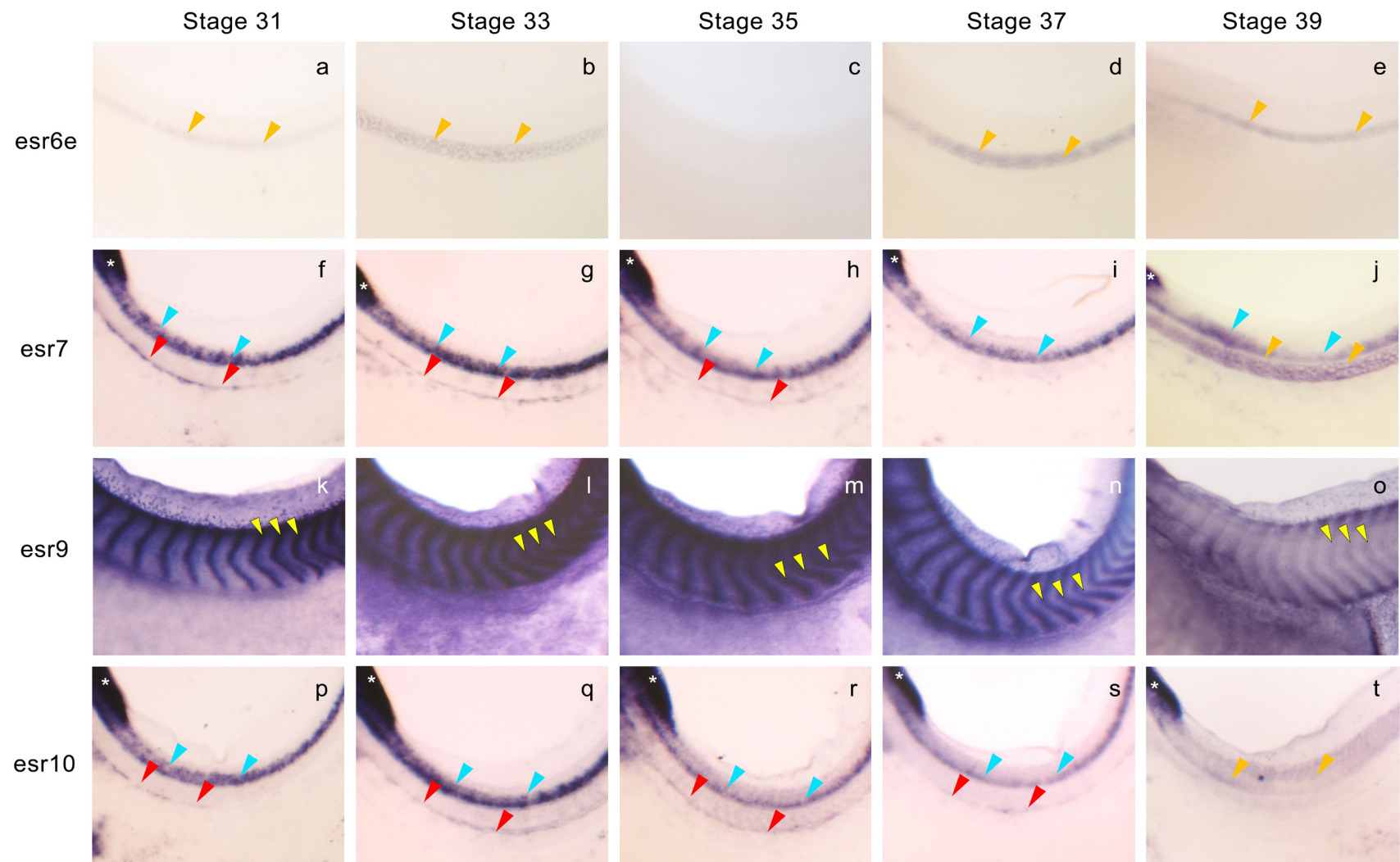


Figure 3.7 Expression analysis of *esr6e-esr10* in the Xenopus DA

Esr7 and *esr10* are expressed in the Xenopus DA; expression of both *esr7* and *esr10* initiates at stage 31, although *esr7* is initially stronger than *esr10*. *Esr7* expression is maintained in the DA to stage 35, whilst *esr10* expression is maintained until stage 37.

Embryos were cleared with 2:1 BB:BA and are visualised laterally, with anterior to the left and dorsal to the top. Orange arrowheads mark notochord expression, blue arrowheads mark the neural tube, red arrowheads mark the DA, yellow arrowheads mark somites and white asterisks mark the hindbrain.



However, expression at stage 31 is much weaker for *esr10* than it is for *esr7*, and *esr10* expression is maintained longer, being clearly visible still at stage 37, whilst *esr7* DA expression is fading by stage 35.

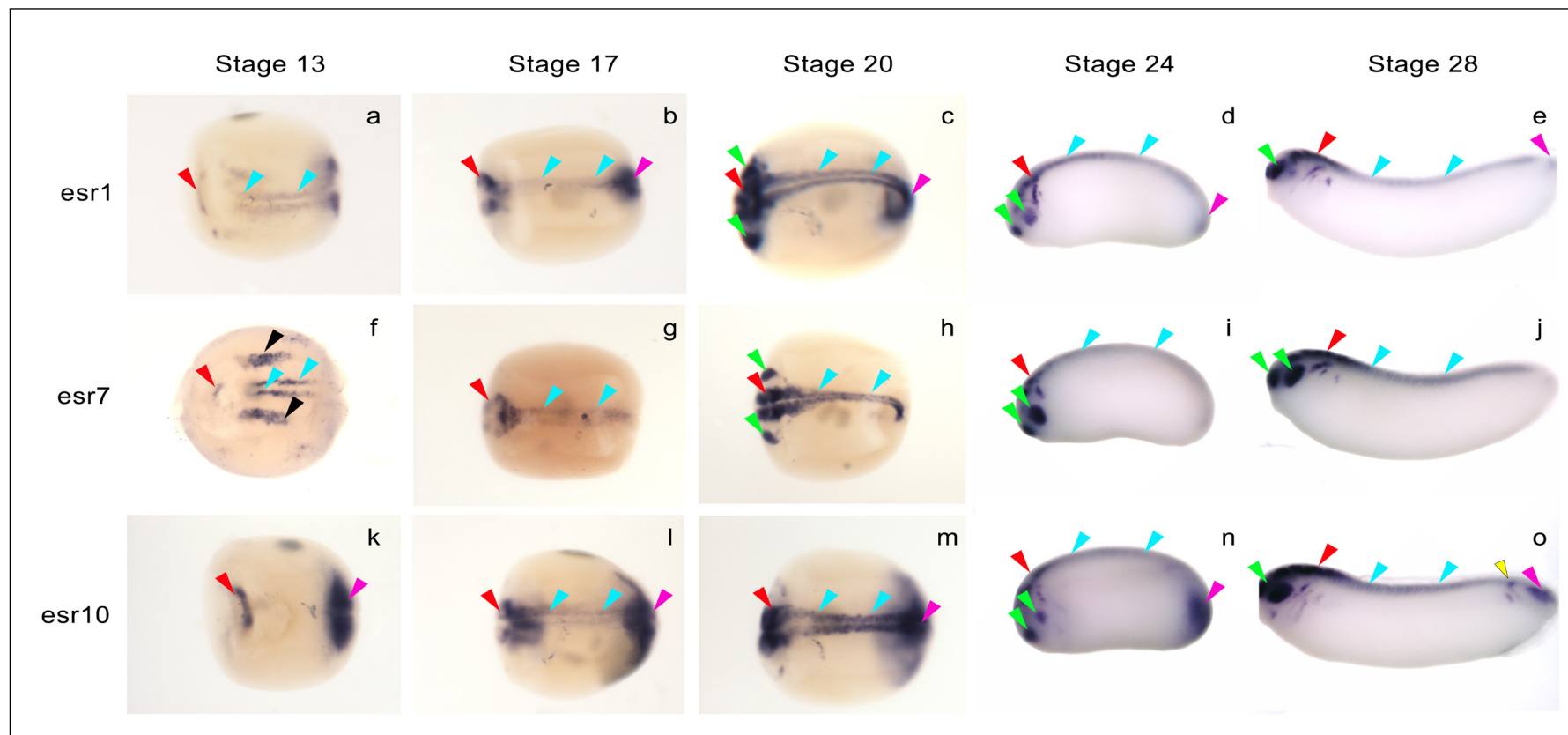
Out of the eight known *esr* genes in *Xenopus laevis*, three are expressed in the DA, all of which are paralogues of mouse *hes5*. *Esr7* and *esr10* are both expressed from stage 31, at the same time *notch4* expression initiates in the DA, whilst *esr1* expression is observed from stage 35, immediately after *notch1* expression initiates in the DA. These three *esr* genes are good candidates for notch effector genes involved in arterial and haemogenic endothelium specification. As such, knocking down these genes using morpholinos may aid the understanding of notch signalling in the DA (see Chapter 4 and Chapter 5). Expression patterns of *esr1*, *esr7* and *esr10* during early development and specification of the DLP reveal expression in the neural tube (Figure 3.8, green arrowheads), brain (red arrowheads) and eye (blue arrowheads) for *esr1*, *esr7* and *esr10*. Expression in the presomitic mesoderm can be seen for *esr1* and *esr10* (black arrowheads), and in somitomeres for *esr10* (yellow arrowheads). Importantly, no expression is seen in lateral mesoderm containing the presumptive DA and HSC cells, and thus injection of morpholinos from the 2-cell stage should not have any early effect on the haemangioblast (Figure 3.8).

Figure 3.8 Expression analysis of *esr1*, *esr7* and *esr10* during early *Xenopus* development and DLP specification

No expression of *esr1*, *esr7* or *esr10* is seen in cells destined to become the DA or HSCs.

Embryos in panels a-c, f-h, k-m were cleared with 2:1 BB:BA and were visualised from the dorsal view, with anterior to the left. Embryos in panels d-e, i-j, and n-o were not cleared and were visualised laterally, with dorsal to the top and anterior to the left.

Blue arrowheads mark neural tube expression, pink arrowheads mark presomitic mesoderm, yellow arrowheads mark somitomeres, red arrowheads mark the brain, black arrowheads mark primary neurons and green arrowheads mark eye expression.



3.4.3. Hairy and enhancer of split (hes) genes

Seven hairy and enhancer of split (hes) genes have been identified in *Xenopus laevis*; *hes1a/hes1b*, *hes2*, *hes3-like-a/hes3-like-b*, *hes4a/hes4b*, *hes6*, *hes7.1* and *hes8*. *Hes1a* and *hes1b* (also known as *hairy1a/hairy1b*), sharing sequence identity of 91% (1148/1263, E-value 0.0), are paralogues of mouse *hes1*. Expression analysis of *hes1a* reveals expression in the neural tube from stage 31-35 (Figure 3.9a-e, blue arrowheads), and in the somites (yellow arrowheads) from stage 31-39, with expression weakening from stage 35. Expression of *hes2* is seen in the notochord (Figure 3.9f-j, orange arrowheads) and in the eye (inset pictures). No expression of *Hes3-like-a* is seen at stages 31-39 (Figure 3.9k-o). *Hes4a* and *hes4b* (also known as *hairy2a/hairy2b*) share sequence similarity of 99% (702/704, E-value 0.0) and expression is seen in the neural tube and notochord (Figure 3.9 blue arrowheads and orange arrowheads, respectively). *Hes6* expression is seen in the somites at stage 31 (Figure 3.10a-e, yellow arrowheads), and in the neural tube and notochord from stage 31-39 (blue arrowheads and orange arrowheads, respectively). Expression in the neural tube decreases from stage 35-39. Expression of *hes7* (also known as *hr1*) is seen in the notochord (Figure 3.10f-j, orange arrowheads), and in the brain (inset pictures). *Hes8* is weakly expressed in the neural tube at stages 31-33 (Figure 3.10k-l, blue arrowheads) and in head tissues (k-o, inset pictures). Thus, no hes genes are expressed in the *Xenopus* DA.

Figure 3.9 Expression analysis of *hes1-4* in the *Xenopus* DA

No expression is seen in the DA for *hes1*, *hes2*, *hes3-like* or *hes4*.

Embryos were cleared with 2:1 BB:BA and visualised from the lateral view, with anterior to the left and dorsal to the top. Blue arrowheads mark neural tube expression, yellow arrowheads mark the somites and orange arrowheads mark the notochord. Expression of *hes2* in the eye is seen in pictures inset.

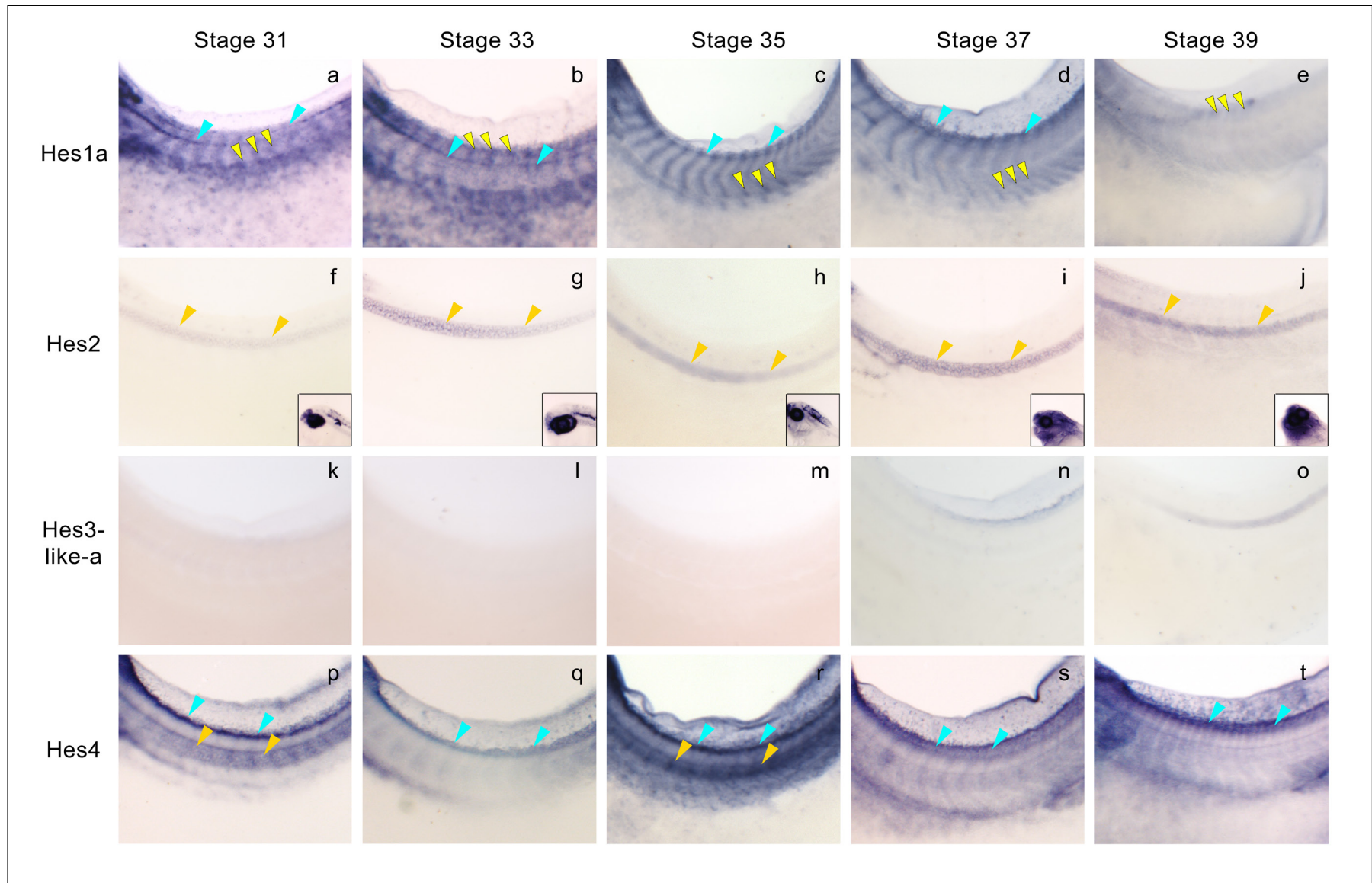
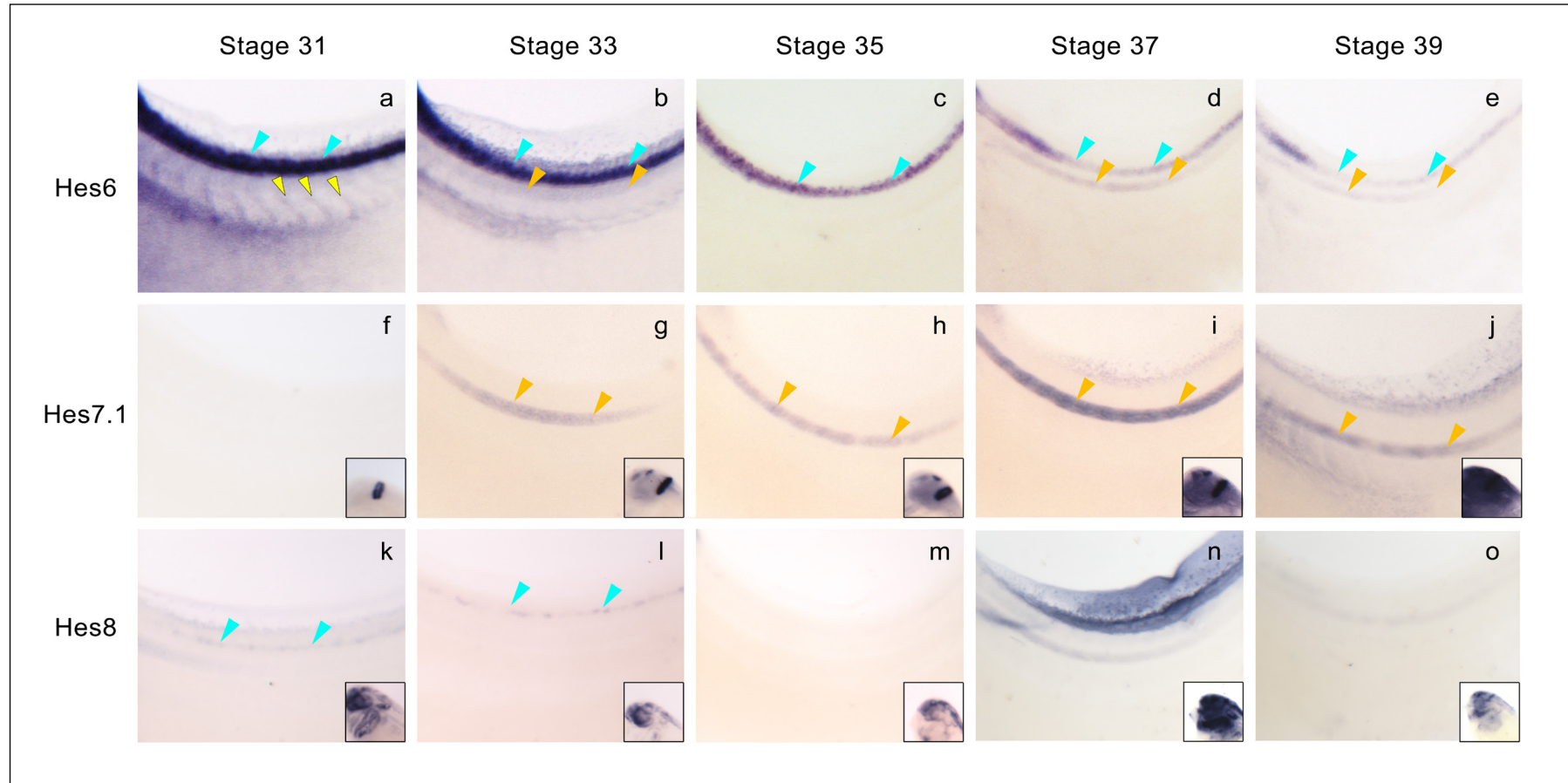


Figure 3.10 Expression analysis of *hes6-8* in the *Xenopus* DA

No expression is seen in the DA for *hes6*, *hes7.1* or *hes8*.

Embryos were cleared with 2:1 BB:BA and visualised from the lateral view, with anterior to the left and dorsal to the top. Blue arrowheads mark neural tube expression, orange arrowheads mark the notochord and yellow arrowheads mark somites. Expression of *Hes7.1* in the brain is seen in inset pictures (f-j) and expression of *Hes8* in the eye is seen in inset pictures (k-o).



3.5. Discussion

3.5.1. Overview

Notch receptors *notch1* and *notch4*, and canonical notch ligands *jag-1*, *jag-2* and *dll-4*, are known to be expressed in the mouse DA at the time of DA programming and subsequent HSC emergence (Robert-Moreno et al., 2005). Active notch signalling in the murine DA was initially presumed due to the expression of notch target genes *hes1*, *hesr1* and *hesr2*. Here, the expression analysis of notch receptors, ligands, and target genes in the *Xenopus* DA has been evaluated for the first time (summarised in Table 3.1). Three notch receptors (*notch1*, *notch3* and *notch4*) three notch ligands (*jag-1*, *jag-2* and *dll4*) and five hairy and enhancer of split genes (*hesr1*, *hesr2*, *esr1*, *esr7* and *esr10*) have been identified in the *Xenopus* DA in a temporally dynamic fashion (Figure 3.11). The expression of *notch4* and *notch1* can be associated with the arterial programme and the HSC programme, respectively (Figure 3.12), whilst *notch3* expression is initiated after initial arterial and HSC specification.

3.5.2. *Notch4* expression is initiated as haemangioblast cells reach the midline, and is associated with arterial gene expression

Notch4 is expressed in the DA from stage 31, initiating as the migrating haemangioblast cells reach the midline of the embryo to form the dorsal aorta. Expression is maintained in the DA at all stages investigated, and becomes expressed in intersomitic vessels from stage 34. Prior to initiation at stage 31, *notch4* is not identified in lateral mesodermal precursors of haemangioblast cells or in haemangioblasts of the DLP. At the same time as *notch4* expression is initiated in the DA, the expression of arterial genes is also initiated, including *ephrinB2* (Figure 3.12). As such, based on expression analysis, it is possible that *notch4* could also be an arterial specific gene required for arterial programming.

Xenopus gene name(s)	Zebrafish gene name(s)	Mouse gene name(s)	Xenopus expression
Notch1	Notch1a AND Notch1b	Notch1	Somites, hindbrain, neural tube, presomitic mesoderm, eye, notochord and DA
Notch2	Notch2	Notch2	Neural tube, notochord, lateral mesoderm, somites, head ectoderm and pronephric ducts
Notch3	Notch3	Notch3	Neural tube, eye, pronephros, somites, notochord, lateral mesoderm and DA
Notch4	-	Notch4	DA, somites (early only), midbrain-hindbrain boundary (early only), ISVs and notochord
Serrate-1/Jagged1	Jagged 1a AND Jagged1b	Jagged1	DA, lateral mesoderm, neural tube. somites and notochord
Serrate-2/Jagged-2	Jagged-2	Jagged-2	Somites and DA
Delta-like1 (Dll1)	Delta-a (dla) AND Delta-d (dld)	Delta-like1 (Dll1)	Neural tube, notochord and endoderm
Delta-like3 (Dll3)	Delta-b (dlb) AND Delta-c (dlc)	Delta-like3 (Dll3)	Neural tube, hindbrain and notochord
Delta-like4 (Dll4)	Delta-like4 (Dll4)	Delta-like4 (Dll4)	DA, ISVs and notochord.
Hesr1/Hey1/Hrt1	Herp1/Hey1	Hey1/CHF2/HRT1/Herp2/Hesr1	Somites, neural tube, DA and migrating hypaxial muscle cells
Hesr2/Hey2/Hrt2	Gridlock/Herp2/Hey2	Hey2/CHF1/HRT2/Herp1/Hesr2	Somites, neural tube, DA endoderm and notochord.
Esr1/Hes5 AND esr1b/Hes5.1	Hes5-like/Her15.1	Hes5	Neural tube, notochord, and DA
Esr2/Hes3.3	-	Hes3	Neural tube and notochord

Esr4/Hes7.2	Her5*	Hes7	Notochord, presomitic mesoderm and somitomere
Esr5	Her1*	Hes7*	Notochord, presomitic mesoderm and somitomere
Esr6e/Hes3.1	Her3	Hes3	Notochord
Esr7/Hes5.2a AND Esr3/Esr7b/Hes5.2b	Her15.1	Hes5	DA, neural tube, hindbrain and notochord
Esr9/hes9.1 AND esr9b	Her12*	Hes5*	Somites and ventral endoderm
Esr10/Hesr5	Her12*	Hes5	Neural tube, notochord, hindbrain and DA
Hes1a/Hairy1a AND Hes1b/Hairy1b	Hes6	Hes1	Neural tube and somites
Hes2	Hes2*	Hes2	Notochord and eye
Hes3-like-a AND Hes3-like-b	Her3	Hes3	No expression
Hes4a/Hairy2a AND Hes4b/Hairy2b	Her9*	Hes4	Neural tube and notochord
Hes6	Hes6	Hes6	Somites, neural tube and notochord
Hes7/Hr1	Her5*	Hes7	Notochord and brain
Hes8	Her12*	Hes5*	Neural tube and head

Table 3.1 List of *Xenopus* Notch pathway genes

Table showing *Xenopus* Notch receptors, notch ligands, canonical notch target genes, their corresponding genes in zebrafish and mouse, and their expression location in *Xenopus*. Asterisks denote orthology assessed by BLAST analysis only, synteny not available.

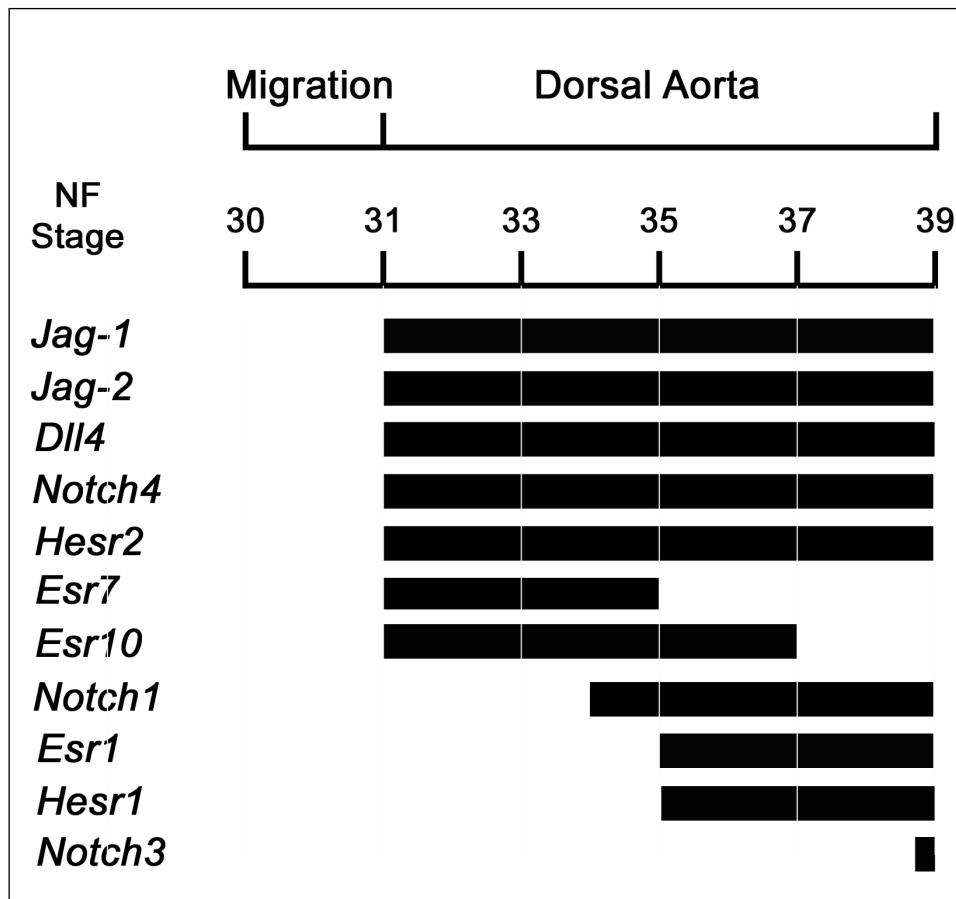


Figure 3.11 Notch receptors, ligands and target genes are expressed in the Xenopus DA in a temporally dynamic fashion

Schematic diagram representing the expression of notch receptors, notch ligands, and notch target genes from the hairy and enhancer of split family in the Xenopus DA.

Three notch ligands are expressed in the DA at the same time *notch4* expression initiates; *jag-1*, *jag-2* and *dll4*. As receptor-ligand binding specificity is a result of post-translational modification rather than each notch receptor having a bias towards a particular DSL ligand (see chapter 1.8), it is therefore potentially possible for any of these three ligands to bind to, and activate, *notch4* (and later to *notch1* and *notch3*).

At the same time as *notch4* expression initiates, three notch target genes become expressed in the DA, suggesting *notch4* expression is synonymous with active notch signalling. *Esr7*, *esr10* and

hesr2, all members of the hairy and enhancer of split related family of notch target genes, become expressed in the DA from stage 31, likely due to active *notch4* signalling. *Hesr2* has been identified in the mouse DA (Robert-Moreno et al., 2005), and in the zebrafish DA (Zhong et al., 2000), and a role for *hesr2* in arterial specification has been demonstrated in zebrafish (Rowlinson and Gering, 2010; Zhong et al., 2001; Zhong et al., 2000). Based on the expression data, *hesr2* appears to be a good candidate for a notch target gene controlling aortic specification in *Xenopus*. However, *hesr2* has been demonstrated to be upstream of notch signalling in the zebrafish (Rowlinson and Gering, 2010), and so it may also be upstream of notch signalling in *Xenopus*, rather than having the traditional downstream target gene role. *Esr7* and *esr10*, together with *esr1*, are paralogues of mouse *hes5*. *Hes5* expression has not previously been documented in the dorsal aorta, and so these may be novel dorsal aorta notch target genes.

3.5.3. *Notch1* expression is initiated at stage 35, and is found at the top of the haematopoietic stem cell genetic cascade.

Notch1 expression is initiated in the *Xenopus* DA at stage 34, and is maintained at all subsequent stages studied. Following expression of *notch1*, a cascade of genes associated with haematopoietic stem cell development is initiated (Figure 3.12). Immediately following *notch1* expression in the DA, HSC marker *runx1* expression is observed, and subsequently *scl*, *lmo2*, *gfi1* and *spiB* expression is initiated. Thus, based on expression data, it is probable that *notch1* is an upstream genetic regulator of the HSC programme in the DA. Two hairy and enhancer of split related notch target genes are expressed in the DA following *notch1* expression: *esr1* and *hesr1* are expressed from stage 35. As previously discussed, *esr1* is a paralogue of *hes5*, expression of which has not previously been identified in the DA.

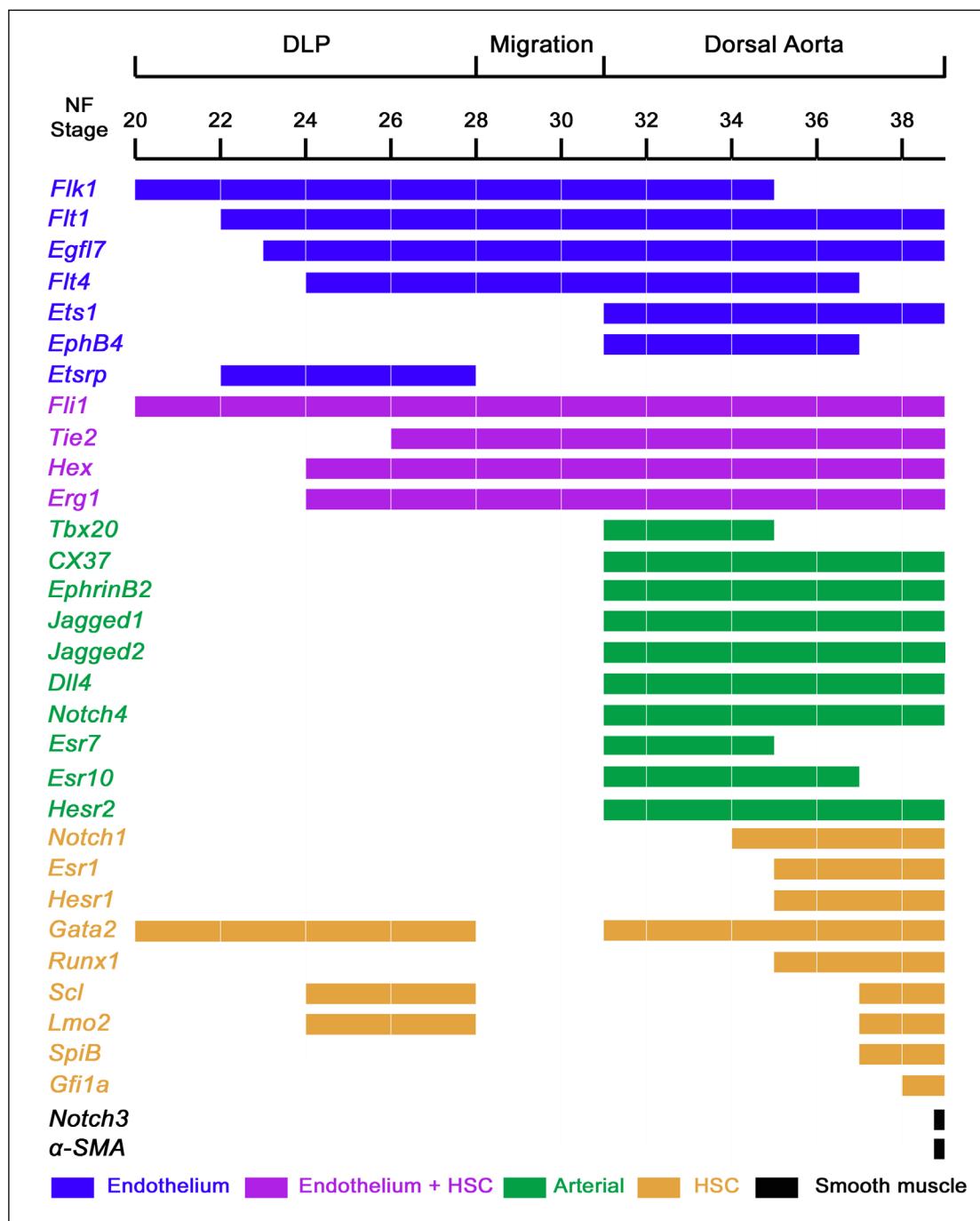


Figure 3.12 Expression of notch receptors is associated with arterial and HSC specification in the Xenopus

DA

Schematic diagram representing the expression of endothelial, arterial, HSC, and smooth muscle genes in the DA, including the expression of *notch4* with the initiation of arterial genes, and the expression of *notch1* immediately prior to the onset of the HSC gene cascade (Ciau-Uitz et al., 2010a; Ciau-Uitz et al., 2010b; Ciau-Uitz et al., 2000; Liu et al., 2008; Walmsley et al., 2002; and Ciau-Uitz, personal communication)

Hesr1 expression has been identified in the murine DA, and has been shown to have a *hesr2* redundant role in vasculogenesis (Fischer et al., 2004; Robert-Moreno et al., 2005). Therefore, both *esr1* and *hesr1* are good candidates for downstream regulation of notch signalling in the *Xenopus* DA.

3.5.4. *Notch3* is associated with smooth muscle development

Notch3 is expressed in the DA from stage 39, after the initial specification of the DA and haemogenic endothelium. At this stage smooth muscle development is beginning in the *Xenopus* DA, as demonstrated by the expression of α -smooth-muscle-actin (α -SMA, Aldo Ciau-Uitz, personal communication). Smooth muscle is found associated with all major arteries in vertebrates, and is essential for the regulation of blood flow and pressure throughout the body. *Notch3* has been described in adult smooth muscle regulation, and mutations in the gene have been associated with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a genetic stroke disorder caused by degeneration of the smooth muscle in blood vessels (reviewed in Wang et al., 2008). Whilst *notch3*^{-/-} mouse embryos show only mild vascular malformations, *notch2/notch3* double knockouts are embryonic lethal, demonstrating severe vascular abnormalities, suggesting *notch2* can compensate for *notch3* in vascular development (Wang et al., 2012). It is possible that the expression of *notch3* in the *Xenopus* DA is associated with smooth muscle development, rather than DA or HSC specification.

Chapter 4. Characterising the role of *notch4* and its target genes in dorsal aorta development and HSC specification

4.1. Introduction

Notch4 is expressed in the dorsal aorta of mouse and *Xenopus* embryos (Robert-Moreno et al., 2005; Uyttendaele et al., 1996; Villa et al., 2001, and Chapter 3.2, respectively). In mice with homozygous null *notch4* mutations, no endothelial or haematopoietic defects have been found, and mice were viable and fertile (Krebs et al., 2000). However, *notch1/notch4* double mutants exhibited greater vascular defects than *notch1* mutants alone, suggesting redundancy between *notch1* and *notch4* during vascular development (Krebs et al., 2000). Overexpression of constitutively active *notch4* under the endothelial specific *flk1* promoter in murine embryos leads to embryonic lethality due to vascular abnormalities (Uyttendaele et al., 2001). E9.5 embryos showed a failure to make branched vessels, instead having large unstable, and mostly unbranched vessels (Uyttendaele et al., 2001). Whilst analysis of definitive haematopoiesis was not undertaken in this study due to embryonic lethality at E9.5-10.5 (prior to HSC emergence), it has suggested that a specific *notch4* dosage is required for vascular branching, patterning and stability. Further analysis using tetracycline (tet) repressible overexpression of constitutively active *notch4* in endothelial cells (under the *tie2* promoter) reveals a larger dorsal aorta and aortic arch arteries, and an underdeveloped cardinal vein lacking a lumen (Kim et al., 2008). In addition, arteriovenous shunts were observed in the vasculature. The number of endothelial cells observed in the DA was 14.5% higher in mutant embryos when compared to wild type, and this increase could not be attributed to proliferation or reduced apoptosis, as analysed by BrdU-labelling and TUNEL assay, respectively. Instead, the distribution of endothelial cells between the cardinal vein and dorsal aorta was found to be disrupted; in the constitutively active *notch4* mutant more endothelial cells were observed in the DA and fewer were observed in the cardinal

vein. This suggests a fate change in these endothelial cells through the promotion of arterial fate by notch4 (Kim et al., 2008).

Using the same constitutively active *notch4* line to temporally activate notch4 signalling in the endothelium of adult mice, it has been demonstrated that higher than normal levels of notch4 signalling causes reversible arteriovenous malformations, shunts and haemorrhages (Carlson et al., 2005; Miniati et al., 2010). Arteriovenous malformations can be found in any tissue and are characterised by enlarged and destabilised vessels which often fuse with veins to cause shunting of circulating blood, and are believed to underlie many clinical disorders. Cessation of constitutively active *notch4* signalling in these mice reversed the physiological findings, so that high-flow arteriovenous shunt containing vessels converted to capillary-like vessels (Murphy et al., 2012). However, continued notch4 signalling led to lethality (Carlson et al., 2005). Thus, notch4 can be directly linked to vessel size and stability in adult mice. In addition to the role of notch4 in primary vasculogenesis, a role in angiogenesis has also been reported (Leong et al., 2002). However, the results overexpression experiments may not represent the endogenous function of the protein. In order to understand what role endogenous notch4 signalling has on vascular or haematopoietic programming, specific notch4 knock-out is needed.

Whilst an endothelial role for notch4 is proposed, the functional redundancy between notch1 and notch4, and the embryonic lethality associated with endothelial-specific overexpression of *notch4* in mouse, has prevented the study of the role of notch4 in haematopoietic stem cell development (Krebs et al., 2000; Uyttendaele et al., 2001). However, a role for notch4 in human haematopoietic stem cell maintenance has been reported (Vercauteren and Sutherland, 2004). Overexpression of *notch4* in human marrow or cord cells reduces the short-term proliferation potential of haematopoietic cells and reduces the colony-formation of monocyte and erythroid

lineages, and of granulocyte-macrophage lineages. In contrast, the primitive progenitor content of long-term cultures is increased. There was also an increase in the level of engraftment of cord cells into NOD/SCID mice when *notch4* was overexpressed. Subsequent B-cell development of these cells was blocked whilst immature T-cell development was increased, suggesting *notch4* may act to decide the fate of the common lymphoid progenitor (Vercauteren and Sutherland, 2004). Retroviral transduction of constitutively active *notch4* into human myeloid leukaemia (HL-60) cells has validated the role of *notch4* in promoting the expansion of haematopoietic progenitor cells at the expense of haematopoietic differentiation (Ye et al., 2004).

To understand the role of *notch4* in *Xenopus* arterial and haematopoietic stem cell development, morpholino oligonucleotide technology was employed. Here, I show for the first time that *notch4* is essential for haematopoietic stem cell programming in *Xenopus*. Notch4 controls expression of *esr7* and *esr10* in the DA immediately after *notch4* expression initiates, and later controls expression of *esr1* and *hesr1*. *Esr7* and *esr10* were also found to be required for haematopoietic stem cell development, whilst expression of *hesr2* in the DA was found to be not dependent on notch signalling.

4.2. Notch4

4.2.1. Designing a morpholino to target *notch4*

The only known sequence for *Xenopus laevis notch4* was a short EST obtained through sequence alignment to mouse *notch4*. This EST, unfortunately, does not contain the 5' UTR or ATG translation start site sequence, so it was not possible to design a translation blocking morpholino with the available sequence. In addition, there was no published *Xenopus laevis* genome sequence available to identify intron/exon boundaries in *notch4* to which a splice morpholino could be designed.

Initially, in order to specifically knock-down *notch4*, a molecular approach known as 5' Rapid Amplification of cDNA Ends (5' RACE – see section 2.2.1.12) was undertaken with the aim of amplifying the 5' UTR to which an ATG morpholino could be designed. RNA was isolated from stage 39 wild type embryos (see section 2.2.4) and using primers notch4 F, R1 and R2 (see Table 2.1 for primer sequences), an additional 437bp sequence 5' to the EST was obtained (Figure 4.1A). When blasted using tblastx, the newly identified sequence was shown to have sequence similarity to *notch4* in several different species. Unfortunately, this newly identified region did not contain the ATG or therefore the 5' UTR and, due to the repetitive nature of the notch gene, further amplification using 5' RACE was unsuccessful.

In autumn 2011, permission was given to access an unpublished preliminary version of the *Xenopus laevis* genome sequence (thanks to Dr. R Harland). A series of short genomic unassembled and unannotated scaffolds was available to download, after which scaffolds were accessed through a UNIX platform (thanks to Dr. J Zuo for his UNIX knowledge and help in accessing these scaffolds). Using SequenceBLAST Locally, a scaffold (Scaffold403602) of 5.3kb was identified as containing regions of sequence identical to the *notch4* EST (E-value 3e-113). Manual annotation of this scaffold identified six exons and seven introns. The identified exons were assigned numbers from 1 to 6 (Figure 4.1B) – these numbers do not reflect their true exon number as the position of these exons in relation to the rest of the exons in the gene is not currently known. Sequence results from 5' RACE were confirmed after identification of the 437bp of additional sequence obtained by 5' RACE in the genomic scaffold, directly 5' to the EST sequence, consisting of exons 1 and 2. These exons are all internal to the protein, containing EGF-like repeats of the extracellular domain of the notch4 receptor. As the scaffold identified was relatively short, it was not possible to find exons 5' to the known sequence, and so it was still not possible to design a morpholino targeting the 5' UTR or translation start site.

Figure 4.1 Sequence analysis of *notch4* to identify potential morpholino binding sites.

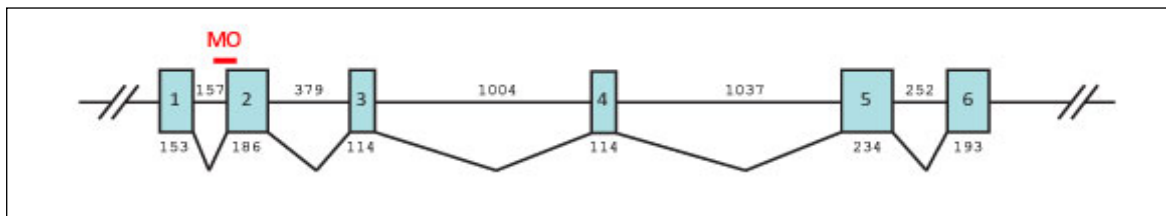
A. 437bp of *notch4* sequence generated by 5' RACE. Highlighted in purple is the *notch4* F primer, used as an internal control, and highlighted in green is the *notch4* R1 primer, used during the nested PCR step of 5' RACE. Highlighted in yellow is the oligo d(T)-anchor primer sequence as provided with the RACE kit (Roche). All sequence highlighted in blue is new *notch4* sequence obtained through 5' RACE. **B.** Schematic showing the six exons and seven introns identified during analysis of genomic scaffold 403602. A morpholino (MO) was designed to target the splice acceptor site at the second identified exon, labelled as '2'. Exon and intron sizes are indicated. **C.** Sequence of *notch4* morpholino (red) compared to *notch4* pre-mRNA. Black indicates intronic sequence whilst green indicates exonic sequence.

A.

```

GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTCTAACATTGATGACTGT 55
GCAACTGCAACCTGTGCAGAAGGGGCTACCTGCATTGATCAAGTGGGAATGTACT 110
TCTGTTTGTGTCCTCCTGGAAAGATTGGTCTGCTGTGTACAACCCAGATGGCTG 165
TAGTAATAACCCCTGCCACCCAGAAGCCACTTGTGATAGTCAGCTAACATCAGTT 220
GCTGTATGTCGGTGCCAGCCTGGATATACTGGCCAACTTGCCACCAAGATCTAG 275
ATGAATGCGGGCTGGGTTCTCCCTTGTGAACATGGTGGGCAGTGTGTCAACAC 330
GCCGGGTTCATTCTATTGCAGCTGTCCTCGGGGGTTTACTGGTGCAAGGTGTGAA 385
ACTGTCCAAGCTGAATGTCAGCCAAATCCTTGTCAACAACAAAGCAACCTGCTTGG 440
ATCTGCTGGGACATTTTGAGTGTGTGTGTTCCTGGATACAAAGGGGACAGATG 495
CGAGTTGGAAGTAGATGAATGTCAGAGCTCTCCATGTCAGAAATGGGGGAAGCTGC 550
ACAGATCTGCTCAATGACTACAACCTGTTCTTGCCACAAGGATTTTCAGGGACCAC 605
AGTGTGAGGAGGATGTTGATGAATGTACCAGCTCCCCCTGTAAAAACAGTGGACA 660
GTGCCACAATCAACCAGGTTCTTACAACCTGTAGCTGCCCTTGGGGTTTCCAAGGT 715
TCAGTGTGTGAGAC 729
  
```

B.



C.

```

notch4 - CTTTCTATGTTTTTCAATCAGGTTCTCCCTTGT
          |||||
          ATACAAAAGTTAGTCCAAGGAGGG
  
```

Instead, intron/exon boundary sequences were sent to Gene Tools for the identification of a suitable splice morpholino site. Whilst several potential splice morpholinos were identified, it was predicted that a morpholino targeting the splice acceptor site for exon 2 would have the best potential of truncating or altering the *notch4* protein to reduce *notch4* signalling (Figure 4.1B and C). *Notch4* morpholino sequence can be found in Table 2.3.

4.2.2. Characterisation of *notch4* splice morpholino

To assess the effect of *notch4* depletion on *Xenopus* development, embryos were injected at the 2 cell stage with 20-40ng *notch4* morpholino. At 40ng, embryos looked unhealthy, showing signs of non-specific toxicity during early development, including developmental delay, necrosis and oedema, and began to die from stage 24. Embryos injected with 20ng and 30ng looked healthy, showing no gross morphological defects (30ng shown in Figure 4.2A). Observation of *notch4* MO injected embryos at stage 39 revealed normally beating hearts but no circulating blood cells in the tail of the embryos. In wild type embryos, circulation is evident in the tails of embryos from stage 37. As no gross morphological defects other than absent circulation were observed at stage 39 in embryos injected with 30ng *notch4* MO, this was chosen as the optimal dose for analysing *notch4* depletion in *Xenopus*. Although no gross morphological defects were seen, some minor developmental differences were observed. In morphant embryos, the developing eye appeared smaller (Figure 4.2A, red arrowheads), fewer pigment cells were observed (blue arrowheads), and the ventral endoderm showed a small protrusion at, or near, the developing proctodeum (green arrowheads).

In order to determine what effect the *notch4* splice morpholino was having on nascent *notch4* mRNA transcripts, PCR amplification across the splice donor site to which the morpholino was designed was carried out to look for alterations in the mRNA transcripts. First, RT-PCR was carried

out with mRNA from stage 39 embryos injected with 30ng notch4 splice morpholino, and from wild type sibling embryos, to generate cDNA from which PCR amplification could be carried out. Primers were designed to amplify across the splice donor site for exon 2, to which the morpholino was designed (see Figure 4.2C for schematic, and Table 2.2 for primer sequences).

When carrying out PCR using the forward F2 primer (sequence located within intron 2 of *notch4*) and reverse R2 primer, amplification should not be possible in wild type embryos. Introns in wild type embryos are spliced out from pre-mRNA to form mature mRNA. In the absence of introns the F2 primer should be unable to bind and so amplification is not possible (Figure 4.2B). However, in the *notch4* morphants, it is possible to see amplification from the F2 primer, signifying the inclusion of intron 2 in the mature mRNA. The expected band size for this is 368bp, which is roughly what is shown on the gel. To confirm this result, the band amplified from morphant embryos using the F2 primer was cut out and gel purified before being ligated into the pGEM[®]-T Easy vector. Sequence analysis of the insert in the pGEM[®]-T Easy vector confirmed the inclusion of intron 2 in the *notch4* morphants. This intron includes three in-frame stop codons, so inclusion of the intron into mature mRNA would result in the truncation of the protein, in the extracellular domain of notch4 (Figure 4.2E). In addition to the band of expected size, a larger band is seen in both wild type and morphant embryos. When sequenced, this band shows notch4 sequence containing both introns 1 and 2 across the region of PCR amplification, suggesting the amplification of immature, pre-spliced mRNA. This is probably due to selection for RNA containing introns through use of an intronic primer. Importantly, the intensity of the band does not change between wild type and morphant RNA extracts, suggesting notch4 MO does not have an impact on the levels of immature, pre-spliced, mRNAs.

The inclusion of intron 1 into mature mRNA is confirmed when amplifying across the region using primers F1 and R1. In morphants, in addition to a band of equal size to that seen in wild type embryos, a larger band is observed. The inclusion of an intron into this region would anticipate a band of 465bp, which is approximately what is seen on the gel. Again, ligation and sequencing of this band has confirmed that this represents the inclusion of an intron into the mature mRNA, containing three in frame stop codons.

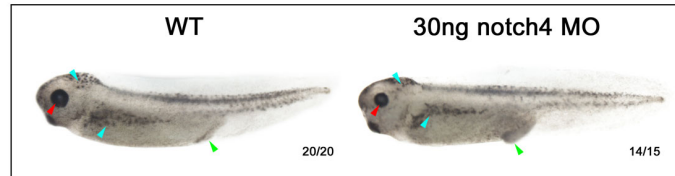
In addition to the inclusion of intron 2 in the *notch4* mRNA in *notch4* morphants, it was possible to see a smaller band in the morphants when compared to wild type, when amplifying from *notch4* morphants across the whole region using primers F1 and R2 (Figure 4.2B). This band was believed to represent the loss of exon 2 in the mature mRNA, and the expected band size of 219bp was in agreement to the band observed on the gel. Again, this band was gel purified before being ligated into pGEM®-T Easy and then sequenced. The sequence data shows that exon 2 is missing from the mRNA transcript in *notch4* morphants. Exon 2 is 186bp in length and, being divisible by three means that deletion of exon 2 would not result in a frameshift mutation of the protein. Instead, this would result in the exclusion of 62 amino acids from the *notch4* protein, including a calcium binding EGF-like repeat (Figure 4.2F). With the sequence information available it is not possible to determine if this is one of the two calcium binding EGF-like repeats essential for notch receptor-ligand interaction (chapter 1.8), but the loss of any EGF-like repeat in the NECD is likely to interrupt the ability of the notch receptors to bind effectively to ligands.

In addition to the alteration in band size observed when amplifying *notch4* using F1 and R2, a band of equal size to that seen for uninjected sibling controls is also observed, suggesting some of the *notch4* mRNA is unaffected by 30ng *notch4* splice MO. This band was again gel purified before being ligated into pGEM®-T Easy and then sequenced.

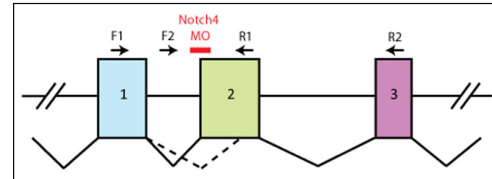
Figure 4.2 Characterisation of *notch4* splice morpholino

A. Photograph showing morphology of stage 39 *notch4* morphant embryos as compared to wild type. **B.** Photograph of gel showing bands from amplification across the *notch4* splice MO boundary in WT and morphant (MO) embryos. Primer combinations of F1/R1, F2/R2 and F1/R2 were used, with ODC as an internal control. Annotations in red dictate the expected size of each band, and the corresponding schematic diagram of mRNA splicing products. **C.** Figure showing the position of primers to amplify across the splice donor for exon 2 in *notch4* transcripts. F1 is a forward primer (*notch4* Ex2Acc MO f1) amplifying from exon 1, F2 is a forward primer (*notch4* Ex2Acc MO f2) amplifying from intron 2, and R2 is a reverse primer (*notch4* Ex2Acc MO r2) amplifying from exon 3. *Notch4* morpholino binding site is shown across the splice donor site for exon 2, labelled as Notch4 MO. Primer sequences can be found in Table 2.2. Dashed line shows cryptic splicing in exon 2. **D.** Wild type composition of mature *notch4* mRNA. **E.** Composition of mature *notch4* mRNA after injection of *notch4* MO, showing inclusion of intron 2 and in-frame stop codons (red crosses). **F.** Composition of mature *notch4* mRNA after injection of *notch4* MO, showing exclusion of exon 2. **G.** Composition of mature *notch4* mRNA after injection of *notch4* MO, showing partial deletion of exon 2 by using a cryptic splice site, and showing a downstream in-frame stop codon (red cross).

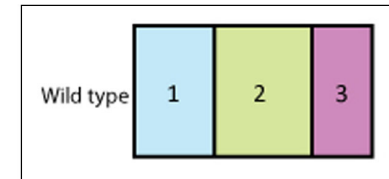
A.



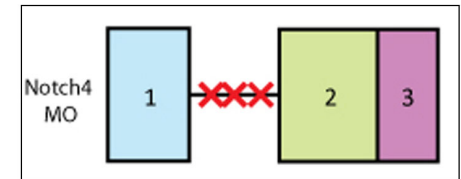
C.



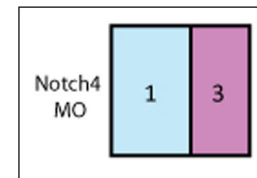
D.



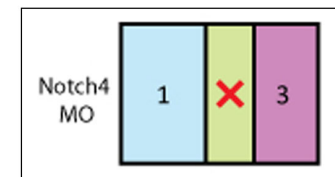
E.



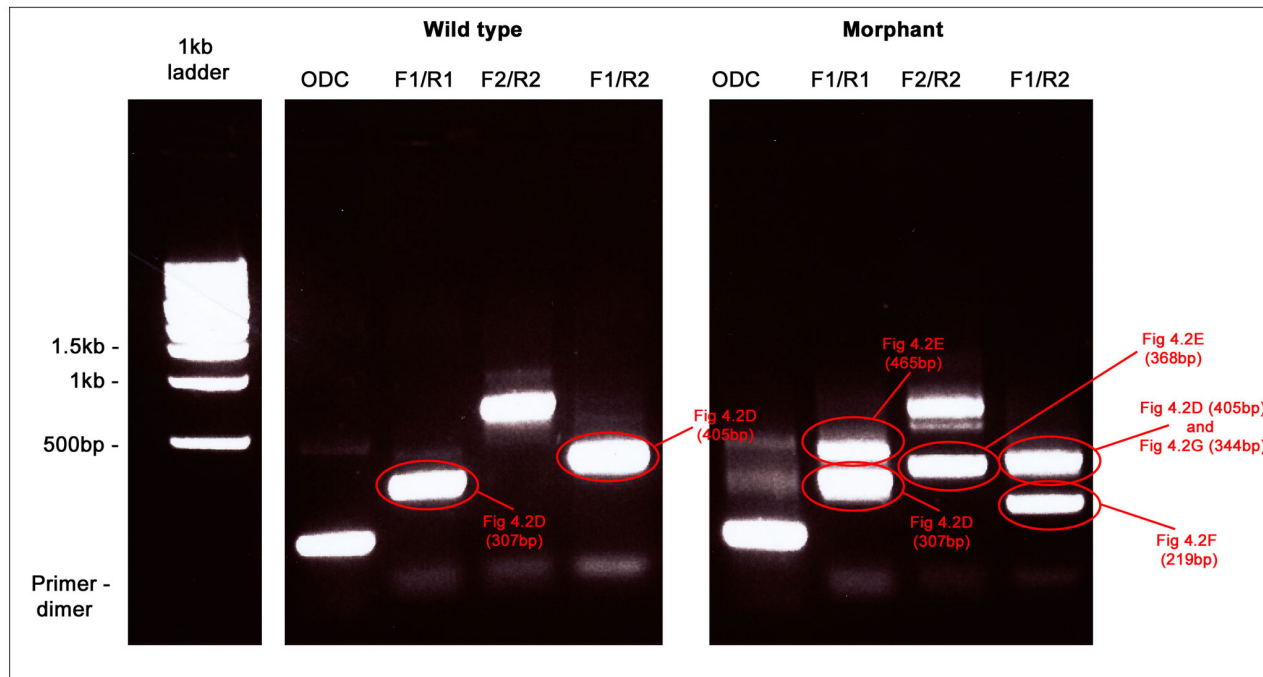
F.



G.



B.



The sequence data shows that whilst the majority of transcripts in this band are wild type (approximately 80%, Figure 4.2D), some of the transcripts show loss of the first 61bp of exon 2, suggesting the mRNA splices to a cryptic splice acceptor site within exon 2. Loss of this 61bp would cause a frameshift mutation, resulting in the generation of a stop codon 30bp downstream of the cryptic splice acceptor site, and thus the truncation of the notch4 protein at this point (Figure 4.2G). When comparing this to the results of F1/R1 amplification, it is mostly in agreement. Whilst it is not expected that a loss of exon 2 in morphant embryos could be detected using primers F1 and R1 due to the R1 sequence being within the exon 2, the splicing to a cryptic splice site within exon 2 should be detected. In F1/R2 amplification of notch4 morphant RNA, this cryptic splicing is not detected by the alteration of a band size, but only through sequence analysis. As this cryptic splicing was observed in only 20% of sequences analysed in F1R2 amplification, it is possible that sequencing of only a few clones has missed the identification in F1/R1 amplification. Another discrepancy observed in this PCR amplification of notch4 in morphant embryos is that the inclusion of intron 1 into mature mRNA is not observed when amplifying using primers F1 and R2, although it is observed when amplifying using both F1 and R1, and F2 and R1 – there is no current explanation for this discrepancy. ODC was used as an internal control throughout the PCR analysis (Figure 4.2B).

4.2.3. Notch4 is essential for haematopoietic stem cell programming

The role, if any, of notch4 in embryonic haematopoietic stem cell programming has yet to be described. To investigate the role of notch4 in *Xenopus* haematopoietic stem cell development, stage 39 embryos injected with 30ng *notch4* MO were analysed by *in situ* hybridisation for the expression of HSC markers. Stage 39 embryos were chosen to analyse to ensure any minor developmental delay caused by morpholino injection does not affect the analysis of DA markers.

For example, *runx1* expression initiates at stage 35 - by analysing expression at stage 39, it is very unlikely that a developmental delay would cause the alteration of HSC marker expression to this point. As previously mentioned, *notch4* morphant embryos show reduced pigmentation. Staging of embryos was not carried out using overall pigment development, but by analysing recession and angle of the ventral yolk and by looking at the migration of pigment throughout the tail, which is not affected by a loss of notch signalling.

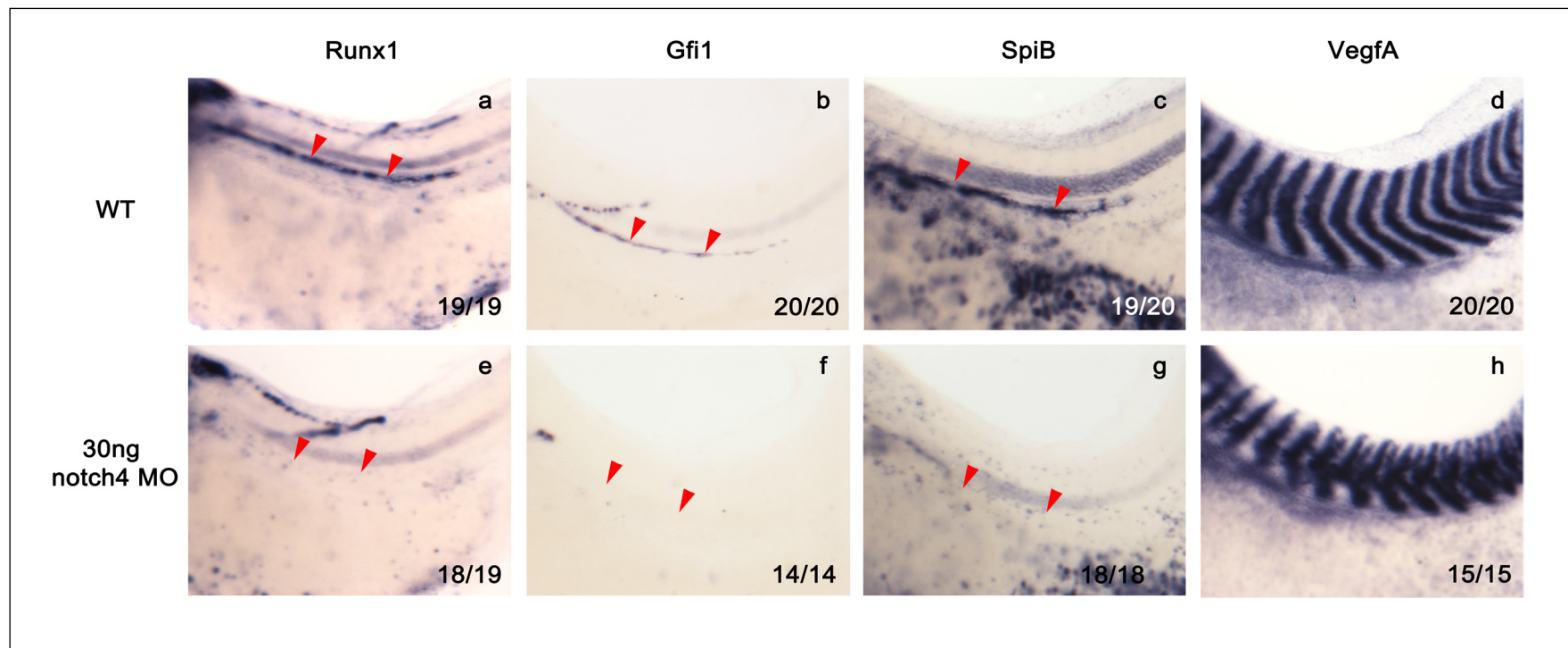
Expression of putative HSC marker *runx1* was abolished in morphant embryos to the point where expression in the DA cannot be detected (Figure 4.3, panel e, red arrowheads). *Sp1B* (a *PU.1* related ETS family member) and *gfi1a* (growth factor independent 1a, a zinc finger transcription factor) are HSC markers expressed after *runx1* expression initiates, and have been found to be *runx1* dependent in *Xenopus* (Aldo Ciau-Uitz, personal communication). Expression of both *sp1B* and *gfi1* was absent in *notch4* morphant embryos, as expected by the loss of *runx1* expression (Figure 4.3, panels f and g, respectively, red arrowheads).

VEGFA (vascular endothelial growth factor A) signalling from the somites to the DA is known to be required for expression of haematopoietic markers including *runx1* (Gering and Patient, 2005; Leung, 2010). As *notch4* is expressed in the somites at stage 20 (see Figure 3.3), it is possible that depletion of *notch4* may alter the expression of *vegfa* in the somites. Expression analysis of *vegfa* in the somites at stage 39 reveals that, although the somites appear somewhat disorganised, expression of *vegfa* is unaffected in *notch4* morphants (Figure 4.3, panel h). As the *vegfa* contribution to haematopoietic programming appears to be maintained, the haematopoietic defects observed are more likely to be due to *notch4* depletion in the DA.

HSC marker *gata2* (GATA binding protein 2, a zinc finger transcription factor) is expressed in the *Xenopus* DA at stage 31, prior to *runx1* expression, and at the same time as *notch4* expression initiates (see schematic, Figure 3.12). The presence of a GATA binding site in the *runx1* promoter suggests that a GATA factor is involved in *runx1* transcription regulation (Nottingham et al., 2007). As *gata2* is the primary definitive haematopoietic GATA factor, and is the only known GATA factor expressed in the *Xenopus* DA during haematopoietic programming, it is probable that *gata2* is involved in the regulation of *runx1* (Aldo Ciau-Uitz, personal communication). At stage 39 expression of *gata2* is notably decreased in *notch4* morphants (Figure 4.4, panel d, red arrowheads), suggesting that HSC programming is disrupted prior to *runx1* expression. Interestingly, *gata2* expression initiates normally, as evidenced by the relatively normal expression in the DA at stage 32 (Figure 4.4, panel c, red arrowheads). As the regulation of *gata2* is not disrupted in *notch4* morphants at stage 32, but is disrupted at stage 39, it suggests two independent modes of *gata2* regulation in the *Xenopus* DA, dependent on developmental stage, with the later stages requiring notch signalling. Loss of haematopoietic markers was used as a positive control for all subsequent *notch4* morphant phenotypes.

Figure 4.3 *Notch4* is required for the expression of haematopoietic stem cell markers.

Notch4 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of HSC markers *runx1*, *spiB* and *gfi1* is lost in *notch4* morphant embryos at stage 39 when compared to WT sibling controls. Expression of *vegfa* in the somites appears normal. Red arrowheads mark the dorsal aorta.



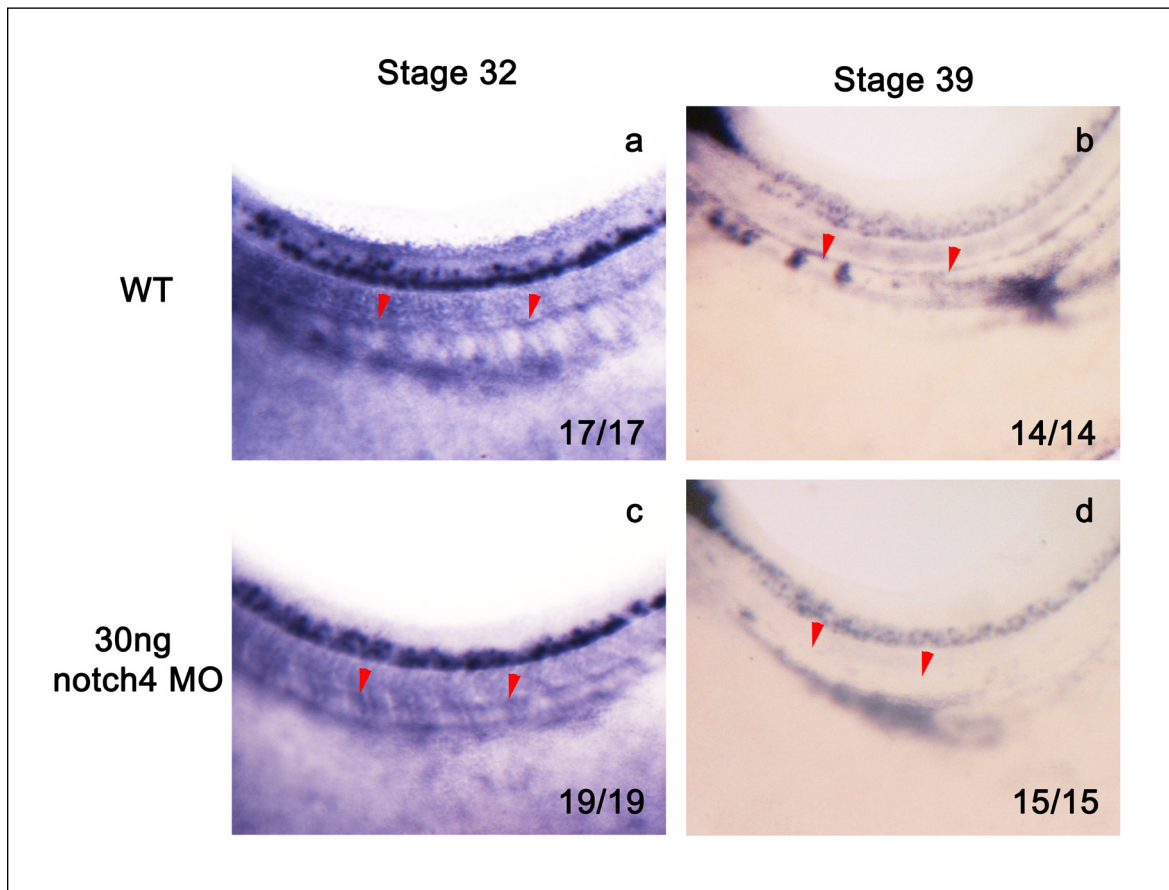


Figure 4.4 Expression of *gata2* is dependent on notch4 at stage 39, but not at stage 32

Notch4 MO was injected into both cells of two-cell embryos which were collected at stage 32 and 38-39 and analysed by *in situ* hybridisation. *Gata2* expression initiates relatively normally in *notch4* morphant embryos, as evident by expression in the DA at stage 32. However, expression is not maintained, as evident by the loss of expression at stage 39. Red arrowheads mark the DA.

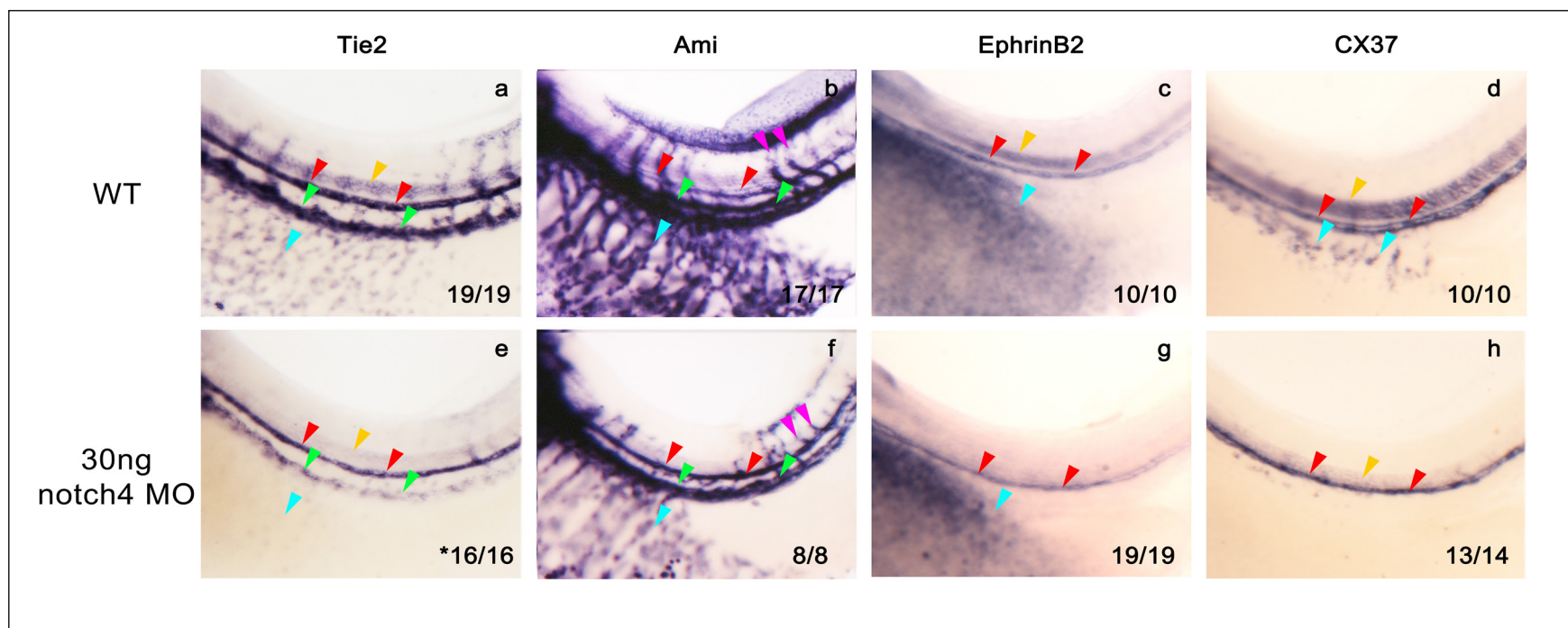
4.2.4. Endothelial and dorsal aorta specification is not affected by loss of *notch4*

A role for *notch4* in vascular programming has been suggested in mouse; overexpression of *notch4* expands arterial vessels, and causes arteriovenous malformations and shunts (Kim et al., 2008; Krebs et al., 2000; Miniati et al., 2010; Murphy et al., 2012; Uyttendaele et al., 2001). In order to understand if the haematopoietic defect found in *notch4* *Xenopus* morphants is the direct result of a *notch4* deficiency on haematopoiesis, or is due to an earlier vascular programming defect, it is essential to look at endothelial and arterial programming. Expression of endothelial markers *tie2* (Tunica interna endothelial cell kinase-2, an angiopoietin receptor) and *ami* (meaning 'mesh' in Japanese, similar to human complement factor D, CFAD), revealed normal expression in the DA of stage 39 *notch4* morphants (Figure 4.5, panels e and f, respectively, red arrowheads). Similarly, expression of arterial markers *ephrinB2* (EfnB2, a transmembrane arterial specific protein) and *cx37* (connexin 37, or gap junction protein alpha 4, an arterial specific protein) is also normal in the DA of *notch4* morphant embryos (Figure 4.5, panels g and h, respectively, red arrowheads). However, in the *notch4* morphants, the DA appears to be thinner, suggesting either a problem with lumen formation or fewer cells in the DA.

Expression of *tie2*, *ami*, *ephrinB2* and *cx37* in the vitelline vessels is massively reduced, and fewer vessels are apparent here, suggesting *notch4* has a role in the development of the vitelline vasculature (Figure 4.5, panels e, f, g and h, respectively, blue arrowheads). Development of the intersomitic vessels from the vein is also disrupted in *notch4* morphants, as marked by expression of *tie2* and *ami* (Figure 4.5, panels f and g, respectively, purple arrowheads). Intersomitic vessels (ISVs) initially sprout from the posterior cardinal vein in *Xenopus*, at stage 35, before intersomitic arteries sprout from the DA at stage 37 in the anterior region of the embryo (Levine et al., 2003).

Figure 4.5 Expression of endothelial and arterial genes is normal in *notch4* morphants

Notch4 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation.. Expression of endothelial markers *tie2* and *ami*, and of arterial markers *ephrinB2* and *cx37* are normal in the dorsal aorta of *notch4* morphants. However, the vessel appears marginally thinner than in wild type sibling controls, and intersomitic vessel defects are apparent. Red arrowheads mark dorsal aorta, green arrowheads mark the vein, orange arrowheads mark the notochord, blue arrowheads mark vitelline vasculature, and purple arrowheads mark intersomitic vessels.



Here, intersomitic vessel sprouts from the cardinal vein are seen most clearly in the wild type embryos, but are lost in the morphants. This suggests that whilst *notch4* is dispensable for programming of the DA, it has a functional role in angiogenesis of intersomitic vessels.

Runx1 has been shown to downregulate the expression of *flk1* in ES cell differentiation studies, and thus it was proposed that *runx1* could do the same during development of haemogenic endothelium (Hirai et al., 2005). During development of the *Xenopus* DA, the timing of *flk1* downregulation is concomitant with initiation of *runx1* expression, thus the timing is appropriate for this theory. However, when knocking down *runx1* using morpholinos in *Xenopus*, it was not possible to maintain the expression of *flk1* in the DA (Fernandez, 2007), indicating that *runx1* does not repress expression of *flk1* in *Xenopus*. Several other studies have suggested that the notch target gene *hesr1* is responsible for down-regulating expression of *flk1* (Henderson et al., 2001; Holderfield et al., 2006; Taylor et al., 2002). The interaction of Hey proteins with promoters lacking a TATA box but containing an initiator element, such as the *vegfr2/flk1* promoter, leads to transcriptional repression in an E-box independent manner (Holderfield et al., 2006; reviewed in Siekmann et al., 2008). Artificial introduction of a TATA box to these promoters overcomes the ability of Hey proteins to repress transcription from these promoters, suggesting that they act to prevent transcription machinery binding at the promoter (Holderfield et al., 2006). In the *Xenopus* DA, expression of *hesr1* and a second hairy and enhancer of split gene, *esr1*, initiates at the same time *flk1* expression is downregulated, and thus one or both may be controlling *flk1* downregulation (Figure 3.12). As *notch4* controls the expression of both *esr1* and *hesr1* in the DA (see chapter 4.2.6), it is possible that *flk1* is downregulated in *notch4* morphants. To analyse this, expression analysis of *flk1* was carried out in stage 39 *notch4* morphants. At stage 39, expression of *flk1* has been downregulated and cannot be seen in the DA of wild type embryos (Figure 4.6,

panel a, red arrowheads). In *notch4* morphant embryos, expression of *flk1* is also not seen (Figure 4.6, panel c, red arrowheads), suggesting *notch4* does not control the downregulation of *flk1*. Another initiator containing TATA-less gene expressed in the DA is *flt4/vegfr3*, and expression analysis has indicated that expression is also downregulated in the DA, slightly after expression of *flk1* is downregulated (Figure 3.12), and cannot be seen in the DA at stage 39 (Figure 4.6, panel b, red arrowheads). In *notch4* morphants, expression of *flt4* is also not maintained (Figure 4.6, panel d, red arrowheads). Similar to expression analysis for *tie2* and *ami*, expression analysis of *flt4* in *notch4* morphants reveals defective intersomitic vessel spouting when compared to wild types (Figure 4.6, panel d and b, respectively, green arrowheads).

4.2.5. Primitive haematopoiesis is normal in *notch4* morphant embryos, but primitive blood does not circulate normally

Expression of *notch4* has not been observed in the VBI in *Xenopus*, and therefore should not be involved in primitive haematopoiesis. In addition, a role for *notch4* in primitive haematopoiesis in other organisms has not been reported. To analyse programming of the VBI in *notch4* morphants, expression analysis of *alpha-T4 globin* was carried out in stage 28 embryos. An embryonic form of globin, alpha-T4 globin, is necessary for the correct function of primitive erythrocytes in *Xenopus*. Expression of *globin* in the VBI of *notch4* morphants is normal (Figure 4.7A, panel c, orange arrowheads), suggesting that VBI programming is normal.

In addition to primitive erythrocytes, early primitive myeloid cells are also specified in the *Xenopus* VBI (see chapter 1.9.2.1), and express *myeloperoxidase* (*mpo*, or *pox*). Expression analysis of *mpo* in *notch4* morphant embryos reveals normal programming of myeloid cells at this stage (Figure 4.7A, panel d), again demonstrating that *notch4* is not required for VBI programming. Therefore, *notch4* can be regarded as not essential for primitive haematopoiesis.

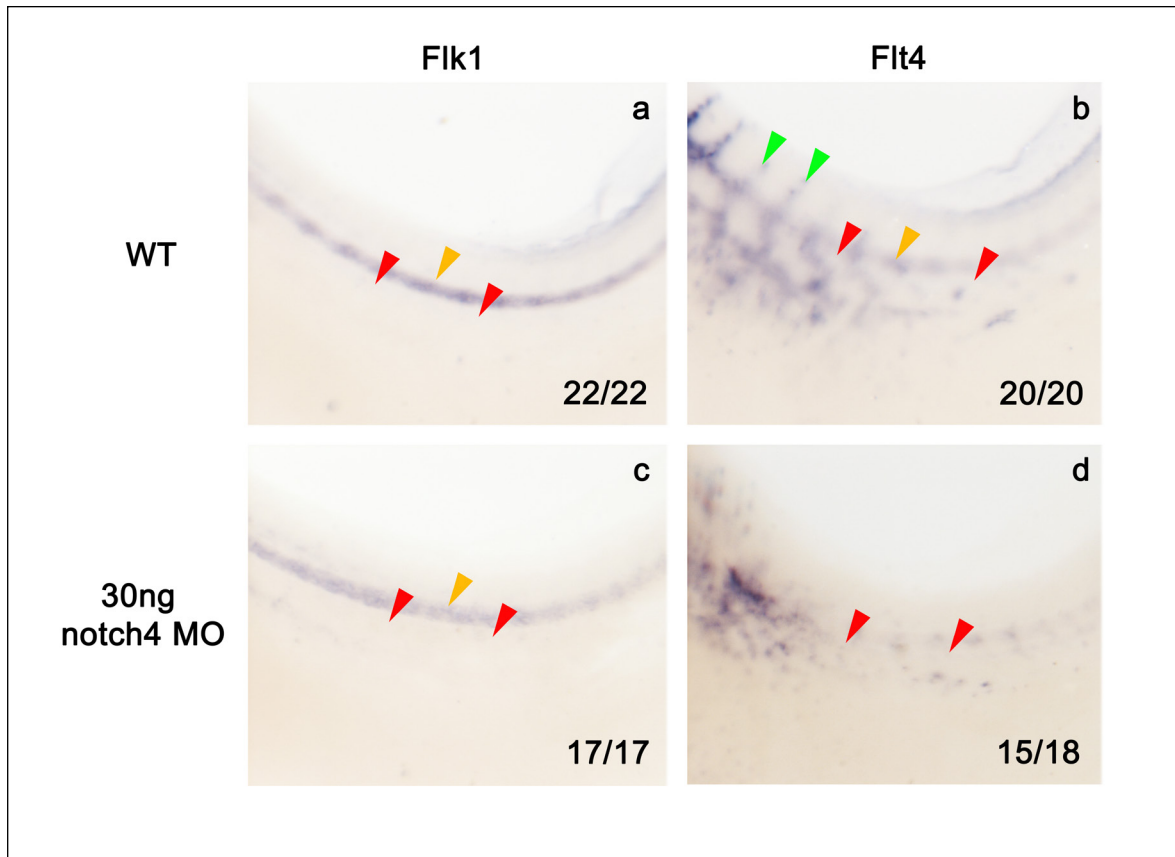


Figure 4.6 Expression of endothelial genes *flk1* and *flt4* is not maintained in *notch4* morphants.

Notch4 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of *flk1* and *flt4* in the DA of *notch4* morphants is not maintained. Red arrowheads mark the location of the dorsal aorta, orange arrowheads mark the notochord, green arrowheads mark intersomitic vessels.

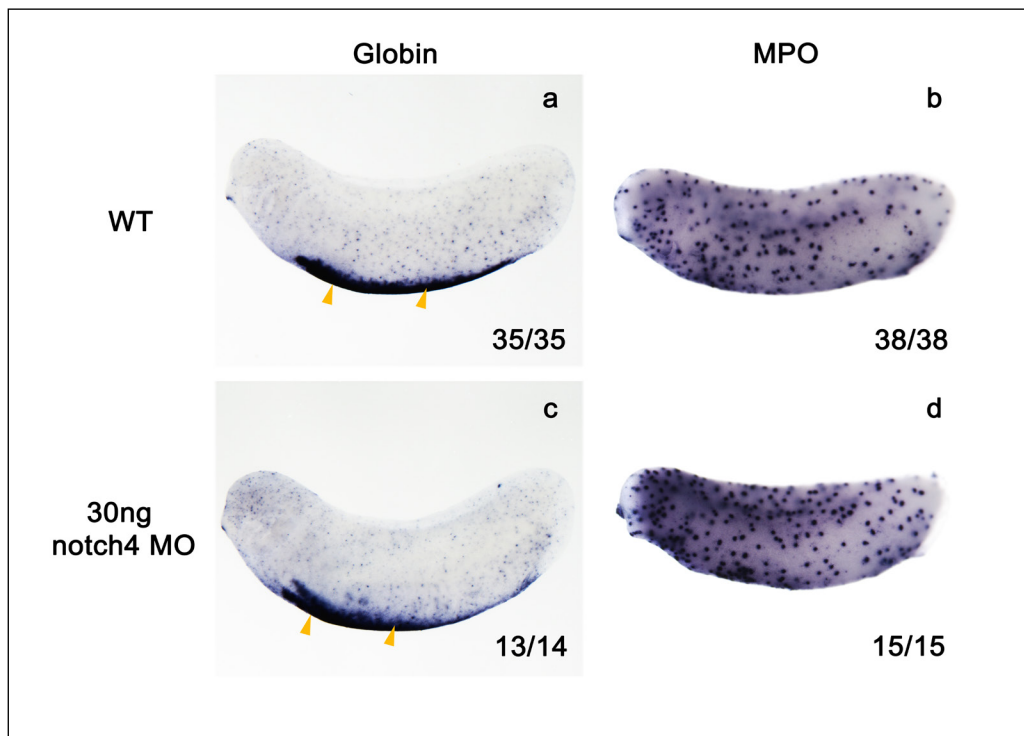
Analysing the expression of primitive haematopoietic markers later in development can provide essential information about circulation in embryos. At stage 39, primitive erythrocytes can mainly be seen circulating throughout wild type embryos, as analysed by the expression of *globin* and *gata-1* (Figure 4.7B, panels a and b, respectively). Primitive erythrocytes can be seen in the dorsal aorta (red arrowheads), cardinal vein (green arrowheads), and within the heart (blue arrowheads). However, in *notch4* morphants, primitive erythrocytes are seen pooling in the VBI (Figure 4.7B, panels c and d, orange arrowheads). This is consistent with the observation that blood cells are not seen circulating through the tail in living stage 39 *notch4* morphant embryos (chapter 0). As the heart is beating normally in *notch4* morphant embryos, the lack of circulation is likely due to a vascular defect. A reduced number of vitelline vessels are seen in *notch4* morphants stained for endothelial marker *ami* at stage 39 (Figure 4.5, panel f, blue arrowheads). Connection of the VBI to the rest of the circulatory network through the vitelline vasculature is essential to allow primitive erythrocytes to circulate throughout the embryo, and thus, this reduced number of vitelline vessels may account for the lack of circulation. As previously discussed, *notch4* has been demonstrated to have a role in controlling lumen size in the mouse. In *notch4* morphants, the vessel often appears thinner than in wild type sibling controls. For example, in embryos stained for *ephrinB2* the DA appears thinner in *notch4* morphants (Figure 4.5, panel g, red arrowheads). It is possible that *notch4* is required for the lumenisation of vessels, and thus lumens may not be formed in *notch4* morphants, inhibiting the circulation of erythrocytes through vessels. In order to fully understand whether *notch4* is required for lumenisation of the *Xenopus* DA, it is essential for *in situ* hybridisation on sections to be carried out, whereby the vessel diameter can be studied.

Blood flow is known to regulate expression of haematopoietic markers in the zebrafish DA (North et al., 2009). Detection of sheer stress through nitric oxide (NO) signalling alters maintenance of *runx1* expression in the zebrafish DA from 36hpf, which is after initial stem cell emergence from the DA at 30hpf. However, prior to 36hpf, *runx1* expression is not affected by sheer stress and NO signalling, and thus it is maintenance, rather than initiation, of *runx1* that is controlled by blood flow (Wang et al., 2011). In zebrafish, HSCs begin to emerge from the DA at 30hpf (Kissa and Herbomel, 2010), so maintenance of *runx1* expression only becomes dependent on blood flow after initial HSC emergence. In support of this, the absence of blood flow does not hinder the emergence of HSCs from the DA of murine embryonic sections (Boisset et al., 2010). Furthermore, in *Xenopus*, *runx1* expression initiates in the DA at stage 35, prior to circulation of primitive erythrocytes at stage 37. Thus, circulation cannot be controlling initiation of *runx1*. In *notch4* morphant embryos, *runx1* expression was analysed at stage 39, before the emergence of stem cells, but after the onset circulation. This is analogous to 26-28hpf in zebrafish, at a time when zebrafish *runx1* expression is not dependent on blood flow. Thus, it is unlikely that the haematopoietic defect seen in *notch4* morphants is due to a circulatory problem. Furthermore, a loss of *runx1* and other haematopoietic markers was evident in *esr7* morphant embryos (Figure 4.14), where primitive erythrocytes circulate as normal (Figure 4.18), and thus this haematopoietic defect cannot be due to a loss of circulation.

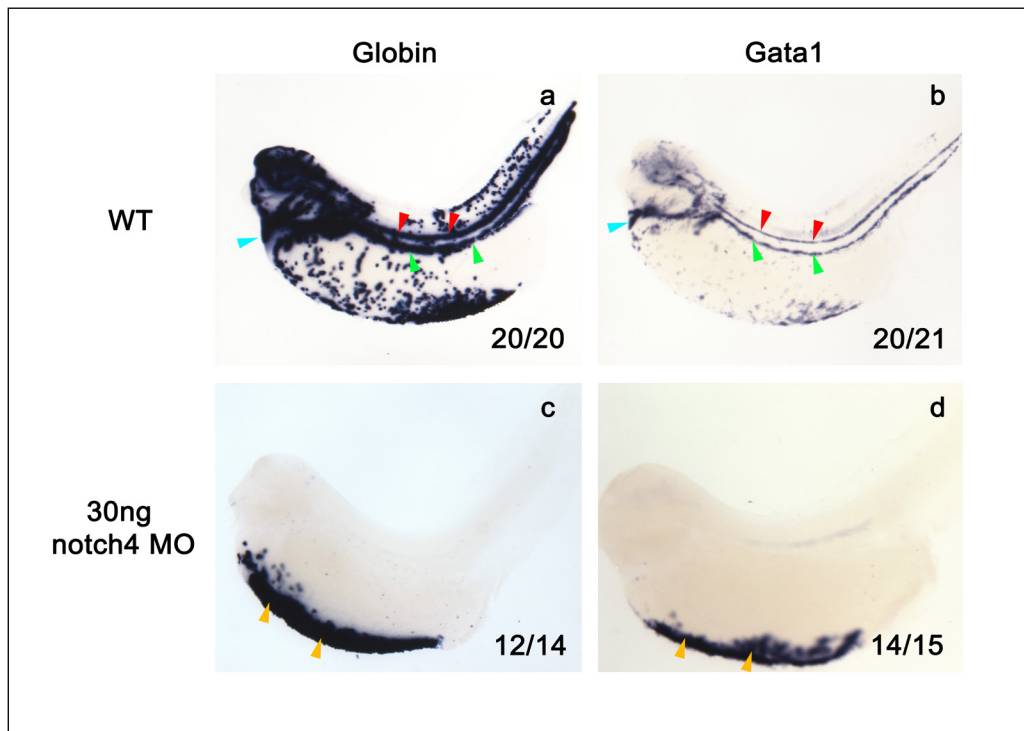
Figure 4.7 Primitive haematopoiesis is not affected by depletion of *notch4*, but primitive blood cells do not circulate in a *notch4* deficient embryo.

Notch4 MO was injected into both cells of two-cell embryos which were collected at stage 28 or 38-39 and analysed by *in situ* hybridisation. **A.** At stage 28, expression of α -T4 globin, a marker of primitive erythrocytes, is normal in *notch4* morphants, as is *mpo*, a marker of primitive myeloid cells. **B.** At stage 39, erythrocytes can be seen circulating throughout wild type embryos, but circulating cells are not evident in *notch4* morphants. Red arrowheads mark the DA, green arrowheads mark the cardinal vein, orange arrowheads mark primitive erythrocytes in the VBI and blue arrowheads mark the heart.

A.



B.



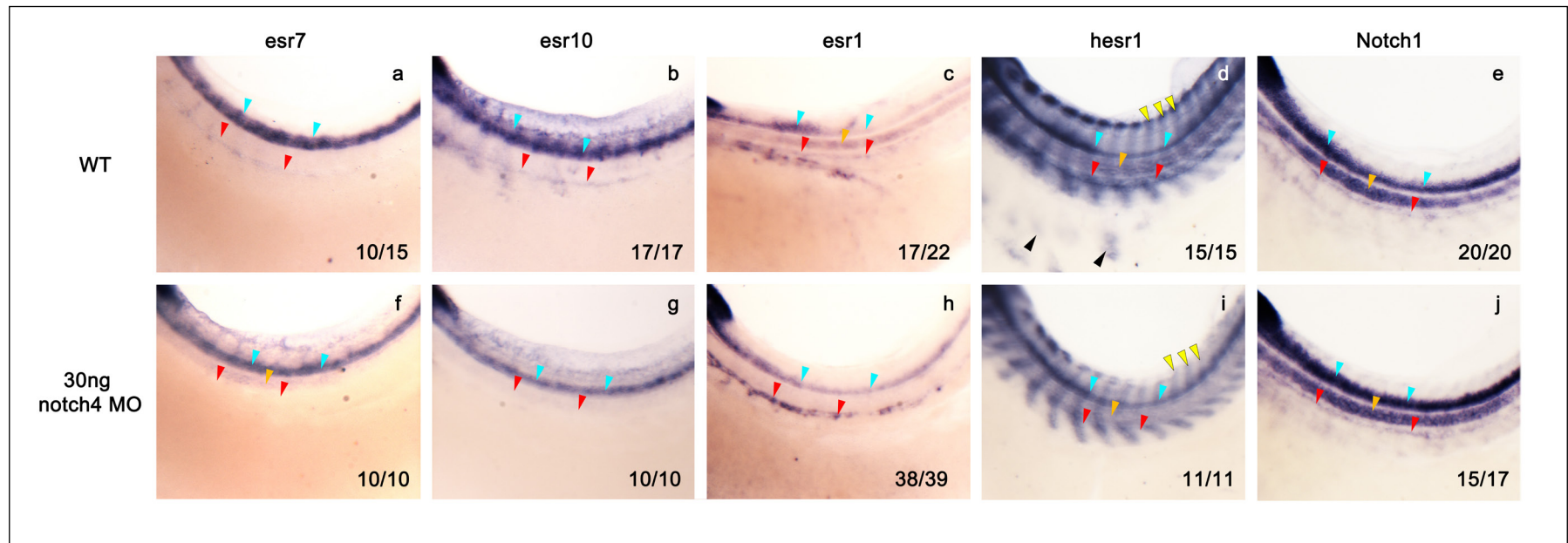
4.2.6. *Notch4* is required for the expression of hairy and enhancer of split genes

Genes from the hairy and enhancer of split related family are well documented as direct targets of notch signalling (see chapter 1.8.2). In the *Xenopus* DA, expression of five hairy and enhancer of split related genes has been identified. These five genes are activated in two waves, corresponding to the initiation of expression of two notch receptors (see chapter 3.5). Expression of *esr7*, *esr10* and *hesr2* is initiated at stage 31, at the same time that *notch4* expression initiates. Therefore, these three genes are good candidates for *notch4* target genes in the *Xenopus* DA. In *notch4* morphant embryos, expression of *esr7* and *esr10* is lost in the DA at stage 33 (Figure 4.8, panels f and g, respectively, red arrowheads), suggesting they are targets of *notch4*. Expression of *esr1* is upregulated in *notch4* morphants at stage 39 (Figure 4.8, panel h, red arrowheads), suggesting *notch4* either directly or indirectly negatively regulates expression of *esr1*. In contrast, expression of *hesr1* is downregulated at stage 39 in *notch4* morphants (Figure 4.8, panel i, red arrowheads). This suggests that *notch4* controls expression of *esr7*, *esr10*, *esr1* and *hesr1* in the *Xenopus* DA, in either a direct or indirect manner. However, expression of *hesr2* is unaffected in *notch4* morphants. At both stage 32 and stage 39 expression of *hesr2* can be seen in the DA of *notch4* morphant embryos (Figure 4.9, panel c and d, respectively, red arrowheads), suggesting *hesr2* is not a target gene of *notch4*. Unfortunately, expression of *esr7*, *esr10* and *esr1* is naturally weak in the DA of wild type embryos, making it difficult to decipher after photography on occasion. In addition, whilst *hesr1* and *hesr2* appear to be expressed more strongly in the DA, the expression here is often masked by extremely strong expression in the overlying somites.

Notch1 is expressed after *notch4* in the *Xenopus* DA (Figure 3.12), and has a role in *Xenopus* haematopoietic stem cell development (chapter 5.2). To understand whether the haematopoietic defects observed in *notch4* morphants is due to loss of *notch1* in the DA, *in situ* hybridisation analysis for *notch1* was carried out.

Figure 4.8 *Notch4* regulates the expression of hairy and enhancer of split genes in the DA

Notch4 MO was injected into both cells of two-cell embryos which were collected at stage 33 or 38-39 and analysed by *in situ* hybridisation. Expression of *esr7*, *esr10* and *hes1* is significantly reduced whilst expression of *esr1* is upregulated in *notch4* morphants. *Notch1* expression is not affected in *notch4* morphants. Red arrowheads mark the DA, blue arrowheads mark the neural tube, orange arrowheads mark the notochord and yellow arrowheads mark the somites.



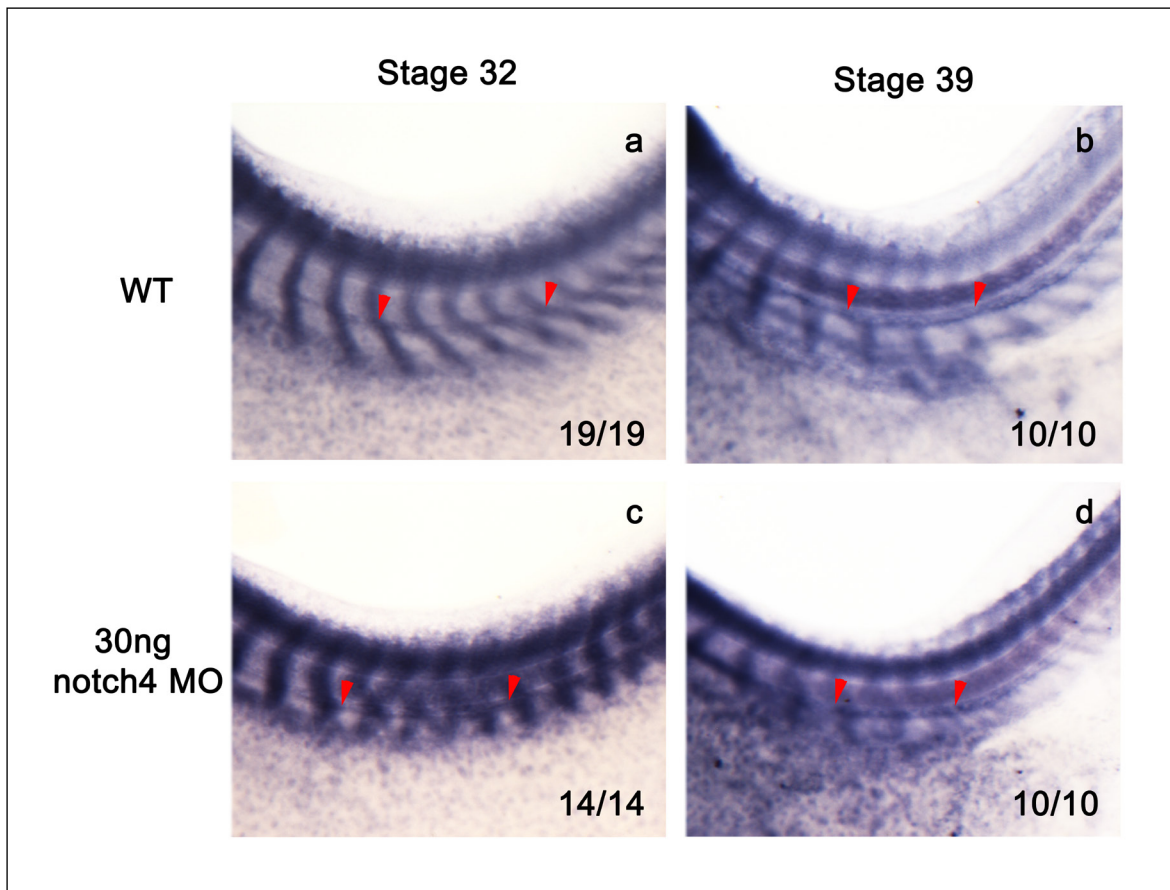


Figure 4.9 Expression of *hesr2* is unaffected in *notch4* morphants

Notch4 MO was injected into both cells of two-cell embryos which were collected at stage 32 or 38-39 and analysed by *in situ* hybridisation. Expression of *hesr2* is evident in the DA of *notch4* morphants at both stage 32 and 39, suggesting it is not a target of *notch4* signalling. Red arrowheads mark the DA.

In *notch4* morphants, expression of *notch1* is normal in the DA (Figure 4.8, panel j, red arrowheads). Therefore, *notch4* does not control the expression of *notch1* in the DA, and the haematopoietic defects observed are not due to a loss of *notch1* signalling.

Notch4 expression is restricted to the DA during arterial and HSC specification (Figure 3.1, panels s-x), and so it is encouraging to note that expression in the neural tube (blue arrowheads) and somites (yellow arrowheads) is unaffected in *notch4* morphants, suggesting little or no non-specific toxic effects altering embryonic patterning.

4.3. *Hesr2*

Hesr2 is a YRPW-containing hairy and enhancer of split related gene and is expressed in the Xenopus DA at the same time *notch4* expression initiates (Figure 3.11). As *hesr2* is a member of a large family of genes mostly thought to act downstream of notch signalling, it was expected that *hesr2* would be a notch target gene in the Xenopus DA also. A paralogue of *hesr2* in zebrafish, named *gridlock*, has been reported as essential for arterial and haematopoietic specification (Rowlinson and Gering, 2010; Zhong et al., 2001; Zhong et al., 2000). In zebrafish, *gridlock* has been shown to act both upstream and downstream of notch signalling, depending on the developmental context (Rowlinson and Gering, 2010).

4.3.1. *Hesr2* is not a notch target gene in the Xenopus DA

Hesr2 was initially believed to be a *notch4* target gene in the Xenopus DA due to the overlapping temporal and spatial expression patterns, and the nature of the family being notch target genes. However, expression analysis of *hesr2* in *notch4* morphants revealed very little, if any, effect on expression in the DA at both stage 32 and 39 (Figure 4.9, panels c and d, respectively, red arrowheads, and Figure 4.10, panel d, red arrowheads). Although temporal expression data suggests *notch4* should regulate *hesr2* from initiation of expression at stage 31, *notch1* expression initiates later in the DA, at stage 34, and may be responsible for maintaining expression of *hesr2*. Analysis of *hesr2* expression in *notch1* morphants revealed expression is relatively unaffected (Figure 4.10, panel e, red arrowheads, see chapter 5.2 for *notch1* MO details). Similarly, using notch inhibitor DAPM to block all notch signalling in the embryo, expression of *hesr2* is unaffected in the DA (Figure 4.10, panel f, red arrowheads, and see Chapter 6 for DAPM details). Thus, *hesr2* is not considered to be a notch target gene in the Xenopus DA.

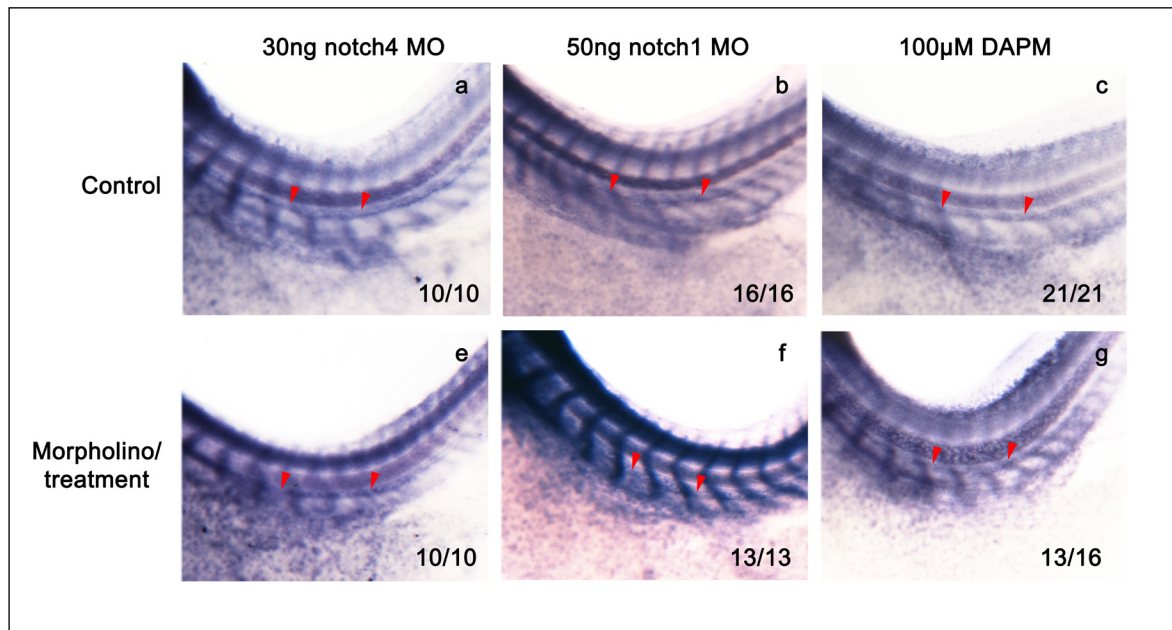


Figure 4.10 *Hes2* is not a notch target gene in the *Xenopus* DA

Notch4 or *notch1* MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. DAPM treatments initiated at stage 20, embryos were collected at stage 38-39, and analysis was carried out using *in situ* hybridisation. Expression of *hes2* is not significantly downregulated in *notch1* or *notch4* morphants, or in embryos treated with notch inhibitor DAPM, and thus is not a notch target in the DA. Red arrowheads mark the DA.

4.3.2. Designing a morpholino to target *hesr2*

In order to understand the role of *hesr2* in arterial and/or haematopoietic development, a morpholino oligonucleotide was designed. The only known sequence for *Xenopus laevis hesr2* is a short EST obtained through sequence alignment to mouse *hesr2*. This EST, unfortunately, does not contain the 5' UTR or ATG translation start site, so it was not possible to design a translation blocking morpholino with the available sequence. In addition, there was no published *Xenopus laevis* genome sequence available to identify intron/exon boundaries in *notch4* to which a splice morpholino could be designed.

5' RACE was undertaken with the aim of amplifying the 5' UTR to which an ATG morpholino could be designed. Using RNA isolated from stage 39 wild type embryos (see section 2.2.4) and primers *hesr2* F, R1, R2 and R3 (see Table 2.1 for primer sequences), 424bp of additional 5' sequence was obtained (Figure 4.11). When blasted using tblastx, the newly identified sequence was shown to have sequence similarity to *hesr2* in *Xenopus tropicalis* (top hit, 2e-46). In this newly identified sequence an in-frame upstream stop codon was identified, suggesting the sequence obtained by 5' RACE contained part, or all, of the *hesr2* 5' UTR. An in-frame ATG site was identified 33bp downstream of the stop codon and was consequently believed to be the translation start site for *hesr2*. A translation blocking morpholino was therefore designed to block this site; morpholino sequence: TGATGAGGTTCCGAGTAGTATATCA. If, however, a further downstream ATG site was the translation start site, this morpholino would still act as a translation blocker as binding anywhere within the 5' UTR prevents translation of the mRNA transcript.

Unfortunately, injection and analysis of this morpholino in order to understand the role of *hesr2* in arterial and/or HSC development was not carried out due to time constraints. However, the

identification of a suitable morpholino binding site and sequencing of the 5' UTR will enable future studies into the role of *hesr2* in haematopoietic development.

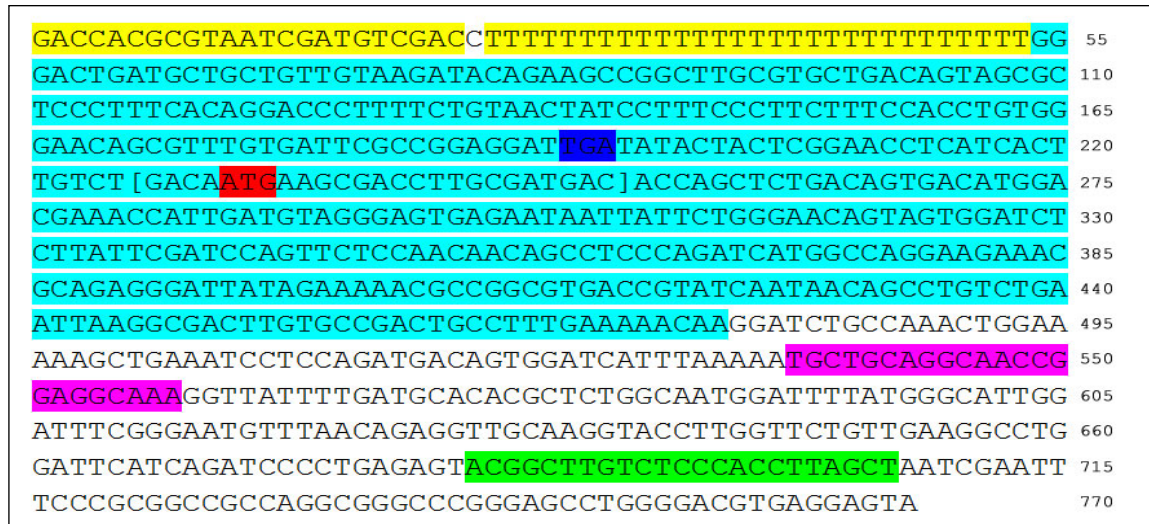


Figure 4.11 Additional sequence of *hesr2*, as identified by 5' RACE.

Figure showing the additional 424bp of sequence generated by 5' RACE. Highlighted in purple is the *hesr2* F primer, used as an internal control. Highlighted in green is the *hesr2* R1 primer used during the nested PCR step of 5' RACE. Highlighted in yellow is the Oligo d(T)-Anchor primer sequence as provided with the 5' RACE kit (Roche). All sequence highlighted in light blue is new *hesr2* sequence obtained through 5' RACE. Highlighted in dark blue is the upstream stop codon and highlighted in red is the first ATG site. Brackets denote the sequence to which the *hesr2* morpholino is targeted.

4.4. *Esr7*

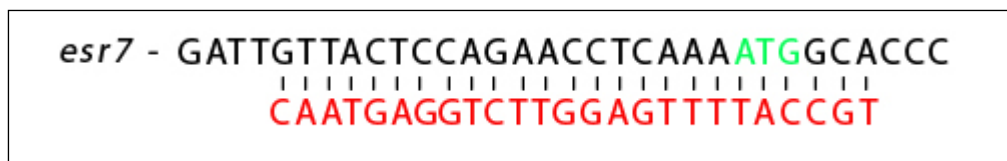
Esr7 is a WRPW-containing enhancer of split related gene expressed in the *Xenopus* DA (Figure 3.7), and an orthologue of mouse *hes5*. *Hes5* expression has not been reported in the developing vasculature or haematopoietic cells, and as such it is not known whether or not it is involved in endothelial, arterial or haematopoietic development. *Hes5* has been found to be a direct notch target gene (Ohtsuka et al., 1999), so it is expected that *esr7* is a direct notch target gene also. Expression of *esr7* initiates at stage 31, at the same time *notch4* expression is initiated (Figure 3.11), and expression of *esr7* is dependent on notch4 signalling (Figure 4.8), and thus, *esr7* may be regarded as a notch4 target gene.

4.4.1. Designing a morpholino to target *esr7*

Using the full-length mRNA sequence for *esr7* (Unigene Xl.586), a morpholino was designed and produced by Gene Tools (see Table 2.3 for sequence, Figure 4.12A). As mentioned in chapter 3.4.2, *esr7* has a pseudoallele referred to as *esr7b* or *esr3*. Available sequence data for *esr7b* lacks the 5' UTR sequence, and thus it is not possible to determine whether or not *esr7* morpholino binds to the pseudoallele. Unfortunately, attempts to sequence the 5' UTR of *esr7b* using 5' RACE were unsuccessful. The coding sequences of *esr7* and *esr7b* share 99% sequence identity (1377/1385, E-value 0.0). Other pseudoalleles with this degree of similarity studied for this thesis (*esr1/esr1b*, and *hesr1/hesr1b*) show strong conservation in the 5' UTR, and both pseudoalleles were targeted by one morpholino in each case; therefore, it is likely that this will be the case for *esr7*. However, this needs to be confirmed by sequencing *esr7b*. The *esr7* MO sequence was blasted against the *X.laevis* nucleotide collection to look for potential off-target effects, and no predicted targets other than *esr7* were detected.

To assess the effect of *esr7* depletion in *Xenopus*, embryos were injected at the 2-cell stage with 20-40ng *esr7* morpholino. At 40ng, embryos looked unhealthy showing signs of non-specific toxicity during early development, including developmental delay, necrosis and oedema, and began to die from stage 20. Embryos injected with 20ng and 30ng looked healthy, showing no gross morphological defects (30ng shown in Figure 4.12B). Observation of *esr7* MO injected embryos at stage 39 revealed normally beating hearts and normal circulation in the tail of the embryos. As no gross morphological defects were observed in embryos injected with 30ng *esr7* MO, this was chosen as the optimal dose for analysing *esr7* depletion in *Xenopus* embryos.

A.



B.

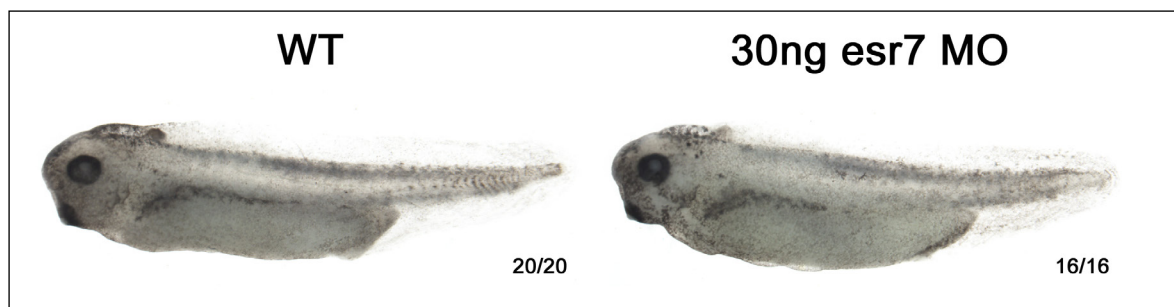


Figure 4.12 *Esr7* MO does not cause gross morphological defects

A. Schematic diagram of *esr7* MO (red) aligned next to *esr7* mRNA sequence (black). Green marks the translation start site for *esr7*. **B.** Stage 37 *esr7* morphant embryos (right) do not show gross morphological defects when compared to wild type sibling controls (left).

4.4.2. *Esr7* morpholino inhibits the translation of *esr7*

In order to test the ability of the *esr7* morpholino to bind to the specified sequence, gateway technology was undertaken to create an *esr7-gfp* construct containing the *esr7* morpholino binding site tagged with *gfp* (see section 2.2.1.11). Injection of 200pg *esr7-gfp* mRNA into the animal pole of 1 cell stage embryos generated a GFP signal, as visualised under a fluorescence microscope (Figure 4.13iii), whilst co-injection of 200pg *esr7-gfp* mRNA with 30ng *esr7* MO produced no visible GFP signal (Figure 4.13iv). This suggests that *esr7* MO is capable of binding to the *esr7* MO target sequence and preventing translation. As a control, co-injection of 30ng *esr7* MO with 200pg *gfp* (Figure 4.13ii) elicited a strong GFP signal, comparable to that of 200pg *gfp* alone (Figure 4.13i), suggesting *esr7* MO does not bind non-specifically to *gfp*.

4.4.3. *Esr7* is required for haematopoietic stem cell programming

A role for *esr7* in embryonic haematopoietic stem cell programming has not been reported. Whilst a role for murine *hes5*, the orthologue of *esr7*, in B- and T-cell development has been reported (Varnum-Finney et al., 2008), embryologically earlier investigations of *hes5* in haematopoietic development have not been described. To investigate if there is a role for *esr7* in *Xenopus* haematopoietic stem cell development, stage 39 embryos injected with 30ng *esr7* MO were analysed by *in situ* hybridisation for the expression of HSC markers. Expression of HSC marker *runx1* was significantly downregulated in morphant embryos to the point where expression in the DA cannot be seen by eye (Figure 4.14, panel d, red arrowheads). Expression of both *spiB* and *gfi1*, HSC markers downstream of *runx1*, is also absent in *esr7* morphant embryos, as expected by the loss of *runx1* expression (Figure 4.14, panels e and f, respectively, red arrowheads). Thus, *esr7* is required for haematopoietic stem cell programming in the *Xenopus* DA. Loss of these haematopoietic markers was used as a positive control for all subsequent *esr7* MO morphant phenotypes.

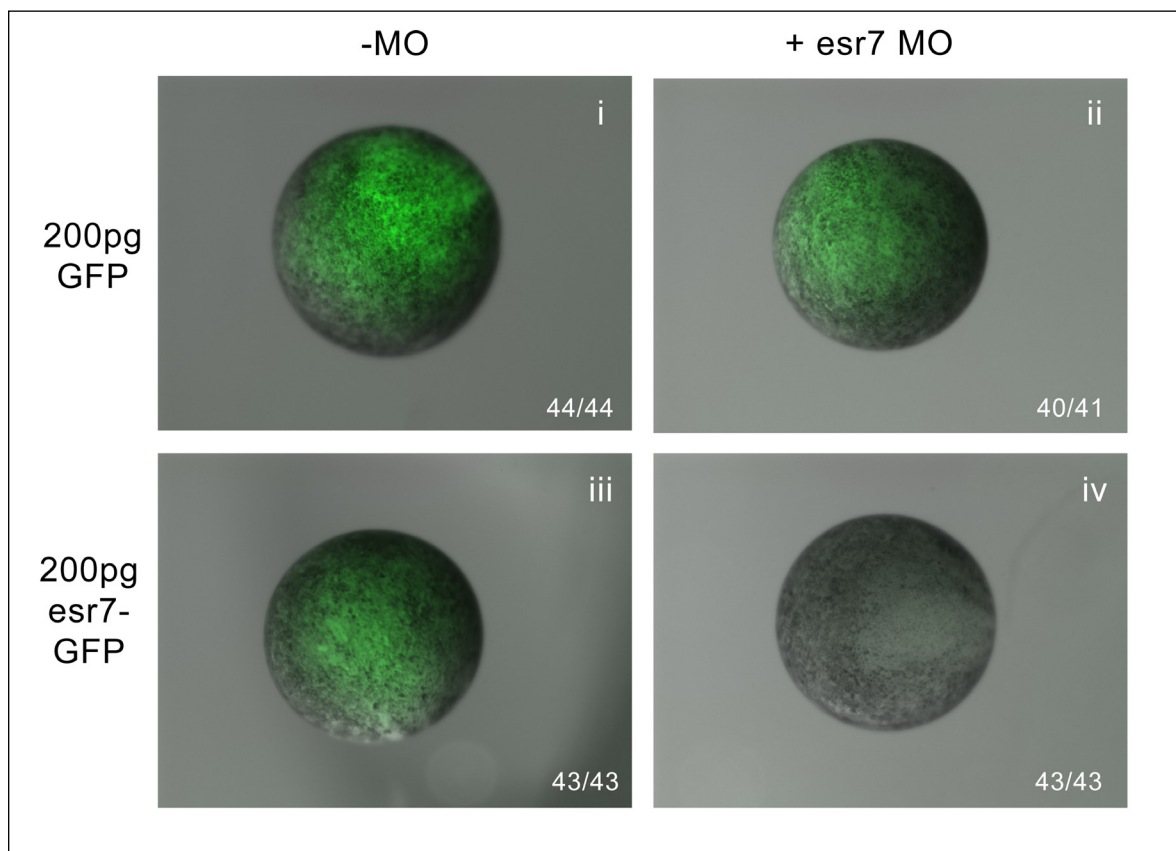


Figure 4.13 *esr7* MO is capable of preventing translation from *esr7* MO binding site

Figure showing *esr7* MO can block translation from the *esr7* MO target site. **i)** 200ng *gfp* mRNA elicits a strong GFP signal. **ii)** 200ng *gfp* mRNA co-injected with 30ng *esr7* MO elicits a strong signal. **iii)** 200ng *esr7*-*gfp* elicits a strong signal. **iv)** 200ng *esr7*-*gfp* co-injected with 30ng *esr7* MO elicits little to no GFP signal.

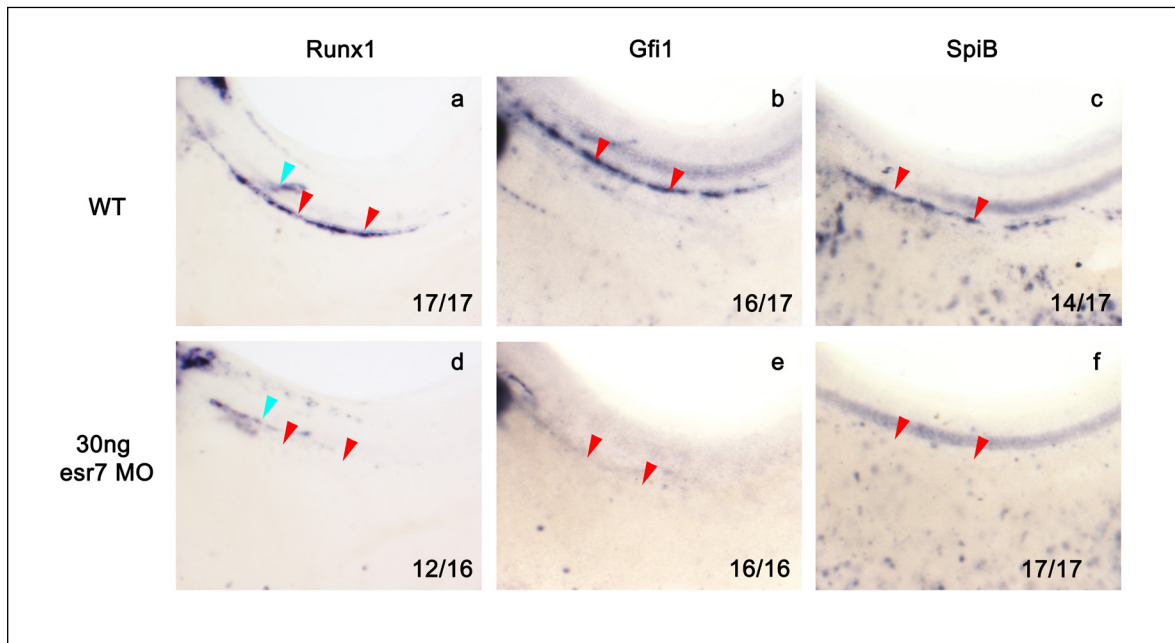


Figure 4.14 *Esr7* is required for the expression of haematopoietic stem cell markers.

Esr7 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of HSC *runx1*, *gfi1* and *spiB* markers is lost in *esr7* morphant embryos at stage 39 when compared to WT sibling controls. Red arrowheads mark the dorsal aorta, blue arrowheads mark the lateral line.

4.4.4. Haemangioblast programming is unaffected in *esr7* morphant embryos

Whilst *esr7* is not expressed in lateral mesoderm populations during early development or haemangioblast programming (Figure 3.7), injection of *esr7* MO at the 2-cell stage provides the possibility that some early programming of HSCs is compromised in *esr7* morphants. In order to investigate if *esr7* has a role in haemangioblast specification, stage 28 embryos injected with 30ng *esr7* MO were analysed for haematopoietic and endothelial markers in the DLP. Expression of haematopoietic marker *scl* in morphant embryos is relatively normal (Figure 4.15, panel d, red arrowheads). In addition, expression is normal for *flk1*, a VEGF receptor expressed in the DLP which is required for migration of haemangioblast cells to the midline (Cleaver and Krieg, 1998, Figure 4.15, panel e, red arrowheads). *Egfl7* has been found to be towards the bottom of the genetic hierarchy controlling haemangioblast specification (Figure 1.11, Aldo Ciau-Uitz, personal communication). Expression of *egfl7* in the DLP of *esr7* morphants is normal (Figure 4.15, panel f, red arrowheads), suggesting expression of crucial genes upstream in the genetic regulatory network is also normal. Expression of *scl*, *flk1* and *egfl7* can also be seen in the VBI at this stage. *Scl* marks haematopoietic cells, and is expressed in the developing primitive erythrocytes of the VBI. Expression of *scl* is normal in the VBI of *esr7* morphants when compared to wild type sibling controls (Figure 4.15, panels d and a respectively, orange arrowheads), suggesting primitive haematopoiesis is unaffected by reduction of *esr7*. Similarly, expression of endothelial markers *flk1* and *egfl7* in the VBI is relatively unaffected in *esr7* morphants (Figure 4.15, panels e and f, respectively, green arrowheads). *Flk1* expression in the VBI is slightly reduced in *esr7* morphants, but the majority of vitelline endothelial cells apparently express *flk1*. This reduction of *flk1* expression in the VBI endothelial cells may result in an incomplete or mis-formed vitelline vasculature later in development. However, expression of *ami* in the vitelline vasculature of *esr7* morphants at stage 39 looks mostly normal, and the vitelline vasculature appears to be intact and fully developed when compared to wild type sibling controls (Figure 4.16, panels f and b,

respectively, blue arrowheads), thus there appears to be no fundamental role for *esr7* in vitelline vasculature development.

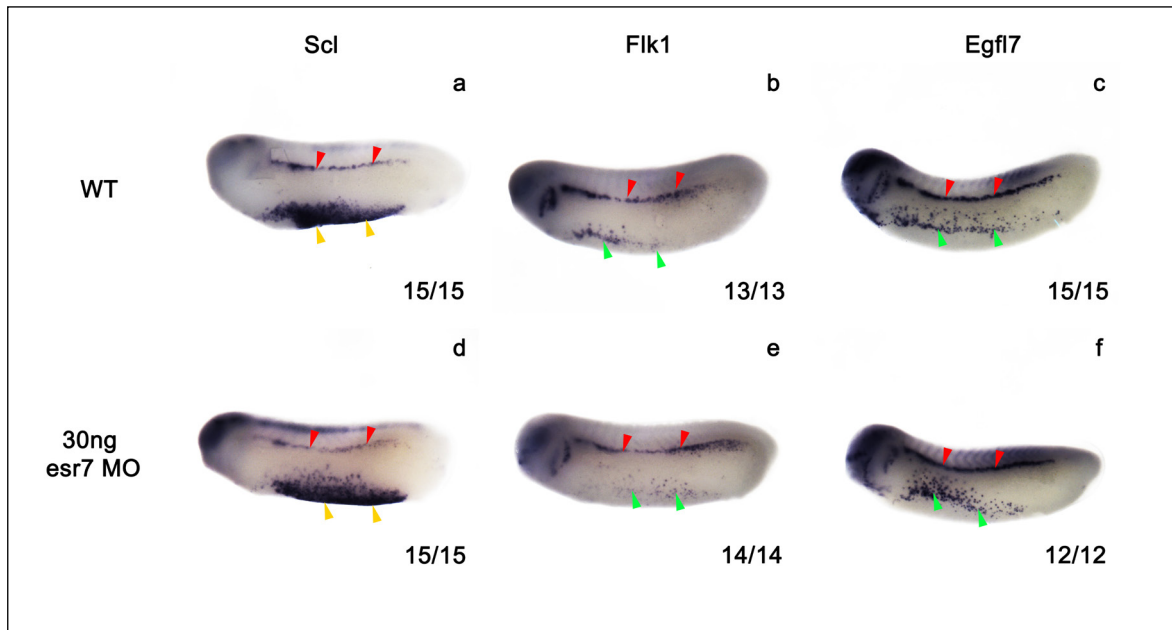


Figure 4.15 Programming of the haemangioblast is normal in *esr7* morphants.

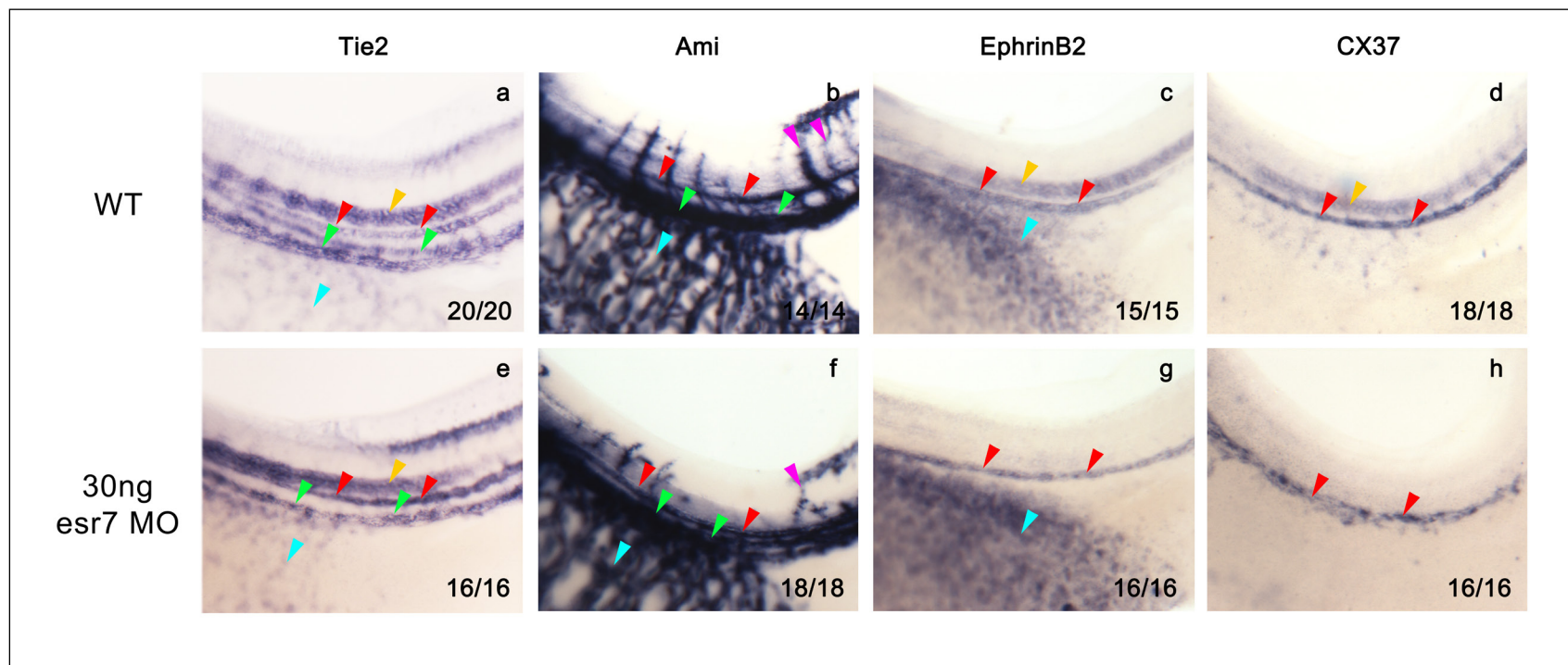
Esr7 MO was injected into both cells of two-cell embryos which were collected at stage 27-28 and analysed by *in situ* hybridisation. Expression of haematopoietic marker *scl*, and endothelial markers *flk1* and *egfl7* is unaffected in *esr7* morphants. Expression can be seen in the DLP (red arrowheads), developing primitive erythrocytes in the VBI (orange arrowheads) and in the developing vitelline vessels (green arrowheads).

4.4.5. Endothelial and dorsal aorta specification is not affected by loss of *esr7*

A role for *esr7*, or its orthologue *hes5*, in vascular programming has not been reported. In order to determine if the haematopoietic defect found in *esr7* *Xenopus* morphants is the direct result of an *esr7* deficiency, it is essential to see if there is an earlier vascular programming defect. To investigate the role of *esr7* in *Xenopus* vascular and arterial development, stage 39 embryos injected with 30ng *esr7* MO were analysed by *in situ* hybridisation for the expression of endothelial and arterial markers. Expression of endothelial markers, *tie2* and *ami*, revealed normal expression in the DA of *esr7* morphants (Figure 4.16, panels e and f, respectively, red arrowheads). Similarly, expression of arterial markers, *ephrinB2* and *cx37*, is also normal in the DA of *esr7* morphant embryos (Figure 4.16, panels g and h, respectively, red arrowheads). However, in the *esr7* morphants, the DA appears to be thinner, suggesting either a problem with lumen formation or fewer cells in the DA. Expression of *tie2*, *ami* and *ephrinB2* in the vitelline vessels is relatively normal, suggesting *esr7* does not have a role in the development of the vitelline vasculature (Figure 4.16, panels e, f and g, respectively, blue arrowheads). Expression of *cx37* shows a slightly disorganised dorsal aorta, with arterial cells seeming to lie outside the main vessel (Figure 4.16, panel h, red arrowheads). Expression analysis of *cx37* in *esr7* morphant embryos has only been undertaken once; as the DA appears structurally normal when analysed with *tie2*, *ami* and *ephrinB2* expression, it is possible that the observation of a disorganised DA in *cx37* expression analysis is not representative. This experiment needs to be repeated to investigate whether there are any structural abnormalities in *esr7* morphant *cx37* expressing cells. Development of the intersomitic vessels from the vein is also disrupted in *esr7* morphants, as marked by expression of *ami* (Figure 4.16, panels f and g, respectively, purple arrowheads).

Figure 4.16 Expression of endothelial and arterial genes is normal in *esr7* morphants

Esr7 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of endothelial markers, *tie2* and *ami*, and of arterial markers, *ephrinB2* and *cx37*, is normal in the dorsal aorta of *esr7* morphants. However, the vessel appears marginally thinner than in wild type sibling controls. Red arrowheads mark dorsal aorta, green arrowheads mark the vein, orange arrowheads mark the notochord, blue arrowheads mark vitelline vasculature, and purple arrowheads mark intersomitic vessels.



The repression of *flk1* is known to be due to the bHLH and the orange domains (see chapter 1.8.2 for domain details), but independent of the YRPW domain (Holderfield et al., 2006). *Esr7* contains both a bHLH and an orange domain, and thus could potentially mediate this repression of *flk1* in the DA. To analyse this, expression analysis of *flk1* and *flt4* was carried out in stage 39 *esr7* morphants. At stage 39, expression of both *flk1* and *flt4* has been downregulated and cannot be seen in the DA of wild type embryos (Figure 4.17, panel a and b, red arrowheads). In *esr7* morphant embryos, expression of *flk1* and *flt4* is also not seen (Figure 4.17, panel c and d, red arrowheads), suggesting *esr7* does not control the downregulation of *flk1* and *flt4*.

4.4.6. Primitive haematopoiesis is normal in *esr7* morphant embryos, and primitive blood circulates relatively normally

Expression of *esr7* has not been observed in the VBI in *Xenopus*, and therefore should not be involved in primitive haematopoiesis. In addition, a role for *esr7* in primitive haematopoiesis in other organisms has not been reported. Expression analysis of endothelial markers *flk1* and *egfl7* in the VBI of *esr7* morphants has already been discussed (chapter 4.4.4), and found to be quite normal. Similarly, expression analysis of *scf* in primitive erythrocytes in the VBI demonstrates relatively normal expression in *esr7* morphants (Figure 4.15, panel d, orange arrowheads). To further analyse programming of the VBI in *esr7* morphants, expression analysis of *globin* was carried out in stage 28 embryos, and was found to be marginally reduced (Figure 4.18A, panel c, orange arrowheads).

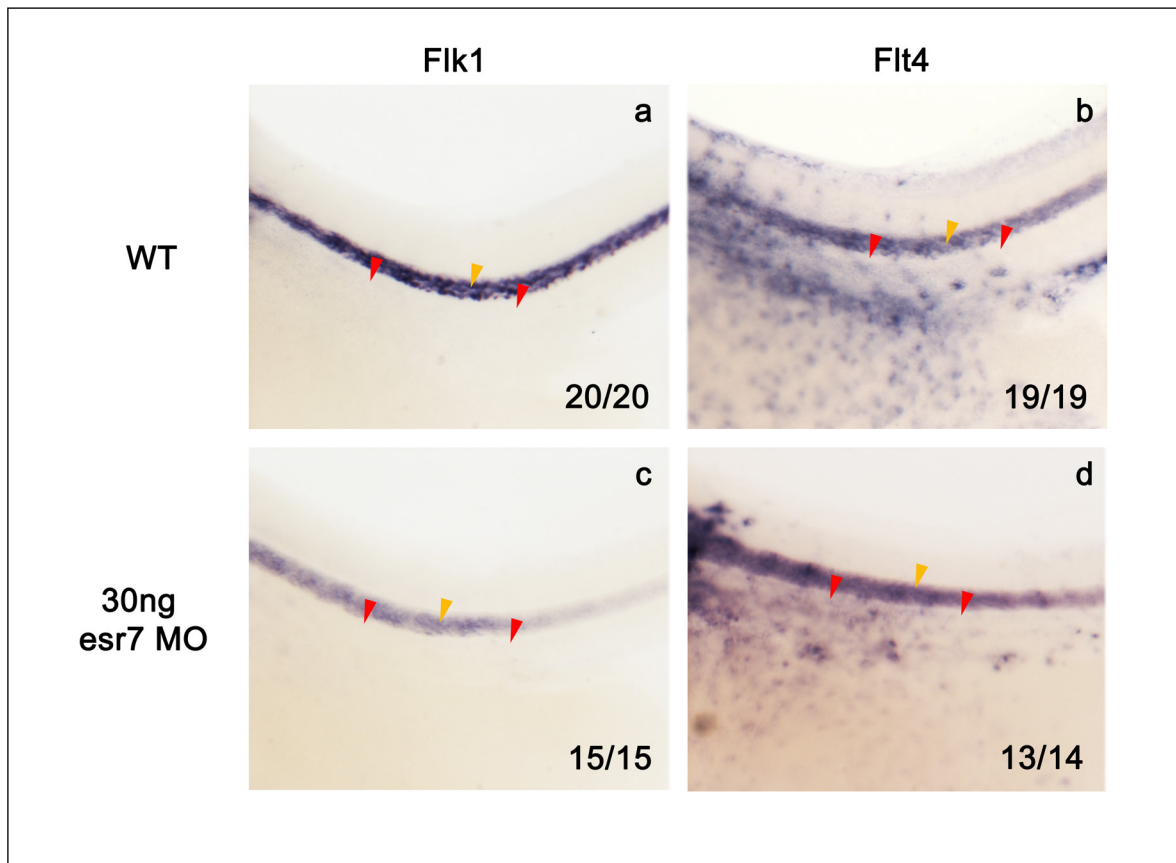


Figure 4.17 Expression of endothelial genes *flk1* and *flt4* is not maintained in *esr7* morphants.

Esr7 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of *flk1* and *flt4* in the DA of *esr7* morphants is not maintained. Red arrowheads mark the location of the dorsal aorta.

As expression of *scl* is relatively normal, but *globin* is slightly reduced in *esr7* morphants, this suggests the primitive erythroid progenitors are present in the VBI but not differentiating. In addition to primitive erythrocytes and vasculature, early primitive myeloid cell specification is unaffected as demonstrated by the normal expression of *mpo* (Figure 4.18A, panel d). Therefore, *esr7* can be regarded as not essential for endothelial or myeloid development in the VBI, but is required for full programming of primitive erythrocytes.

Analysing the expression of primitive haematopoietic markers later in development can provide essential information about circulation in embryos. At stage 39, primitive erythrocytes can be seen circulating throughout wild type embryos, as analysed by the expression of *globin* and *gata-1* (Figure 4.18B, panels a and b, respectively). Erythrocytes can be seen in the dorsal aorta (red arrowheads), cardinal vein (green arrowheads), and can be seen within the heart (blue arrowheads). Similarly, in *esr7* morphants, erythrocytes are seen circulating in the embryo (Figure 4.18B, panels c and d), thus *esr7* is not required for the circulation of primitive erythrocytes. However, it is apparent that there are fewer erythrocytes in the *esr7* morphant embryos at stage 39, confirming the earlier observation that the programming of primitive erythrocytes in the VBI is defective.

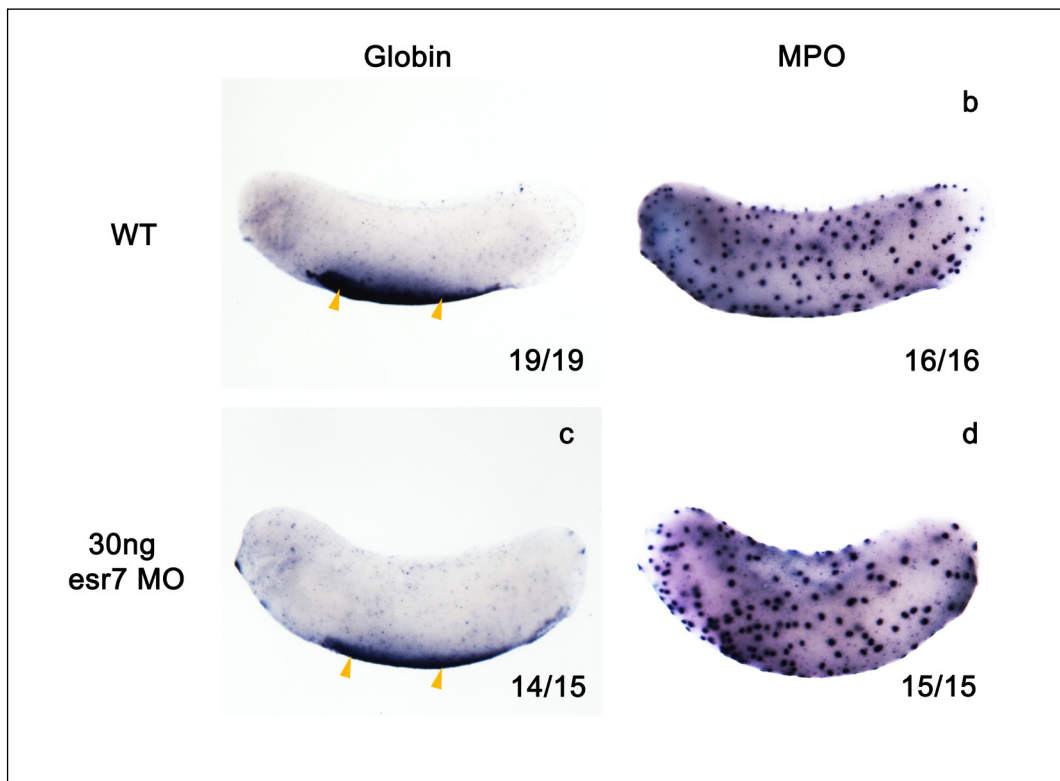
4.4.7. *Esr7* is required for the expression of downstream hairy and enhancer of split genes

Five genes from the hairy and enhancer of split related family are expressed in the DA, with four of these seeming to be notch target genes, as analysed by depletion of notch4 signalling (chapters 4.2.6 and 4.3.1). To determine if these genes interact at a genetic level, expression analysis was carried out in embryos injected with 30ng *esr7* MO.

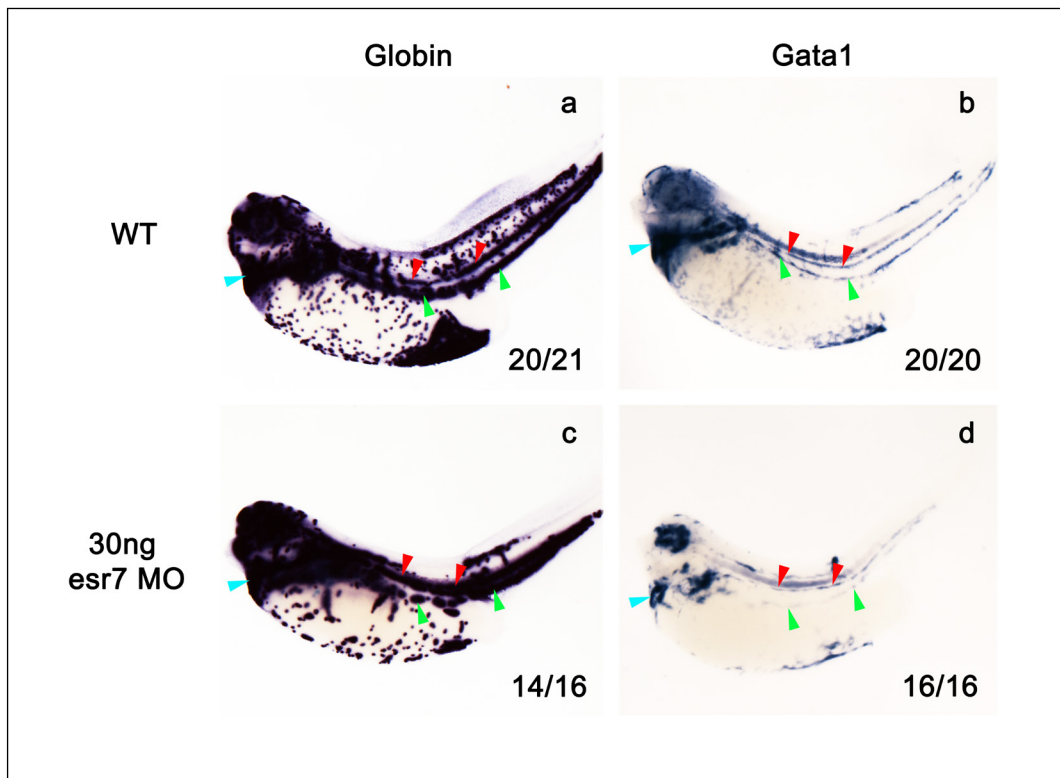
Figure 4.18 Primitive haematopoiesis is not affected by depletion of *esr7*, and primitive erythrocytes circulate relatively normally in the embryo

Esr7 MO was injected into both cells of two-cell embryos which were collected at stage 28 or 38-39 and analysed by *in situ* hybridisation. **A.** At stage 28, expression of α -T4 globin, a marker of primitive erythroid cells, is normal in *esr7* morphants, as is *mpo*, a marker of primitive myeloid cells. **B.** At stage 39 erythrocytes can be seen circulating throughout embryos. Red arrowheads mark the DA, green arrowheads mark the cardinal vein, orange arrowheads mark primitive erythroid cells in the VBI and blue arrowheads mark the heart.

A.



B.



In *esr7* morphant embryos, expression of *esr7* and *esr1* is upregulated in the *Xenopus* DA (Figure 4.19A, panels d and e, respectively, red arrowheads), whilst expression of *hes1* is absent in the DA (Figure 4.19A, panel f, red arrowheads). It is possible that *esr7* MO stabilises *esr7* mRNA, making expression in the DA seem higher. Alternatively, *esr7* may negatively regulate its own expression, and therefore depletion of *esr7* protein in *esr7* morphants may relieve repression on the *esr7* promoter. This negative regulation of gene expression by the corresponding protein has previously been demonstrated in our lab; morpholino knockdown of *gata1* in zebrafish erythroid cells causes a massive upregulation of *gata1* expression. This is thought to be a genuine effect, and not an artefact caused by morpholino stabilisation, as it is seen with multiple *gata1* morpholinos (Rui Monteiro, personal communication). Upregulation of *esr1* expression in the DA of *esr7* morphant embryos suggests *esr7* negatively regulates expression of *esr1*, although, in the absence of ChIP experiments, it is unclear whether this is a direct interaction.

In addition, expression analysis of *notch4* and *notch1* was carried out in *esr7* morphant embryos, to determine if *esr7* has a role in regulation of notch receptor expression. Expression of both *notch4* and *notch1* is unaffected in *esr7* morphant embryos (Figure 4.19B, panels c and d, respectively, red arrowheads), and thus *esr7* does not play a role in notch receptor expression in the DA.

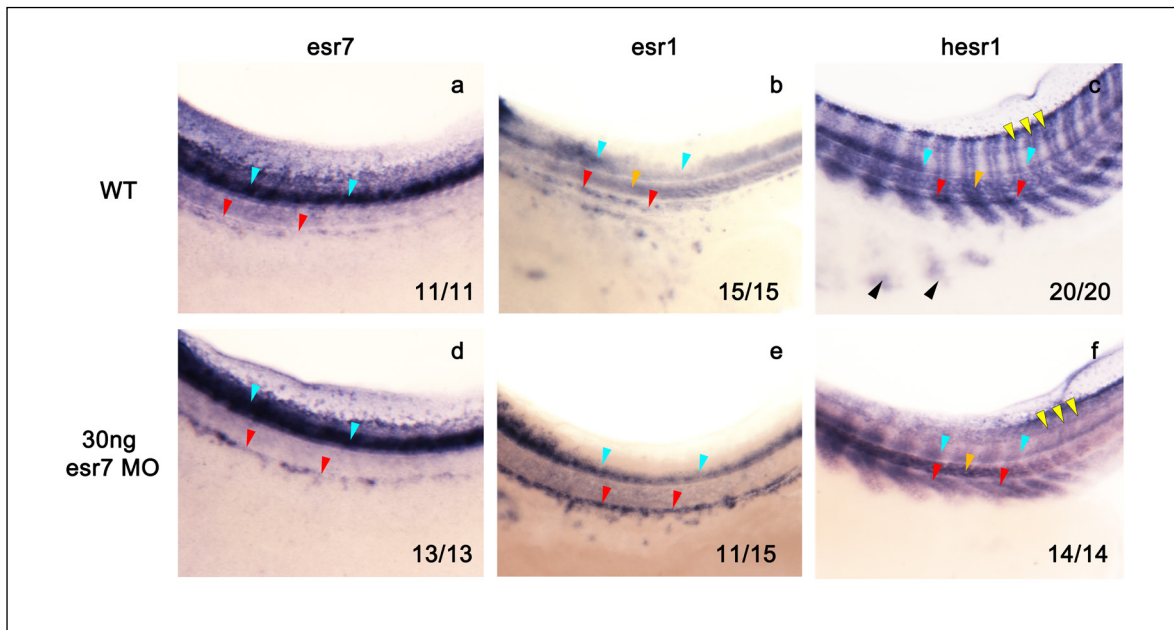
4.5. *Esr10*

Like *esr7*, *esr10* is a WRPW-containing enhancer of split related gene expressed in the *Xenopus* DA (Figure 3.7), and a paralogue of mouse *hes5*. As *hes5* is a direct target of notch signalling (Ohtsuka et al., 1999), and *esr10* is downregulated in *notch4* morphants, it is expected that *esr10* will be a direct notch target gene in the *Xenopus* DA.

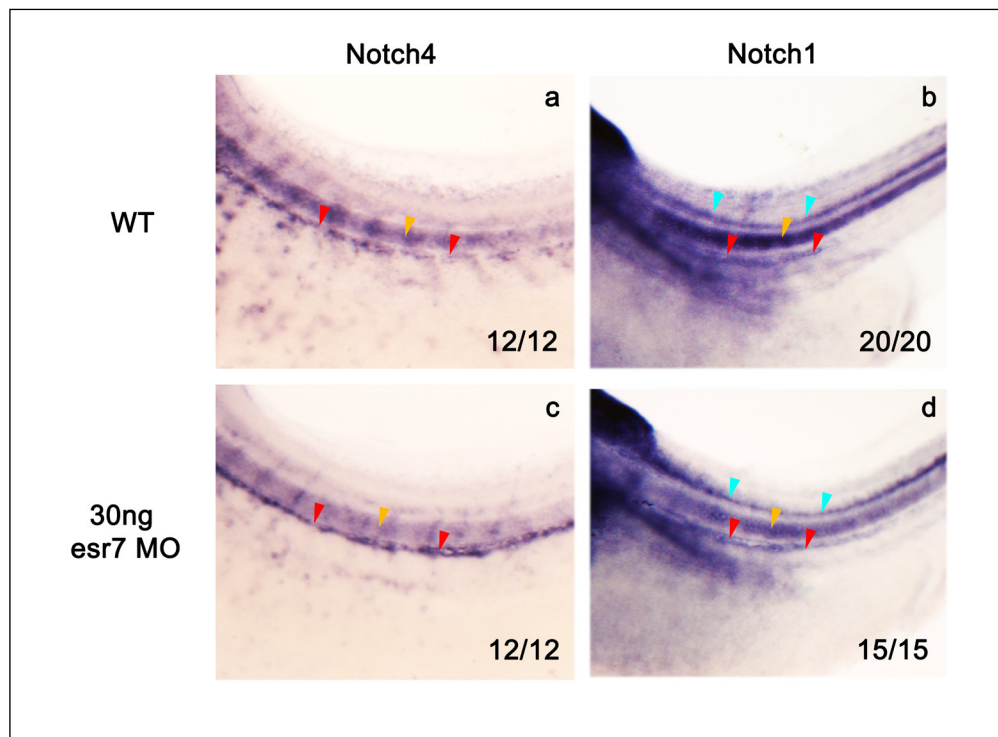
Figure 4.19 *esr7* regulates the expression of hairy and enhancer of split genes in the DA

Esr7 MO was injected into both cells of two-cell embryos which were collected at stage 33 or 38-39 and analysed by *in situ* hybridisation. **A.** Expression of *hesr1* is significantly reduced whilst expression of *esr7* and *esr1* is upregulated in *esr7* morphants. **B.** Expression of both *notch4* and *notch1* is normal in the DA of *esr7* morphants. Red arrowheads mark the DA, blue arrowheads mark the neural tube, orange arrowheads mark the notochord and yellow arrowheads mark the somites.

A.



B.



4.5.1. Defects in *esr10* morphant embryos

A morpholino for *esr10* has been tested and published (Taelman et al., 2006), and so this sequence was used for morpholino studies in this thesis (see Table 2.3 and Figure 4.20A). To assess the effect of *esr10* depletion on *Xenopus*, embryos were injected at the 2-cell stage with 20-40ng *esr7* morpholino. At 40ng, embryos looked unhealthy showing signs of non-specific toxicity during early development, including developmental delay, and failure to close the neural tube. Embryos injected with 20ng, 30ng and 35ng looked healthy, showing no gross morphological defects (35ng shown in Figure 4.20B). Observation of *esr10* MO injected embryos at stage 39 revealed normally beating hearts but no circulating blood cells in the tail of the embryos. In wild type siblings, circulation was evident from stage 37. As no gross morphological defects were observed in embryos injected with 35ng *esr10* MO, this was chosen as the optimal dose for analysing *esr10* depletion in *Xenopus* embryos. Although no gross morphological defects were observed, some minor developmental differences were observed. In morphant embryos, the developing eye appeared smaller (Figure 4.20B, red arrows), and fewer pigment cells were observed (blue arrows).

4.5.2. *Esr10* morpholino inhibits the translation of *esr10*

In order to test the ability of the *esr10* morpholino to bind to the specified sequence, gateway technology was undertaken to create an *esr10-gfp* construct containing the *esr10* morpholino binding site tagged with *gfp* (see section 2.2.1.11). Injection of 200pg *esr10-gfp* mRNA into the animal pole of 1 cell stage embryos generated a GFP signal, as visualised under a fluorescence microscope (Figure 4.21iii), whilst co-injection of 200pg *esr10-gfp* mRNA with 35ng *esr10* MO produced no visible GFP signal (Figure 4.21iv). This suggests that *esr10* MO is capable of binding to the *esr10* MO target sequence and preventing translation.

A.



B.

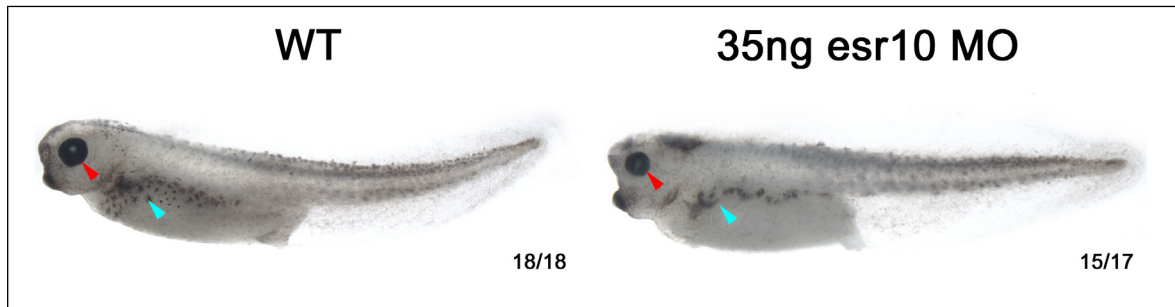


Figure 4.20 *Esr10* MO does not cause gross morphological defects

A. Schematic diagram of *esr10* MO (red) aligned next to *esr7* mRNA sequence (black). The morpholino binds in the 5' UTR and the translation start site is found 3' to this sequence. **B.** Stage 39 *esr10* morphant embryos (right) show a smaller eye (red arrowheads), and fewer pigment cells (blue arrowheads) when compared to wild type sibling controls (left).

As a control, co-injection of 35ng *esr10* MO with 200pg *gfp* (Figure 4.21ii) elicited a strong GFP signal, comparable to that of 200pg *gfp* alone (Figure 4.21i), suggesting *esr10* MO does not bind non-specifically to *gfp*.

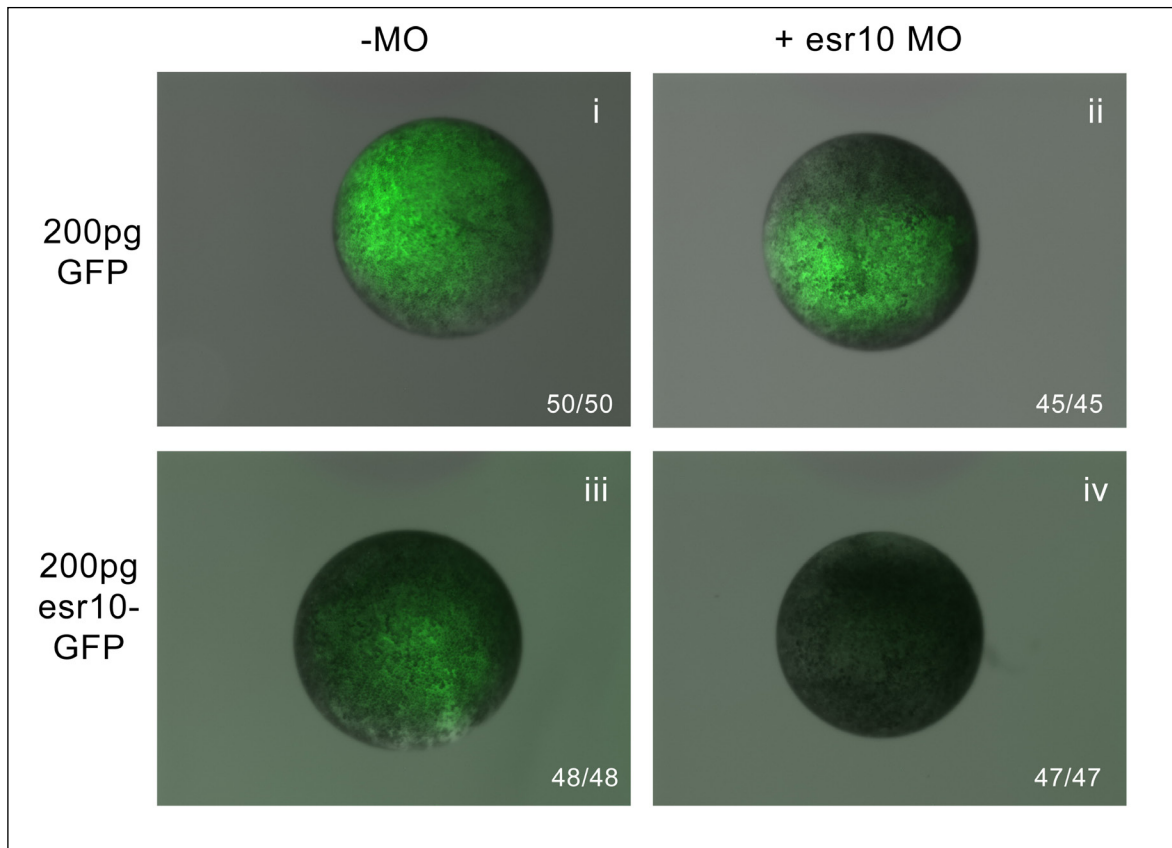


Figure 4.21 *esr10* MO is capable of preventing translation from *esr10* MO binding site

Figure showing *esr10* MO can block translation from the *esr10* MO target site. **i)** 200ng *gfp* mRNA elicits a strong GFP signal. **ii)** 200ng *gfp* mRNA co-injected with 35ng *esr10* MO elicits a strong signal. **iii)** 200ng *esr10-gfp* elicits a strong signal. **iv)** 200ng *esr10-gfp* co-injected with 35ng *esr10* MO elicits little to no GFP signal.

4.5.3. *Esr10* is required for haematopoietic stem cell programming

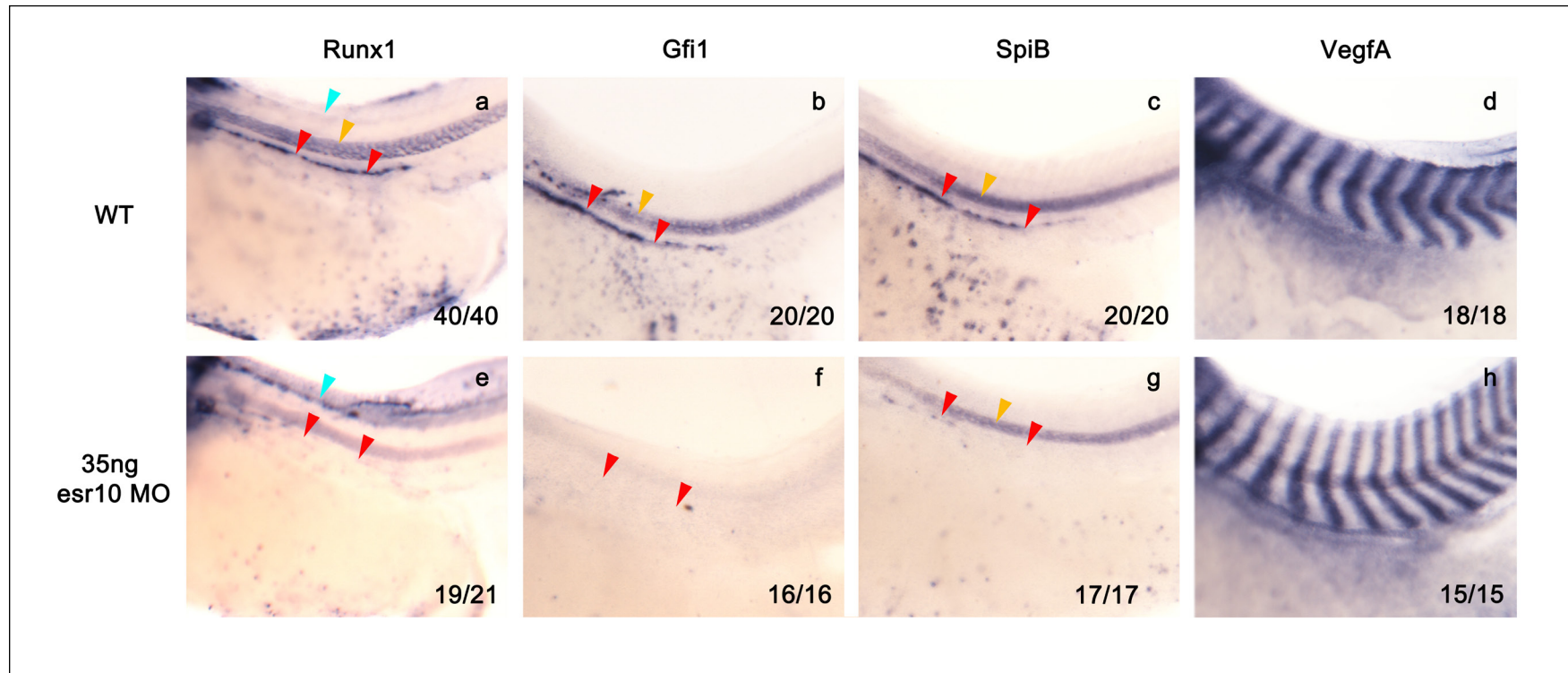
The role, if any, of *esr10* in embryonic haematopoietic stem cell programming has yet to be elucidated. Whilst a role for murine *hes5*, the orthologue of *esr10*, in B- and T-cell development has been reported (Varnum-Finney et al., 2008), earlier investigations of *hes5* in haematopoietic development have not been reported. To investigate the role of *esr10* in *Xenopus* haematopoietic stem cell development, stage 39 embryos injected with 35ng *esr10* MO were analysed by *in situ* hybridisation for the expression of HSC markers. Expression of putative HSC marker *runx1* was significantly downregulated in morphant embryos so far as expression in the DA cannot be seen by eye (Figure 4.22, panel e, red arrowheads). Expression of both *spiB* and *gfi1*, HSC markers downstream of *runx1*, is absent in *esr10* morphant embryos, as expected from the loss of *runx1* expression (Figure 4.22, panels f and g, respectively, red arrowheads).

VEGF signalling from the somites to the DA is known to regulate the expression of *runx1* and haematopoietic genes downstream of *runx1* (Leung, 2010). As *esr7* is expressed in the presomitic mesoderm (Figure 3.8), it may have a role in somitogenesis. Expression analysis of *vegfa* in *esr7* morphant embryos reveals normal expression of *vegfa* in the somites, and normal somitic patterning (Figure 4.22, panel h). Therefore, the requirement for *esr7* in HSC programming is restricted to the DA, and is not due to a somitic defect perturbing *vegfa* expression.

Similarly to the expression analysis of *gata2* in *notch4* morphants, *gata2* is not disrupted in *esr10* morphants at stage 32, but is disrupted at stage 39 (Figure 4.23, panel c and d, respectively, red arrowheads). This again points to two independent methods of *gata2* regulation in the *Xenopus* DA, dependent on developmental stage. Loss of haematopoietic markers was used as a positive control for all subsequent *esr10* MO morphant phenotypes.

Figure 4.22 *Esr10* is required for the expression of haematopoietic stem cell markers.

Esr10 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of HSC markers *runx1*, *gfi1* and *spiB* is lost in *esr10* morphant embryos at stage 39 when compared to WT sibling controls. Expression of *vegfa* in the somites is also normal. Red arrowheads mark the dorsal aorta, blue arrowheads mark neural expression and orange arrowheads mark the notochord.



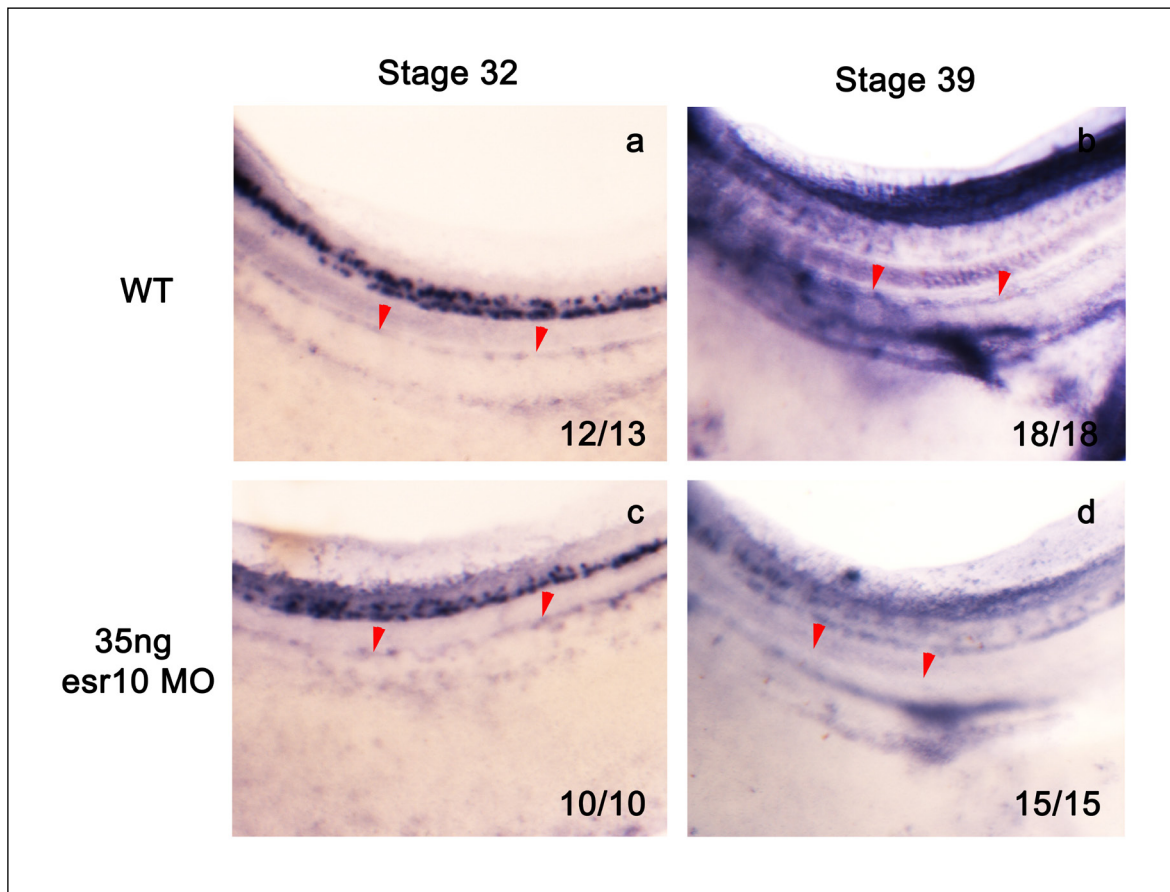


Figure 4.23 Depletion of *esr10* reduces *gata2* expression at stage 39.

Esr10 MO was injected into both cells of two-cell embryos which were collected at stage 32 or 39 and analysed by *in situ* hybridisation. *Gata2* expression initiates normally in *esr10* morphant embryos, as evident by expression in the DA at stage 32, but it is not maintained, as evidenced by the loss of expression at stage 39. Red arrowheads mark the DA.

4.5.4. Definitive haemangioblast programming is unaffected in *esr10* morphants

Whilst *esr10* is not expressed in lateral mesoderm populations during early development or haemangioblast programming (Figure 3.7), injection of *esr10* MO at the 2-cell stage provides the possibility that some early programming of HSCs is compromised in *esr10* morphants. In order to investigate if *esr10* has a role in haemangioblast specification, stage 28 embryos injected with 35ng *esr10* MO were analysed for haematopoietic and endothelial markers in the DLP. Expression of haematopoietic marker *scl* in morphant embryos is relatively normal (Figure 4.24, panel d, red arrowheads). In addition, expression is normal for *flk1*, a VEGF receptor expressed in the DLP which is required for migration of haemangioblast cells to the midline (Figure 4.24, panel e, red arrowheads). Expression of *egfl7*, a gene toward the bottom of the genetic hierarchy controlling haemangioblast specification, is normal in the DLP of *esr10* morphants (Figure 4.24, panel f, red arrowheads). This suggests expression of crucial genes upstream in the genetic regulatory network is also normal. Expression of *scl*, *flk1* and *egfl7* can also be seen in the VBI at this stage. *Scl* marks haematopoietic cells, and is expressed in the developing primitive erythroid cells of the VBI. Expression of *scl* is normal in the VBI of *esr10* morphants when compared to wild type sibling controls (Figure 4.24, panels d and a respectively, orange arrowheads), suggesting primitive haematopoiesis is unaffected by reduction of *esr10*. Similarly, expression of endothelial markers *flk1* and *egfl7* in the VBI is relatively unaffected in *esr10* morphants (Figure 4.24, panels e and f, respectively, green arrowheads).

4.5.5. Endothelial and dorsal aorta specification is not affected by loss of *esr10*

A role for *esr10*, or its orthologue *hes5*, in vascular programming has not been reported. In order to understand if the haematopoietic defect found in *esr10* *Xenopus* morphants is the direct result of an *esr10* deficiency, it is essential to see if there is an earlier vascular programming defect.

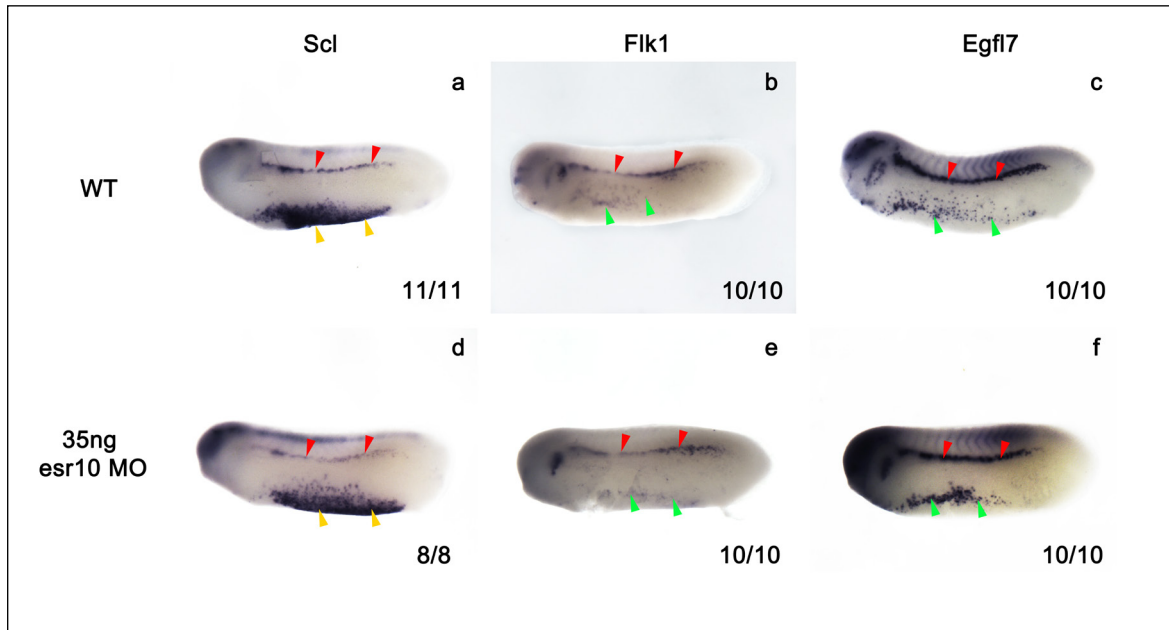


Figure 4.24 Programming of the haemangioblast is normal in *esr10* morphants.

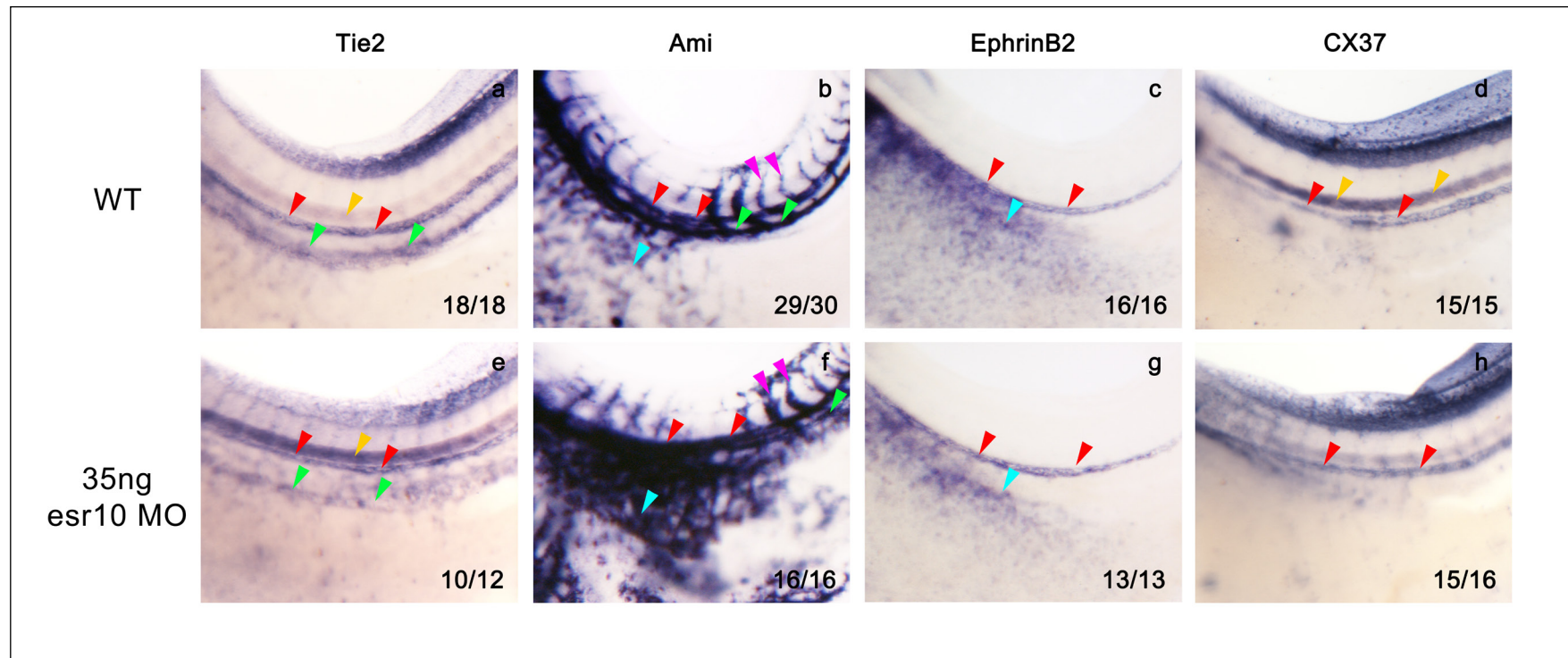
Esr10 MO was injected into both cells of two-cell embryos which were collected at stage 27-28 and analysed by *in situ* hybridisation. Expression of haematopoietic marker *scl*, and endothelial markers *flk1* and *egfl7* is unaffected in *esr10* morphants. Expression can be seen in the DLP (red arrowheads), developing primitive erythroid cells in the VBI (orange arrowheads) and in the developing vitelline vessels

To investigate the role of *esr10* in *Xenopus* vascular and arterial development, stage 39 embryos injected with 35ng *esr10* MO were analysed by *in situ* hybridisation for the expression of endothelial and arterial markers. Expression of endothelial markers, *tie2* and *ami*, revealed normal expression in the DA of *esr10* morphants (Figure 4.25, panels e and f, respectively, red arrowheads). Similarly, expression of arterial markers, *ephrinB2* and *cx37*, is also normal in the DA of *esr10* morphant embryos (Figure 4.25, panels g and h, respectively, red arrowheads). Expression of *tie2*, *ami* and *ephrinB2* in the vitelline vessels is relatively normal, suggesting *esr10* does not have a role in the development of the vitelline vasculature (Figure 4.25, panels e, f and g, respectively, blue arrowheads). Development of the intersomitic vessels from the vein also appears normal in *esr10* morphants, as marked by expression of *ami* (Figure 4.25, panels f and g, respectively, purple arrowheads).

Esr10 contains both a bHLH and an orange domain, and thus could potentially mediate repression of initiator-containing TATA-less endothelial genes in the DA. To analyse this, expression analysis of *flk1* and *flt4* was carried out in stage 39 *esr10* morphants. At stage 39, expression of *flk1* and *flt4* has been downregulated and cannot be seen in the DA of wild type embryos (Figure 4.26, panel a and b, red arrowheads). In *esr10* morphant embryos, expression of *flk1* and *flt4* is also not seen (Figure 4.26, panel c and d, red arrowheads), suggesting *esr10* does not control the downregulation of *flk1* and *flt4* in the *Xenopus* DA.

Figure 4.25 Expression of endothelial and arterial genes is normal in *esr10* morphants

Esr10 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of endothelial markers *tie2* and *ami*, and of arterial markers, *ephrinB2* and *cx37*, is normal in the dorsal aorta of *esr10* morphants. Red arrowheads mark dorsal aorta, green arrowheads mark the vein, orange arrowheads mark the notochord, blue arrowheads mark vitelline vasculature, and purple arrowheads mark intersomitic vessels.



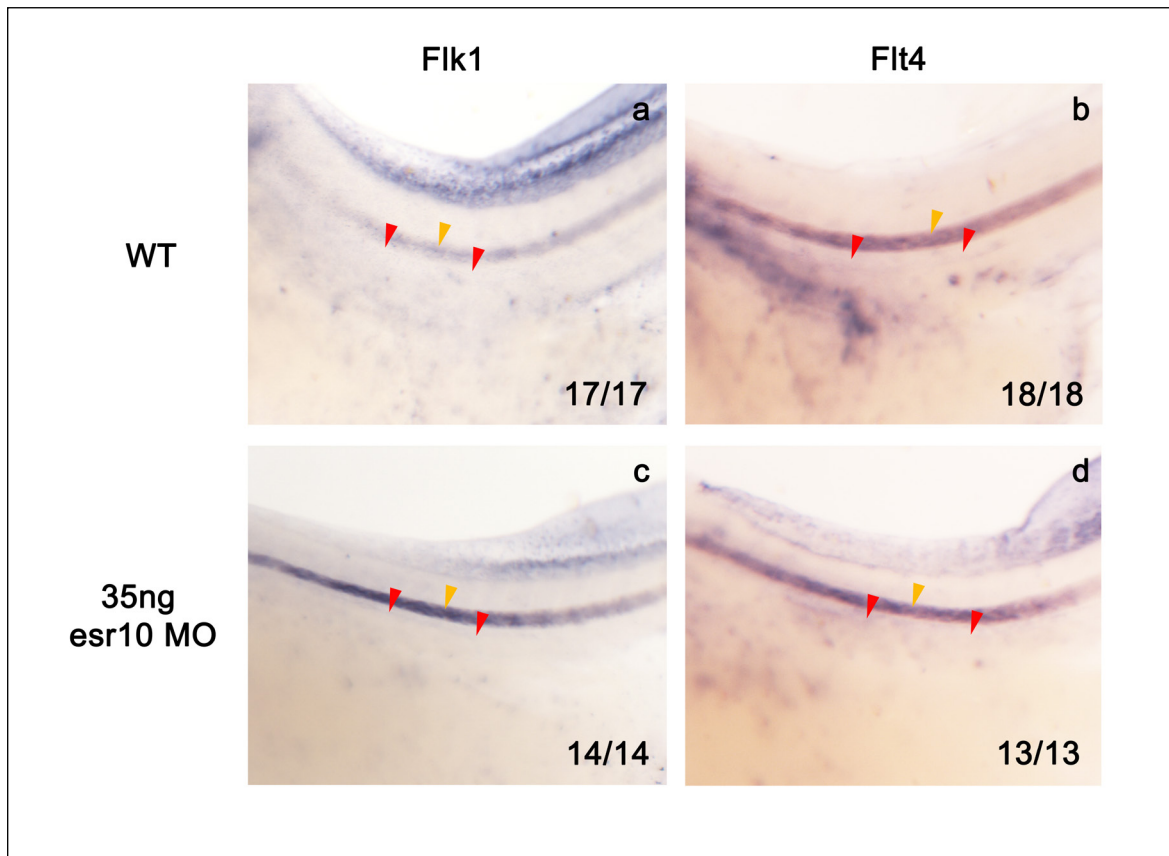


Figure 4.26 Expression of endothelial genes *flk1* and *flt4* is not maintained in *esr10* morphants.

Esr10 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of *flk1* and *flt4* in the DA of *esr10* morphants is not maintained. Red arrowheads mark the location of the dorsal aorta, orange arrowheads mark the notochord.

4.5.6. Primitive erythrocytes do not circulate in *esr10* morphant embryos

Expression analysis of *scl* in stage 28 embryos reveals a normal expression pattern marking primitive erythroid cells in the VBI, and thus primitive blood appears to be forming in *esr10* morphants (Figure 4.24, panel d, orange arrowheads). Analysing the expression of primitive haematopoietic markers later in development can provide essential information about circulation in embryos. At stage 39, primitive erythrocytes can be seen circulating throughout wild type embryos, as analysed by the expression of *globin* and *gata-1* (Figure 4.27B, panels a and b, respectively). Primitive erythrocytes can be seen in the dorsal aorta (red arrowheads), cardinal vein (green arrowheads), and can be seen within the heart (blue arrowheads). However, in *esr10* morphants, primitive erythrocytes are seen pooling in the VBI (Figure 4.27B, panels c and d, orange arrowheads). This is consistent with the observation that blood cells are not seen circulating through the tail in living stage 39 *esr10* morphant embryos (see chapter 4.5.1). As the heart is beating normally, and patterning of vitelline vasculature is relatively normal (see chapter 4.5.1 and chapter 4.5.5), the lack of circulation is likely due to an inability to form a lumen. In *esr10* morphants, the vessel often appears thinner than in wild type sibling controls. For example, in embryos stained for *tie2* or *ephrinB2*, the DA appears thinner in *esr10* morphants (Figure 4.25, panel e and g, red arrowheads). It is possible that *esr10* is required for the lumenisation of vessels, and thus in *esr10* morphants, lumens may not be formed, inhibiting the circulation of erythrocytes through vessels. In order to fully understand whether *esr10* is required for lumenisation of the DA, it is essential for *in situ* hybridisation on sections to be carried out, whereby the vessel diameter can be studied.

Blood flow is known to be required for the maintenance of HSC markers in the zebrafish DA from 36hpf, after initial cluster emergence, without affecting initiation of *runx1* in the DA (North et al., 2009; Wang et al., 2011). The absence of blood flow in *esr10* morphant embryos is not thought to

be a contributing factor to the loss of HSC markers at stage 39, for the same reasoning loss of HSC markers in *notch4* morphants is not thought to be due to loss of circulation (see chapter 4.2.5).

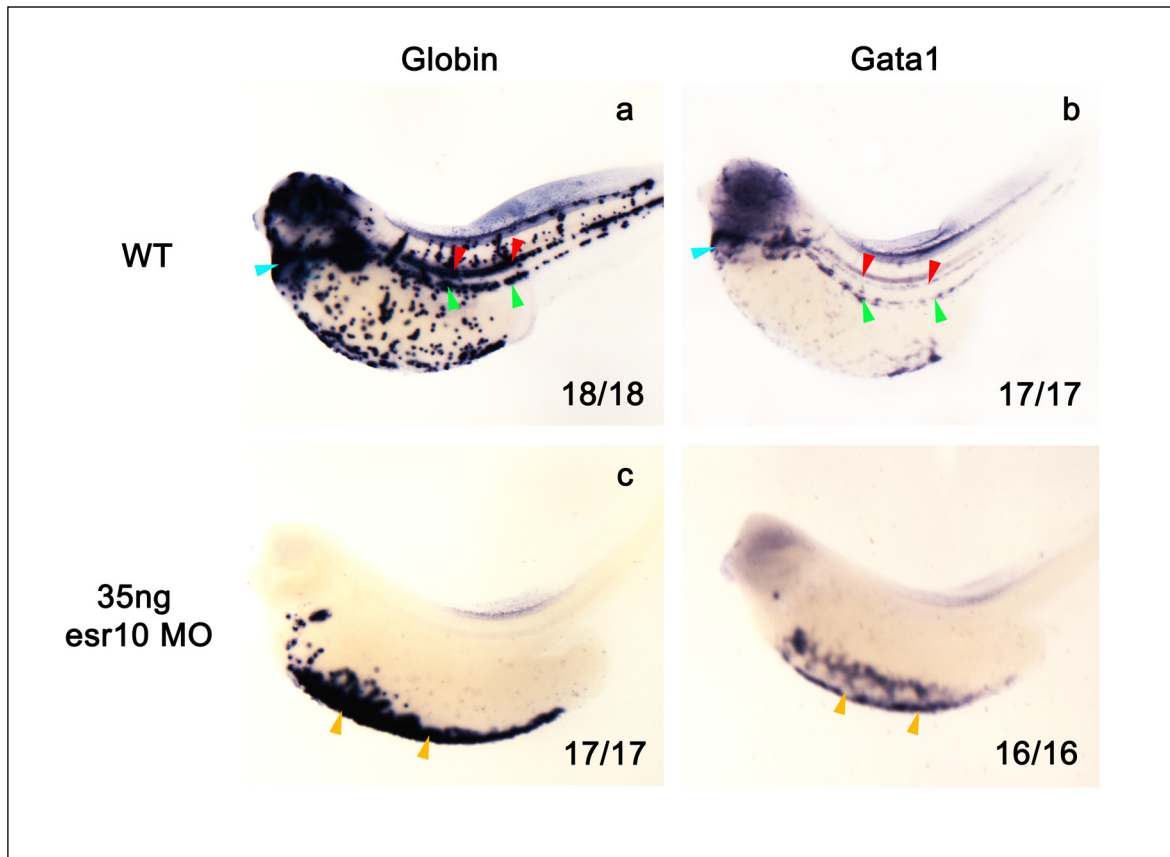


Figure 4.27 Primitive blood cells do not circulate in *esr10* deficient embryo.

Esr10 MO was injected into both cells of two-cell embryos which were collected at stage 38 and analysed by *in situ* hybridisation. At stage 38 erythrocytes can be seen circulating throughout wild type embryos, but circulating cells are not evident in *esr10* morphants. Red arrowheads mark the DA, green arrowheads mark the cardinal vein, orange arrowheads mark primitive erythrocytes in the VBI and blue arrowheads mark the heart.

4.5.7. *Esr10* is required for the expression of downstream hairy and enhancer of split genes

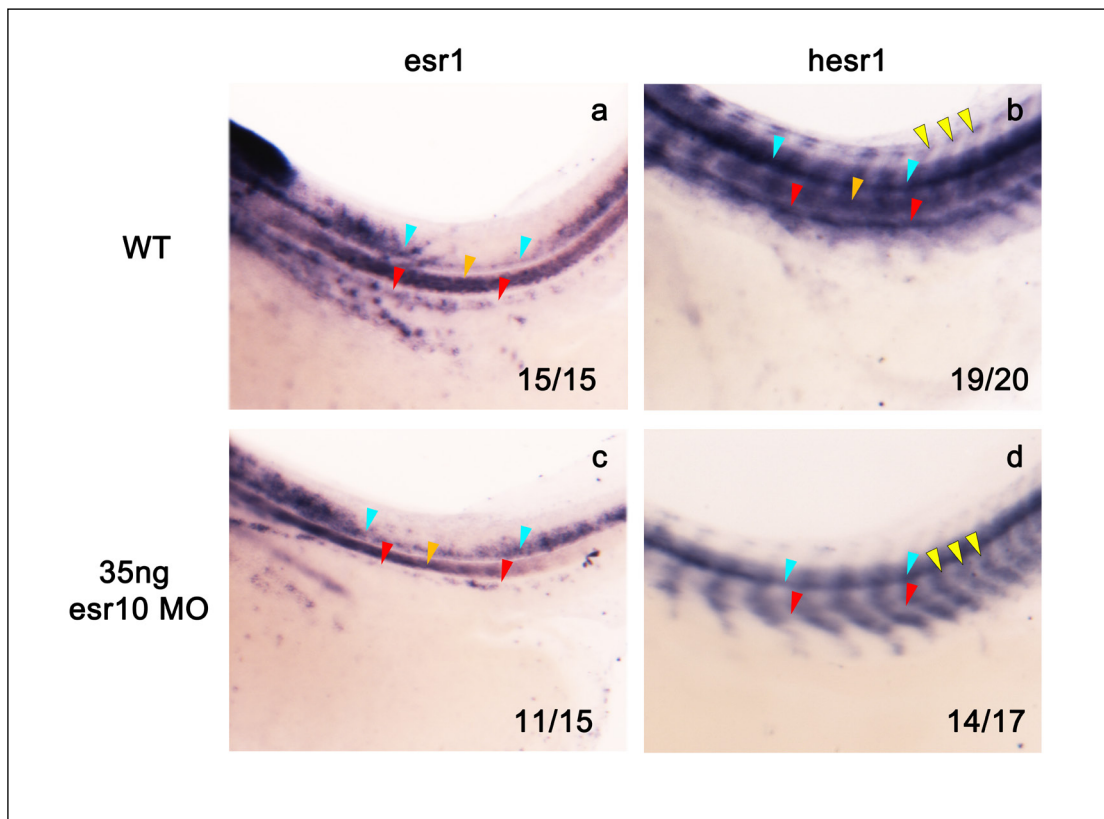
Of the five hairy and enhancer of split related genes expressed in the DA, four have been demonstrated to be notch target genes, as analysed by depletion of notch4 signalling (chapter 4.2.6). In addition, expression of both *esr1* and *hesr1* has been found to be regulated by *esr7*, and thus there appears to be a more complex network underlying notch and notch target gene regulation of haematopoietic stem cell programming. To understand whether expression of downstream hairy and enhancer of split genes is also controlled by *esr10*, expression analysis was carried out in embryos injected with 35ng *esr10* MO. In *esr10* morphant embryos, expression of *esr1* and *hesr1* is downregulated in the *Xenopus* DA at stage 39 (Figure 4.28A, panels c and d, respectively, red arrowheads), suggesting *esr10* acts to positively regulate transcription of them.

In addition, expression analysis of *notch4* and *notch1* was carried out in *esr10* morphant embryos, to determine if *esr10* has a role in regulation of notch receptor expression. Expression of both *notch4* and *notch1* is unaffected in *esr10* morphant embryos (Figure 4.28B, panels c and d, respectively, red arrowheads), and thus *esr10* does not play a role in notch receptor expression in the DA.

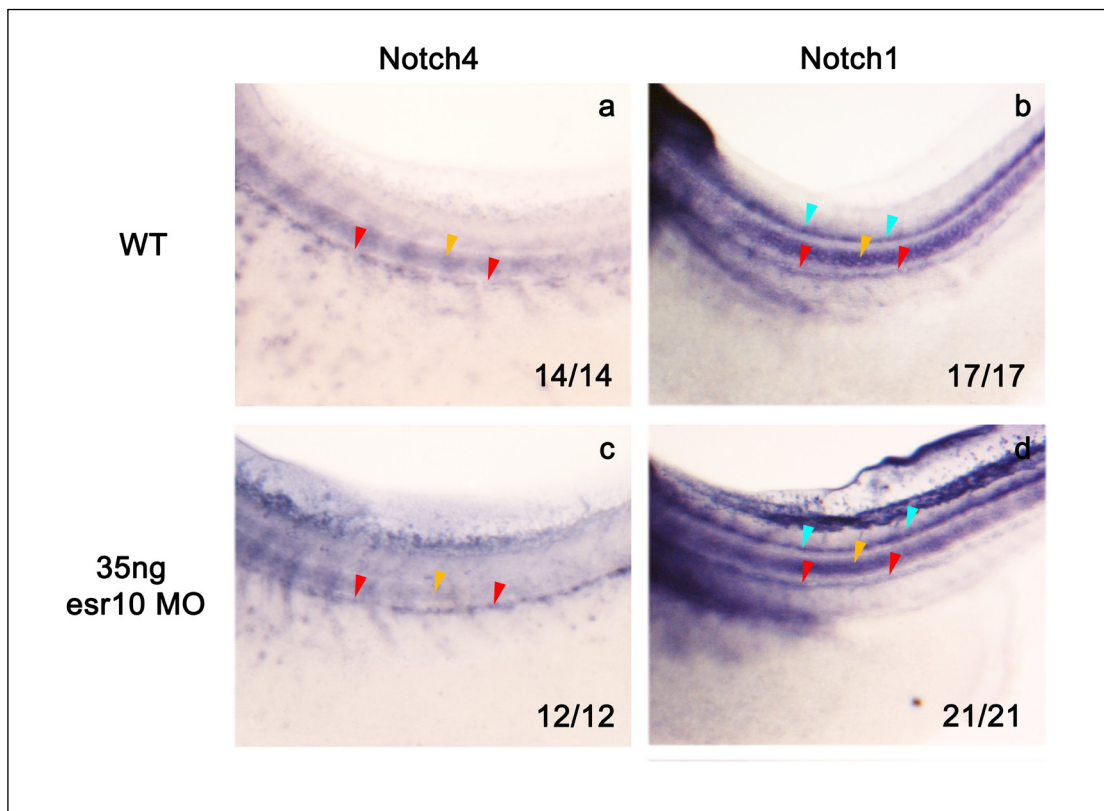
Figure 4.28 *esr10* regulates the expression of hairy and enhancer of split genes in the DA

Esr10 MO was injected into both cells of two-cell embryos which were collected at stage 33 or 38-39 and analysed by *in situ* hybridisation. **A.** Expression of both *hesr1* and *esr1* is significantly reduced in *esr10* morphants. **B.** Expression of both *notch4* and *notch1* is normal in the DA of *esr10* morphants. Red arrowheads mark the DA, blue arrowheads mark the neural tube, orange arrowheads mark the notochord and yellow arrowheads mark the somites.

A.



B.



4.6. Discussion

4.6.1. Overview

Notch4 is known to be an arterial specific gene, essential for vascular programming in both mouse embryos and adults (Carlson et al., 2005; Kim et al., 2008; Krebs et al., 2000; Miniati et al., 2010; Murphy et al., 2012; Uyttendaele et al., 2001; Uyttendaele et al., 1996; Villa et al., 2001). Whilst overexpression of *notch4* in embryonic endothelial cells has suggested an involvement of *notch4* in the regulation of vascular programming, knockout studies have so far only suggested a *notch1*-redundant role for *notch4* (Krebs et al., 2000; Uyttendaele et al., 2001). And, although *notch4* has been implicated in haematopoietic stem cell proliferation and lineage commitment (Vercauteren and Sutherland, 2004; Ye et al., 2004), a role for *notch4* in haematopoietic stem cell specification has not been reported. This study clearly demonstrates, for the first time, a role for *notch4* in haematopoietic stem cell programming. This effect is exhibited without affecting prior haemangioblast, endothelial or arterial programming. In addition to the HSC contribution of *notch4*, some angiogenic deficiencies in *notch4* morphants have been identified in both the definitive (intersomitic vessels) and primitive (vitelline vessels) lineages, along with a circulation problem. Two *notch4* target genes have also been described for the first time: *esr7* and *esr10*. The morphants for *esr7* and *esr10* almost perfectly phenocopy the *notch4* morphant, and thus these two *hes5* paralogues exhibit non-redundant roles in the *Xenopus* DA.

4.6.2. Notch4 signalling directly regulates expression of *esr7* and *esr10*

The expression pattern of *notch4* in *Xenopus* embryos reveals arterial-specific expression initiating in the DA at stage 31, immediately after the haemangioblast cells from the DLP have migrated to the midline (Figure 3.1 and Figure 3.11). At approximately the same time, expression of three hairy and enhancer of split genes *hesr2*, *esr7* and *esr10* initiates in the DA (Figure 3.11).

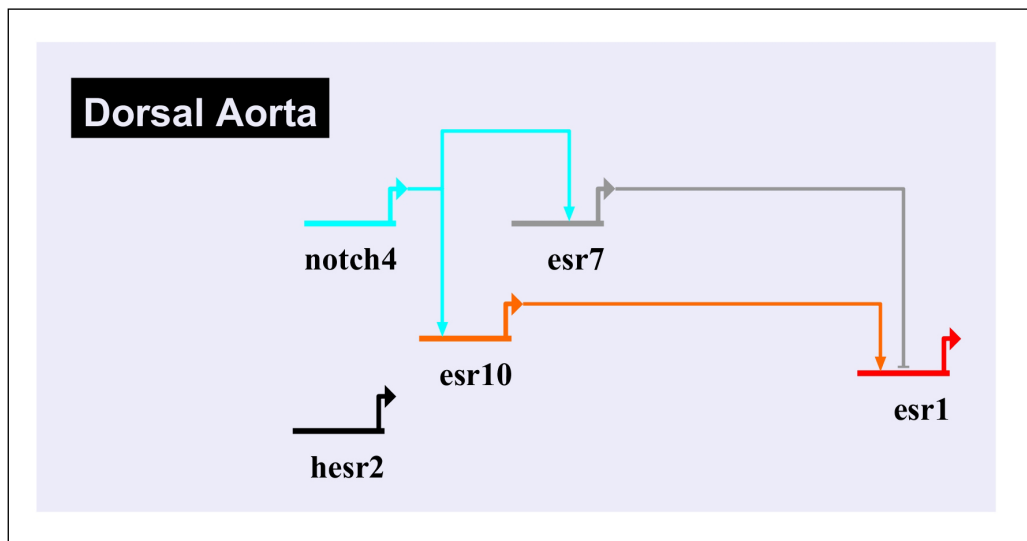
Hesr2 expression was shown to be not dependent on either *notch4* or *notch1* signalling in the DA by morpholino knockdown and chemical inhibition of notch signalling (Figure 4.10). This fits with recent literature suggesting *hesr2*, or *gridlock* as it is named in zebrafish, acts upstream of notch signalling to control arterial and haematopoietic fate in the zebrafish (Rowlinson and Gering, 2010, and reviewed in chapter 1.8.2). What regulates *hesr2* in the *Xenopus* DA is currently unknown, as is the role for *hesr2* in the DA. 5' RACE has uncovered the 5' UTR and ATG translation start site for *hesr2*, enabling a *hesr2* morpholino to be used in future experiments in order to understand the role of *hesr2* in the DA, and to investigate whether, like the zebrafish, it acts upstream of notch.

Esr7 and *esr10*, however, were found to be targets for *notch4*. Expression of both *esr7* and *esr10* was absent in the DA following injection of *notch4* MO. Unfortunately, ChIP-seq experiments to determine if this is a direct regulation are not currently possible in *Xenopus* owing to the lack of available antibodies. However, hairy and enhancer of split genes have long been regarded as direct notch target genes (comprehensively reviewed in chapter 1.8.2). As expression data shows *esr7* and *esr10* expression is initiated at approximately the same time as *notch4* (Figure 3.11), morpholino data shows they are regulated by *notch4* (Figure 4.8), and hairy and enhancer of split genes are widely regarded to be direct notch target genes, it is likely that *esr7* and *esr10* are direct targets of *notch4* signalling in the *Xenopus* DA. However, further experiments, such as ChIP-seq, are needed to confirm a direct interaction.

Expression of both *esr1* and *hesr1* initiates later in the DA, from stage 35, one stage after the initiation of *notch1* expression (Figure 3.11). Thus, *esr1* and *hesr1* were presumed to be *notch1* target genes. However, at the time *esr1* and *hesr1* are expressed, *notch4* expression is also maintained in the DA, thus it is also possible that they are targets of *notch4*. In *notch4* morphant

embryos, expression of *hesr1* is downregulated at stage 39 (Figure 4.8). Thus, *notch4* may also be acting to positively regulate expression of *hesr1* in the DA. However, expression of *esr1* is upregulated in *notch4* morphant embryos, suggesting *notch4* negatively regulates *esr1* expression (Figure 4.8). As *notch4* exerts a positive regulation on *esr7*, *esr10* and *hesr1* (although possibly indirectly), it is more likely that the regulation of *esr1* by *notch4* is indirect, rather than the possibility of *notch4* exerting both negative and positive regulations on very similar genes. This claim is supported by the observation that both *esr7* and *esr10* also regulate the expression of *esr1* (Figure 4.19 and Figure 4.28, respectively). From this data a network has emerged, emanating from *notch4*, in which *esr7* and *esr10* negatively and positively regulate expression of *esr1*, respectively, presumably in order to ensure precise levels of *esr1* expression (Figure 4.29A). However, an alternative network can also be implied from this data (Figure 4.29B). Here, both *esr10* and *esr7* act as negative regulators of transcription, with *esr10* repressing *esr7*, which in turn represses *esr1*. This proposed model fits with the traditional role of hairy and enhancer of split proteins acting as repressors (Dawson et al., 1995). Analysis of *esr7* expression in *esr10* morphant embryos would enable the resolution of these two proposed genetic regulatory networks, as would ChIP-seq experiments. When *notch4* is lost, and thus both *esr7* and *esr10* are lost, expression of *esr1* is upregulated, and thus must be activated by another factor independent of *notch4*. Supporting this evidence, when *esr10* is knocked-down, *esr1* expression is only downregulated, not entirely lost (Figure 4.28).

A.



B.

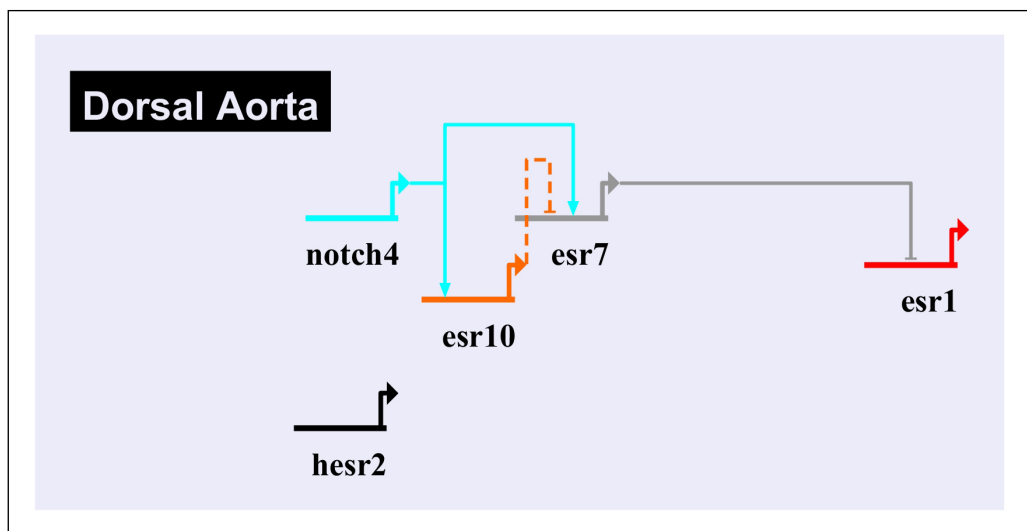


Figure 4.29 Schematic diagrams showing the proposed network of gene regulation controlled by *notch4*

A. Notch4 positively regulates expression of *esr7* and *esr10*; *esr7* negatively regulates *esr1* whilst *esr10* positively regulates *esr1*. *Hesr2* is not regulated by *notch4*. **B.** Alternative model: Notch4 positively regulates expression of *esr7* and *esr10*; *esr10* represses *esr7*, which in turn represses *esr1*. *Hesr2* is not regulated by *notch4*. Dashed line represents proposed interaction not yet investigated.

4.6.3. Notch4 and its target genes *esr7* and *esr10* are specifically required for HSC programming

Whilst a role for *notch4* in haematopoietic stem cell proliferation has been demonstrated (Vercauteren and Sutherland, 2004; Ye et al., 2004), the early lethality of constitutively active *notch4* mutants, the precluding vascular defects, and the redundancy between *notch1* and *notch4* in knockouts studying DA development, has prevented the study of *notch4* in haematopoietic stem cell programming (Krebs et al., 2000; Uyttendaele et al., 2001). Here, a role for *notch4*, and *notch4* target genes *esr7* and *esr10*, in programming of the haemogenic endothelium has been demonstrated.

Runx1 has been well demonstrated to be essential for the formation of definitive haematopoietic clusters (Kissa and Herbomel, 2010; North et al., 1999; North et al., 2002). In *Xenopus*, *runx1* expression initiates at stage 35 in the DA, and is restricted to the ventral wall of the DA, before being expressed in the emerging clusters at stage 43 (Fernandez, 2007, and Aldo Ciau-Uitz, personal communication). It is thought to be toward the top of the genetic regulatory network controlling haematopoietic programming, including being upstream of *scl*, *lmo2*, *spiB*, *gfi1* and *ikaros*, but not *gata2* (Fernandez, 2007). Thus, *runx1* is an ideal marker for the analysis of the haematopoietic programme. In *notch4*, *esr7* and *esr10* morphants, expression of *runx1* is lost in the DA, as is expression of downstream haematopoietic genes, *spiB* and *gfi1*. This suggests that early programming of the HSCs is compromised in notch pathway morphants. *Runx1* is known to be essential for haematopoietic cluster formation (Fernandez, 2007; Kissa and Herbomel, 2010; North et al., 1999), and thus in notch pathway morphants negative for *runx1* expression, haematopoietic clusters are predicted to be absent from the intraluminal space. Carrying out *in situ* hybridisation on stage 43 sectioned embryos is needed to reveal if this is as predicted, and analysis of B-cell development using B-cell q-PCR techniques would enable the analysis of long-term definitive haematopoietic defects in notch pathway morphants.

Haematopoietic stem cells are believed to arise from the ventral wall of the DA, and endothelial cells have been imaged rounding up and emerging from the DA (Kissa and Herbomel, 2010, and reviewed in chapter 1.5). When studying the programming of haemogenic endothelium and the subsequent emergence of HSCs, it is important to consider that a defect in the earlier vascular or arterial programming is likely to influence the programming of haemogenic endothelium and HSCs. Importantly, endothelial and arterial programming appear unaffected in *notch4*, *esr7* and *esr10* morphants, as evidenced by the normal expression of the endothelial markers, *tie2* and *ami*, and arterial markers *ephrinB2* and *cx37*. As such, the haematopoietic defects observed can be regarded as a primary effect of *notch4* depletion. Expression of endothelial markers *tie2* and *ami* revealed reduced sprouting of intersomitic vessels, primarily from the vein, in *notch4*, *esr7* and *esr10* morphants, although this effect was not as apparent in *esr10* morphants. Thus, whilst endothelial and arterial programming was normal, some angiogenic remodelling defects were observed.

However, it is not known if this requirement for notch signalling in HSC programming is cell-autonomous or not. In addition to the long-proposed cell autonomous requirement for notch signalling in HSC programming, a non-cell autonomous requirement for notch ligands has also been demonstrated in zebrafish (Clements et al., 2011), and thus it is possible the loss of *Xenopus* HSC markers presented in this thesis is due to a non-cell autonomous loss of notch signalling.

4.6.4. Initial expression of *gata2* in the DA is independent of *notch4*, *esr7* and *esr10*

Gata2 expression initiates in the *Xenopus* DA at stage 31, at approximately the same time as *notch4*, *esr7* and *esr10* expression initiates (Figure 3.12). It has been found to be upstream or

parallel to *runx1* in HSC development, as *runx1* MO does not affect expression of *gata2* (Fernandez, 2007). Presence of a GATA binding site in the promoter of murine *runx1* suggests that GATA factors are able to directly regulate transcription of *runx1* (Nottingham et al., 2007), and, as the only known GATA factor expressed in the *Xenopus* DA, *gata2* is a strong candidate for *runx1* transcriptional regulation. Expression analysis of *gata2* in the DA of *notch4*, *esr7* or *esr10* morphants reveals that *gata2* expression is not regulated by notch signalling during early specification of the DA (stage 32). However, during later stages of DA development, *gata2* expression is *notch4* and *esr10* dependent. Thus, *gata2* expression is regulated differently during early and later DA programming, with only the later expression requiring notch signalling.

4.6.5. Candidates for the regulation of *notch4*

Notch4 is essential for haematopoietic programming, and, together with genes such as *hesr2* and *gata2*, is expressed as soon as endothelial cells reach the midline. A signal must be activating expression of these genes once the cells reach the midline, although it is currently unknown what that signal is. *Hesr2* is known to be upstream of notch signalling in zebrafish (Rowlinson and Gering, 2010), and could therefore potentially be upstream of *notch4* in *Xenopus*. However, whilst this may be true, it is unlikely to be the initial midline signal activating expression of DA genes due to the fact that it is a transcription factor, not a signalling molecule. Similarly, whilst GATA binding regions have been identified in the *notch4* promoter (Li et al., 1998), and *gata2* is expressed at this time, it is again a transcription factor rather than a signalling molecule, and is therefore extremely unlikely to be the initial specifying midline signal.

VEGF signalling from the somites is known to regulate *notch1* expression in the *Xenopus* DA (Leung, 2010), in the zebrafish DA (Lawson et al., 2002), and in endothelial cells (Liu et al., 2003). Endothelial cells of the DLP are known to migrate to the midline following VEGF cues from the

hypochord, and thus maintain expression of VEGF receptor *flk1* during migration (Cleaver and Krieg, 1998). I hypothesise that paracrine VEGF signalling initiates expression of arterial and haematopoietic genes, including *notch4*, in the DA once the cells reach the midline. There are three known VEGFA isoforms expressed in tissues surrounding the DA, generated by alternative splicing: short VEGFA₁₂₂, medium VEGFA₁₇₀, and long VEGFA₁₉₀ (Leung, 2010). Medium and long isoforms are less diffusible than the short isoforms, due to interaction with heparin at the ECM (Robinson and Stringer, 2001). Using splice-mediated specific isoform knockdown, signalling of medium and long isoforms of VEGFA to endothelial cells undergoing migration has been shown to be required for later *notch1* expression in the DA, whilst not altering expression of *notch4* (Leung et al, submitted). Endothelial cells respond to diffusible short VEGFA signalling from the hypochord in order to migrate to the midline. Acting as a morphogen, short VEGFA isoforms would be detected by *flk1*-expressing endothelial cells at a higher concentration at the midline than in the DLP, and this higher concentration of VEGFA signalling in endothelial cells could activate the expression of arterial genes. Unfortunately, it has not been possible to test this theory; the requirement for VEGF signalling in programming of the DLP (Ciau-Uitz et al., 2010b), and in the migration of these cells to the midline (Cleaver and Krieg, 1998), means that using morpholinos to unconditionally knock-down VEGF signalling would cause earlier defects in programming. In addition, use of specific VEGF inhibitors, which could temporally inhibit VEGF signalling, has not been successful in perturbing VEGF signalling in *Xenopus* (Aldo Ciau-Uitz, personal communication). Photoactivatable morpholinos are a newly released technology, allowing the temporal activation of morpholinos *in vivo*. However, this technology has not yet been demonstrated to work in *Xenopus*. Furthermore, activating a morpholino at the midline of *Xenopus* embryos would be difficult to achieve as *Xenopus* embryos are not transparent like a zebrafish embryo, and earlier activation of the photoactivatable VEGFA morpholino in the DLP would alter migration of cells to the midline. Furthermore, complications would arise in the

uncoupling of a role for VEGFA in migration and arterial programming. As cells begin to express arterial genes immediately after migration, there would be a very small time frame (if any) in which to knock-down VEGF without affecting migration, but before onset of the arterial programme.

4.6.6. A role for *notch* in circulation of primitive erythrocytes

Mice overexpressing *notch4* in endothelial cells exhibit an enlarged vessel (Carlson et al., 2005; Kim et al., 2008; Miniati et al., 2010; Uyttendaele et al., 2001), whilst *notch1/notch4* double mutants exhibit collapsed vessels (Krebs et al., 2000). This suggests notch signalling is regulating vessel and lumen size in the mouse. Analysis of either *notch4* or *esr10* morphant *Xenopus* embryos revealed that circulation is defective. In living embryos, blood cells cannot be seen circulating in the tail, whilst in fixed embryos analysed by *in situ* hybridisation for the expression of primitive erythroid markers, pooling of primitive erythroid cells in the VBI is evident. As the heart is beating normally, this is most likely to be a vasculature defect. In contrast, circulation appears normal in *esr7* morphant embryos, suggesting *notch4* regulates circulation via *esr10*, and not *esr7*. The cause for this lack of circulation is likely due to faulty lumen formation in morphant embryos, as *notch4* has previously been associated with lumen size (Carlson et al., 2005; Kim et al., 2008; Miniati et al., 2010; Uyttendaele et al., 2001). Expression analysis of endothelial and arterial markers in *notch4* and *esr10* morphant embryos reveals a thinner looking DA, and this supports the notion of a collapsed/missing lumen. However, fewer vitelline vessels are seen in *notch4* morphants, and these vessels are essential for the flow of primitive blood to the aorta and cardinal veins. Thus, it is possible that the lack of circulation is due to a vitelline vasculature defect. In *esr7* morphants however, the vitelline vasculature appears relatively normally developed, and thus it is less likely to be the cause of circulation defects. Analysis of lumen size by

in situ hybridisation on sections would reveal whether lumen formation is indeed affected in *notch4* and *esr10* morphants, as predicted.

4.7. Conclusion

In conclusion, the data presented here reveals that *notch4*, and its target genes *esr7* and *esr10*, are specifically required for haematopoietic programming, whilst not affecting earlier development of the haemangioblast or arterial specification.

Chapter 5. Characterising the role of *notch1* and its target genes in dorsal aorta development and HSC specification

5.1. Introduction

Notch1 has been strongly associated with haematopoietic development (reviewed in Bigas and Espinosa, 2012), lymphoblastic leukaemia (reviewed in Koch and Radtke, 2011), and T-cell lineage commitment (reviewed in Rothenberg, 2011). Along with *notch4*, *notch1* is expressed in the dorsal aorta of mouse and *Xenopus* embryos (Robert-Moreno et al., 2005; Villa et al., 2001, and Chapter 3.2, respectively).

The plight to understand the role of *notch1* in haematopoietic stem cell development has been hindered by the relatively early lethality of mutant embryos and the precluding vascular/arterial defects. Mouse embryos homozygous for a null *Notch1* allele die at E9.5-10.5, showing a collapsed DA phenotype and yolk sac vasculature remodelling defects (Kim et al., 2008; Krebs et al., 2000). Endothelial-specific deletion of *Notch1*, as controlled by *Tie2*-Cre-recombination, also results in yolk sac vasculature remodelling defects, a collapsed DA and embryonic lethality at E9.5-10.5, suggesting that the primary defect causing lethality in *Notch1* deficient embryos is vascular (Limbourg et al., 2005). Overexpression of *Notch1* in *Tie2*-expressing endothelial cells causes vascular remodelling and smooth muscle recruitment defects along with haematopoietic defects, and causes embryonic lethality at E9.5-10.5 (Venkatesh et al., 2008). In addition to the early lethality of mutant embryos, the vascular phenotype of both *notch1*-null and *notch1*-overexpressing embryos prevents an understanding of the role of *notch1* in HSC programming: as definitive haematopoietic clusters derive from ventral aortic endothelium during embryogenesis, the aortic endothelium must be correctly specified for subsequent haemogenic endothelium programming and HSC emergence. In order to determine if there is a specific effect of *notch1*

signalling on HSC programming, the notch1 contributions to vascular and HSC programming must be uncoupled.

The first evidence for a role of notch signalling in definitive haematopoiesis came from *Notch1*-null murine embryos with a reduced ability to form transplantable, repopulating LT-HSCs in the AGM (Kumano et al., 2003). Expression of endothelial and haemogenic endothelium markers appeared normal in *Notch1*-null P-Sp cultures by semiquantitative RT-PCR, but there was a marked decrease in expression of haematopoietic markers, and fewer CFCs (Kumano et al., 2003). However, detailed analysis of arterial specification was not carried out, so it still remains possible that the haematopoietic phenotype observed is due to incorrect arterial programming. The haematopoietic potential of *Notch1*^{-/-} P-Sp cultures can be rescued by retroviral transfer of *runx1*, suggesting that the primary requirement of notch1 signalling is to activate *runx1* expression for HSC programming (Nakagawa et al., 2006). Heat-shock activated overexpression of constitutively active *notch1* (N1ICD) in zebrafish causes the expansion of haematopoietic markers from the floor of the DA to the roof of the DA, and the vein (Burns et al., 2005). This suggests that *notch1* confers haematopoietic marker expression ventrally in the DA of a wild type embryo, and has the ability to expand definitive HSCs. Whilst ectopic expression of arterial markers was not observed in the vein during this study, similar studies have found the loss of venous markers in the vein of heat-shock activated N1ICD fish, presenting the possibility that absence of venous markers is a prerequisite of HSC programming (Lawson et al., 2001). Indeed, heat-shock activation of N1ICD cannot rescue expression of *runx1* in embryos with prior endothelial/arterial defects, suggesting that the artery needs to be properly programmed to produce HSCs, even in the presence of *runx1* (Rowlinson and Gering, 2010). In agreement with the proposed role for *Notch1* in haematopoietic development, *Notch1*-deficient ES cells are not capable of colonising the bone marrow in chimeric mice (Hadland et al., 2004). Surprisingly, whilst *notch1* is thought to be essential for HSC

programming, development of primitive erythrocytes and erythromyeloid progenitors (EMPs) is completely normal in *notch1* deficient embryos (Bertrand et al., 2010b; Burns et al., 2005; Gering and Patient, 2005; Kumano et al., 2003).

To understand the role of *notch1* in *Xenopus* arterial and haematopoietic stem cell development, morpholino oligonucleotide technology was used. Here, I show for the first time that *notch1* is essential for haematopoietic stem cell programming in *Xenopus*. Notch1 controls expression of *esr1* and *hesr1* in the DA immediately after *notch1* expression initiates. *Esr1* was also found to be specifically required for haematopoietic stem cell development, whilst *hesr1* acts in parallel to, or downstream of, *runx1* to control expression of later HSC markers.

5.2. *Notch1*

5.2.1. Designing a morpholino to target *notch1*

Using the full length mRNA sequence for *notch1* (Unigene Xl.1057), a morpholino was designed and produced by Gene Tools (see Table 2.3 for sequence, Figure 5.1A). After comprehensive BLAST analysis, no pseudoallele has been identified for *notch1*. The *notch1* MO sequence was blasted against the *X.laevis* nucleotide collection to look for potential off-target effects, and no predicted targets other than *notch1* were detected.

To assess the effect of *notch1* depletion in *Xenopus*, embryos were injected at the 2-cell stage with 20-60ng *notch1* morpholino. At 60ng, embryos looked unhealthy showing signs of non-specific toxicity during early development, including developmental delay, and failure to close the neural tube. Embryos injected with 20ng, 30ng, 40ng and 50ng looked healthy, showing no gross morphological defects (50ng shown in Figure 5.1B). Observation of *notch1* MO injected embryos at stage 39 revealed normally beating hearts and normal circulation in the tail of the embryos. As

no gross morphological defects were observed in embryos injected with 50ng *notch1* MO, this was chosen as the optimal dose for analysing *notch1* depletion in *Xenopus* embryos. Although no gross morphological defects were observed, some minor developmental differences were found, similar to those found in *notch4* morphants: in *notch1* morphant embryos, the developing eye appeared smaller (Figure 5.1B, red arrows) and fewer pigment cells were observed (blue arrows).

A.



B.

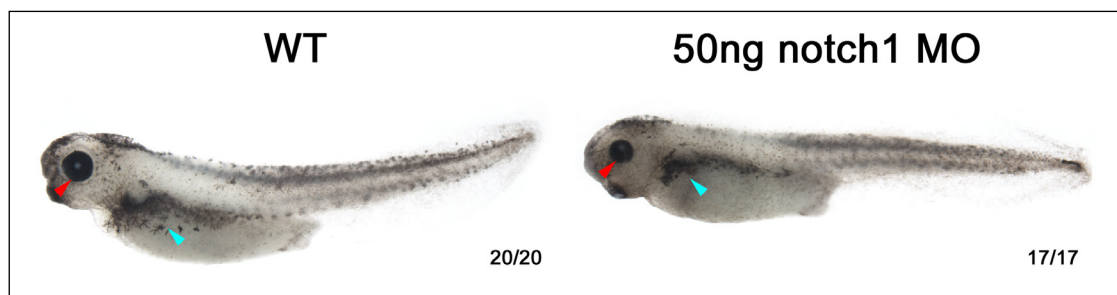


Figure 5.1 *Notch1* MO does not cause gross morphological defects

A. *Notch1* MO (red) aligned next to *notch1* mRNA seq (black). Green marks the translation start site for *notch1*. **B.** Stage 39 *notch1* morphant embryos (right) do not show gross morphological defects when compared to wild type sibling controls (left). However, both the eye (red arrowheads) and pigment cells (blue arrowheads) are underdeveloped.

5.2.2. Notch1 morpholino inhibits the translation of notch1

In order to test the ability of the *notch1* morpholino to bind to its target sequence, Gateway technology was undertaken to create a *notch1-gfp* construct containing the *notch1* morpholino binding site tagged with *gfp* (see section 2.2.1.11). Injection of 200pg *notch1-gfp* mRNA into the animal pole of 1-cell stage embryos generated a GFP signal, as visualised under a fluorescence microscope (Figure 5.2iii), whilst co-injection of 200pg *notch1-gfp* mRNA with 50ng *notch1* MO produced no visible GFP signal (Figure 5.2iv). This suggests that the *notch1* MO is capable of binding to the *notch1* MO target sequence and preventing translation. As a control, co-injection of 50ng *notch1* MO with 200pg *gfp* (Figure 5.2ii) elicited a strong GFP signal, comparable to that of 200pg *gfp* alone (Figure 5.2i), suggesting *notch1* MO does not bind non-specifically to *gfp*.

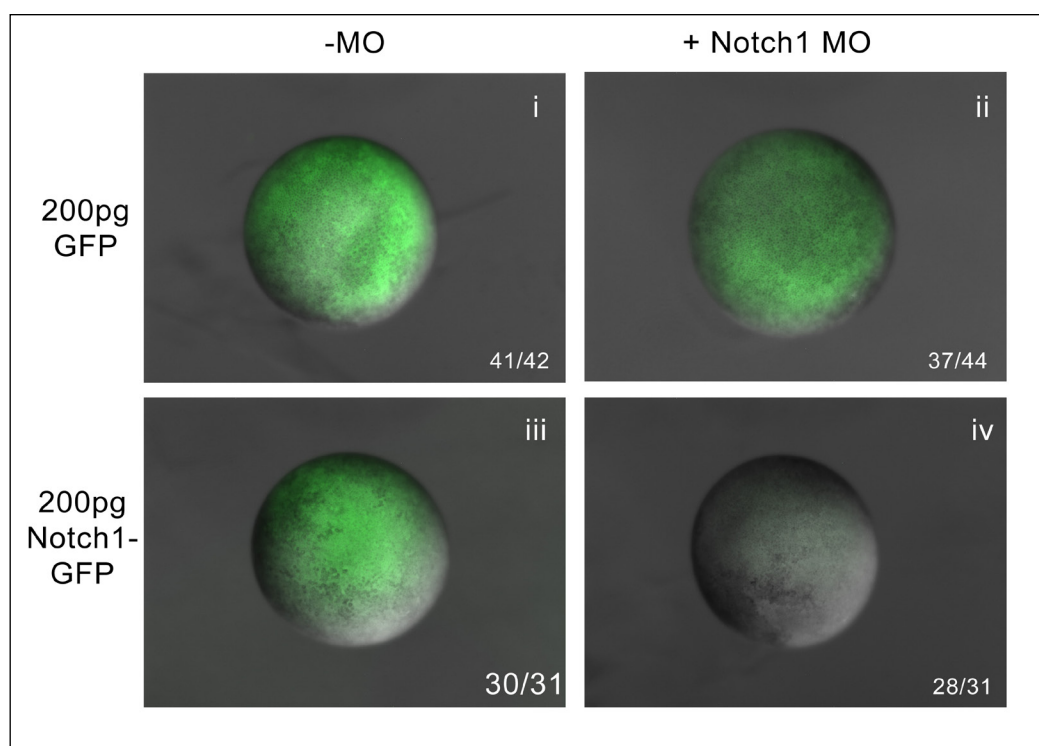


Figure 5.2 *notch1* MO is capable of preventing translation from the *notch1* MO binding site

i) 200ng *gfp* mRNA generates a strong GFP signal. ii) 200ng *gfp* mRNA co-injected with 50ng *notch1* MO generates a strong signal. iii) 200ng *notch1-gfp* generates a strong signal. iv) 200ng *notch1-gfp* co-injected with 50ng *notch1* MO generates little to no GFP signal.

5.2.3. Notch1 is essential for haematopoietic stem cell programming

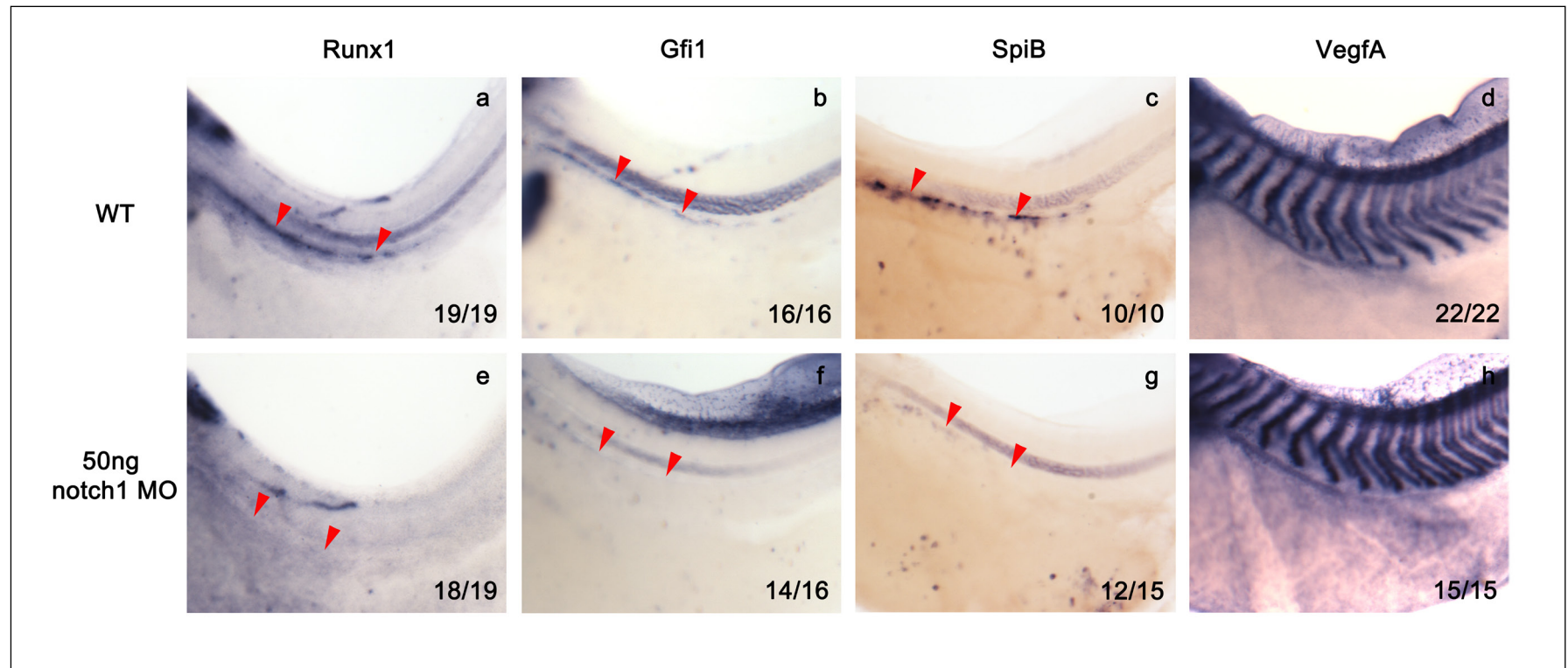
To investigate the role of *notch1* in *Xenopus* haematopoietic stem cell development, stage 39 embryos injected with 50ng *notch1* MO were analysed by *in situ* hybridisation for the expression of HSC markers. Expression of HSC marker, *runx1*, was significantly downregulated in morphant embryos such that expression in the DA cannot be detected (Figure 5.3, panel e, red arrowheads). *SpiB* and *gfi1* are HSC markers expressed after *runx1* expression initiates, and have been found to be *runx1*-dependent in *Xenopus* (Aldo Ciau-Uitz, personal communication). Expression of both *spiB* and *gfi1* is absent in *notch1* morphant embryos, as expected by the loss of *runx1* expression (Figure 5.3, panels f and g, respectively, red arrowheads).

VEGFA (vascular endothelial growth factor A) signalling from the somites to the DA is known to be required for expression of haematopoietic markers including *runx1* (Leung, 2010). As *notch1* is expressed in the somites (see Figure 3.1, Figure 3.2 and Figure 3.3), it is possible that depletion of *notch1* might be altering the expression of *vegfa* in the somites, and thus in turn, perturb HSC programming. However, expression analysis in the somites at stage 39 reveals that *vegfa* is unaffected in *notch1* morphants (Figure 5.3, panel h). As the VEGFA contribution to haematopoietic programming appears normal, the haematopoietic defects observed are due to *notch1* depletion in the DA.

HSC marker, *gata2*, is expressed at stage 31, prior to *runx1* expression in the *Xenopus* DA, and earlier than the initiation of *notch1* (see schematic, Figure 3.12). The presence of a GATA binding site in the *runx1* promoter suggests that a GATA factor is involved in *runx1* transcription regulation (Nottingham et al., 2007). As *gata2* is the only known GATA factor expressed in the *Xenopus* DA during haematopoietic programming, it is probable that *gata2* is involved in the regulation of *runx1*.

Figure 5.3 *Notch1* is required for the expression of haematopoietic stem cell markers.

Notch1 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of HSC markers *runx1*, *gfi1* and *spiB* is lost in *notch1* morphant embryos at stage 39 when compared to WT sibling controls. Expression of *vegfa* in the somites is also normal. Red arrowheads mark the dorsal aorta.



At stage 39, expression of *gata2* is notably decreased in *notch1* morphants (Figure 5.4, panel d, red arrowheads), suggesting that HSC programming is disrupted prior to *runx1* expression. Interestingly, *gata2* expression initiates normally, as evidenced by the relatively normal expression in the DA at stage 32 (Figure 5.4, panel c, red arrowheads). As the regulation of *gata2* is not disrupted in *notch1* morphants at stage 32, but is disrupted at stage 39, it suggests two independent methods of *gata2* regulation in the *Xenopus* DA, dependent on developmental stage, with the later stages requiring notch signalling for expression of *gata2*. Loss of haematopoietic markers was used as a positive control for all subsequent *notch1* MO morphant phenotypes.

5.2.4. Haemangioblast programming is unaffected by loss of *notch1*

Whilst *notch1* is not expressed in lateral mesoderm populations during early development and haemangioblast programming (Figure 3.2 and Figure 3.3), injection of *notch1* MO from the 2-cell stage provides the possibility that early programming of the haemangioblast cells is compromised in *notch1* morphants. In order to investigate if notch1 has a role in haemangioblast programming, stage 28 embryos injected with 50ng *notch1* MO were analysed for haematopoietic and endothelial markers in the DLP. Expression of haematopoietic marker *scl* in *notch1* morphant embryos is relatively normal in the DLP (Figure 5.5, panel d, red arrowheads). Expression of endothelial markers is also normal in the DLP: *flk1*, a VEGF receptor required for haemangioblast cell migration to the midline, is expressed normally in *notch1* morphant embryos (Figure 5.5, panel e, red arrowheads). Similarly, endothelial marker *egfl7*, a gene at the bottom of the haemangioblast gene regulatory network (Figure 1.11), is also expressed normally in the DLP of *notch1* morphant embryos (Figure 5.5, panel f, red arrowheads), suggesting that the genetic regulatory network upstream of *egfl7* is also programmed normally.

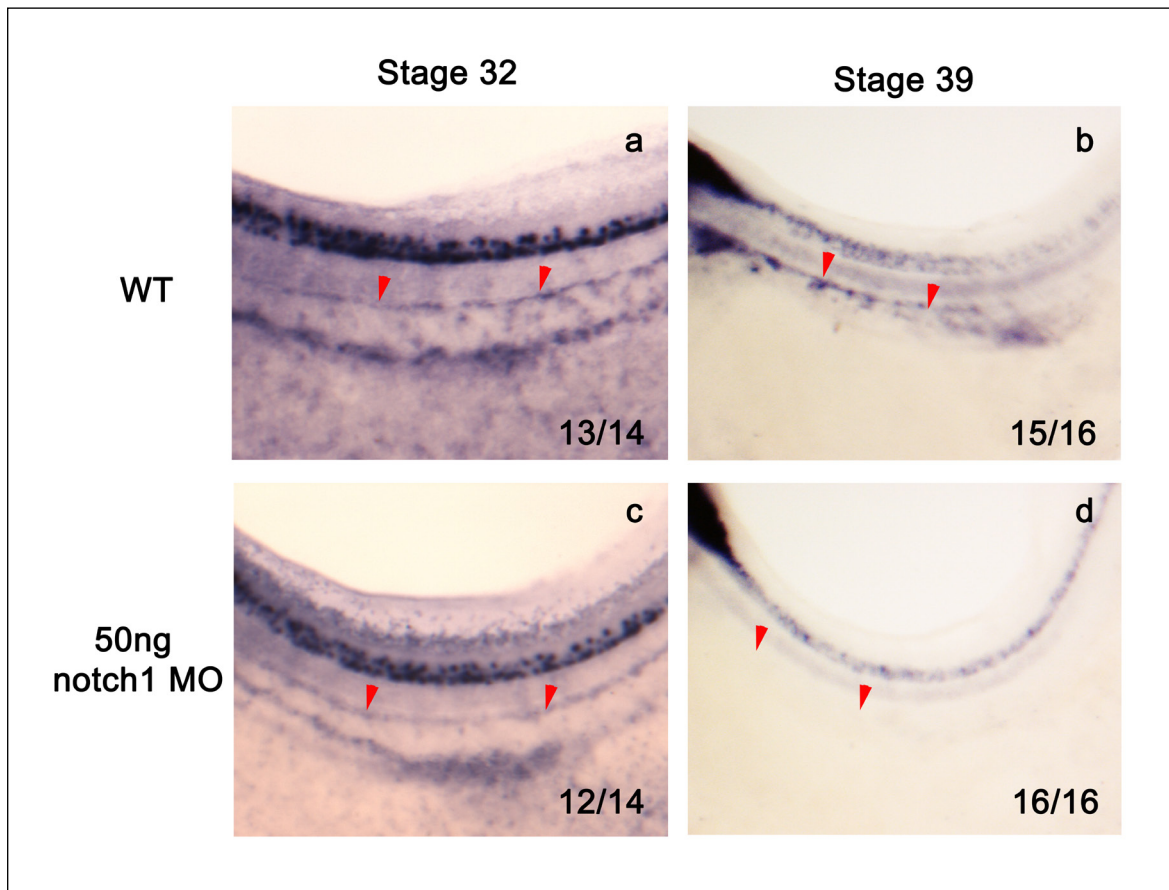


Figure 5.4 Depletion of *notch1* reduces *gata2* expression at stage 39.

Notch1 MO was injected into both cells of two-cell embryos which were collected at stage 32 or 38-39 and analysed by *in situ* hybridisation. *Gata2* expression initiates normally in *notch1* morphant embryos, as evident by expression in the DA at stage 32, but is not maintained, as evident by the loss of expression at stage 39. Red arrowheads mark the DA.

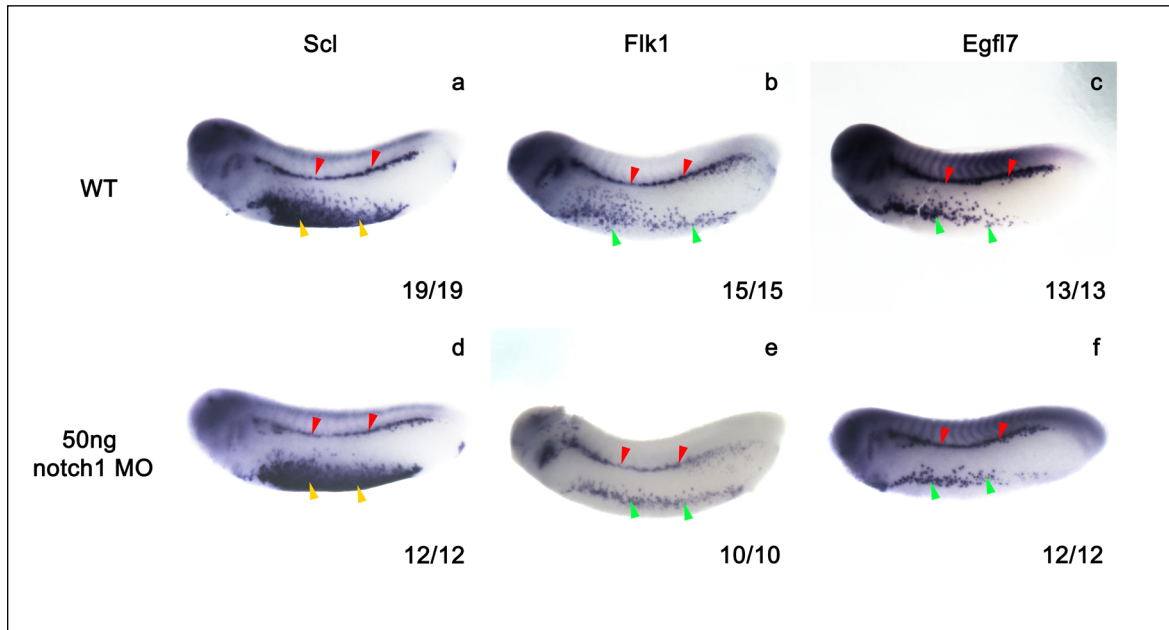


Figure 5.5 Programming of the haemangioblast is normal in *notch1* morphants

Notch1 MO was injected into both cells of two-cell embryos which were collected at stage 27-28 and analysed by *in situ* hybridisation. Expression of haematopoietic marker *scl*, and endothelial markers *flk1* and *egfl7* is unaffected in *notch1* morphants. Expression can be seen in the DLP (red arrowheads), developing primitive erythrocytes in the VBI (orange arrowheads), and in developing vitelline vessels (green arrowheads).

Expression of *vegfa* in the somites is crucial for correct haemangioblast specification. VEGFA signals from the somites to the lateral mesoderm, controlling expression of *scl* and genes downstream of *scl* (Ciau-Uitz et al., 2010b). As *notch1* is strongly expressed in the somites from stage 20, it is essential to determine if *notch1* morphants have a somitic VEGFA deficiency which could impact on haemangioblast development. As *scl* expression in the DLP is normal in *notch1* morphants, it argues that VEGFA signalling from the somites to the DLP is normal at this stage.

Expression of *scl*, *flk1* and *egfl7* is also seen in the VBI, marking primitive erythrocytes (*scl*) and endothelial cells which will form the vitelline vessels (*flk1* and *egfl7*). Expression of *scl* in the VBI is normal (Figure 5.5, panel d, orange arrowheads), suggesting the programming of primitive erythrocytes in the VBI is normal in *notch1* morphant embryos. Similarly, expression of both *flk1* and *egfl7* in the VBI of *notch1* morphant embryos is also normal (Figure 5.5, panels e and f, green arrowheads), and thus early patterning and programming of the vitelline vessels in *notch1* morphant embryos is normal.

5.2.5. Endothelial and dorsal aorta specification is unaffected by loss of *notch1*

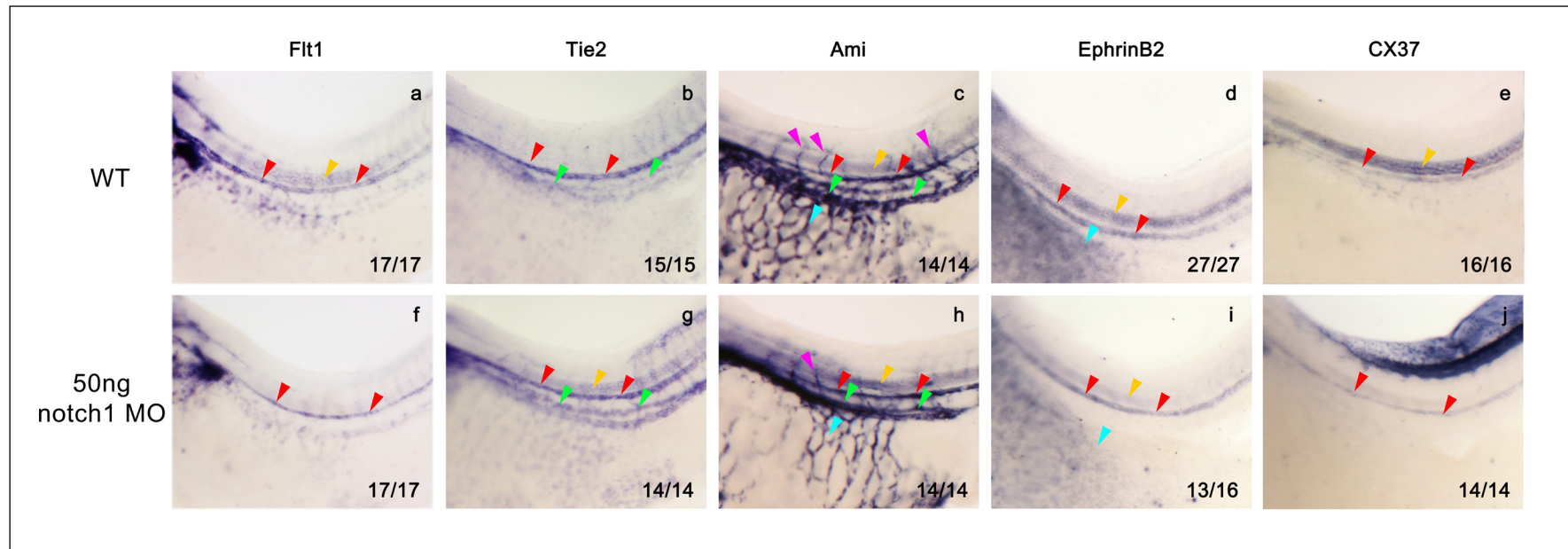
A role for *notch1* in embryonic vascular programming has been demonstrated in mouse: deletion of *notch1* causes the DA and PCVs to have collapsed lumens, whilst overexpression of *notch1* in an endothelial-specific manner causes cardiovascular defects, including a smaller DA (Kim et al., 2008; Krebs et al., 2000; Limbourg et al., 2005; Venkatesh et al., 2008). Similarly, in zebrafish, activated *notch1* signalling causes a loss of venous markers in the PCV, suggesting *notch1* controls suppression of the venous programme (Lawson et al., 2001). In order to determine if the haematopoietic defect observed in *notch1* morphant *Xenopus* embryos is specific to HSC development, or is a consequence of an earlier vascular/arterial programming defect, it is essential to analyse the programming of the DA.

Stage 39 embryos injected with 50ng *notch1* MO were analysed by *in situ* hybridisation for the expression of endothelial and arterial markers. Expression of endothelial markers, *flt1* (a vegf receptor; *vegfr1*), *tie2* and *ami*, revealed normal expression in the DA of *notch1* morphants (Figure 5.6, panels f, g and h, respectively, red arrowheads). Similarly, expression of arterial markers, *ephrinB2* and *cx37*, is also normal in the DA of *notch1* morphant embryos (Figure 5.6, panels i and j, respectively, red arrowheads). Expression of *ami* and *ephrinB2* in the vitelline vessels is reduced, and fewer vessels are apparent here, suggesting *notch1* has a role in the development of the vitelline vasculature (Figure 5.6, panels h and i, respectively, blue arrowheads). This is in agreement with mouse *notch1* knockouts which exhibit vascular remodelling defects in the yolk sac (Kim et al., 2008; Krebs et al., 2000). Development of the intersomitic vessels from the vein is also slightly disrupted in *notch1* morphants, as marked by expression of *ami* (Figure 5.6, panel h, purple arrowheads). This suggests that whilst *notch1* is dispensable for programming of the DA, it has a functional role in angiogenesis of intersomitic vessels.

In the *Xenopus* DA, expression of *hesr1* and *esr1* initiates at the time *flk1* expression is downregulated, and thus one or both may be controlling *flk1* downregulation (Figure 3.12). As *notch1* controls the expression of both *esr1* and *hesr1* in the DA (see chapter 5.2.7), it is possible that *flk1* could be downregulated in *notch1* morphants. At stage 39, expression of *flk1* and *flt4* has been downregulated and cannot be seen in the DA of wild type embryos (Figure 5.7, panel a and b, red arrowheads). In *notch1* morphant embryos, expression of *flk1* and *flt4* is also not seen (Figure 5.7, panel c and d, red arrowheads), suggesting that *notch1* alone does not control the downregulation of *flk1* and *flt4* in the *Xenopus* DA.

Figure 5.6 Expression of endothelial and arterial genes is normal in *notch1* morphants

Notch1 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of endothelial markers, *flt1*, *tie2* and *ami*, and of arterial markers, *ephrinB2* and *cx37*, is normal in the DA of *notch1* morphants. Formation of the intersomitic vessels and the vitelline vasculature appears slightly affected by depletion of *notch1*. Red arrowheads mark dorsal aorta, green arrowheads mark the vein, orange arrowheads mark the notochord, blue arrowheads mark the vitelline vasculature and purple arrowheads mark intersomitic vessels.



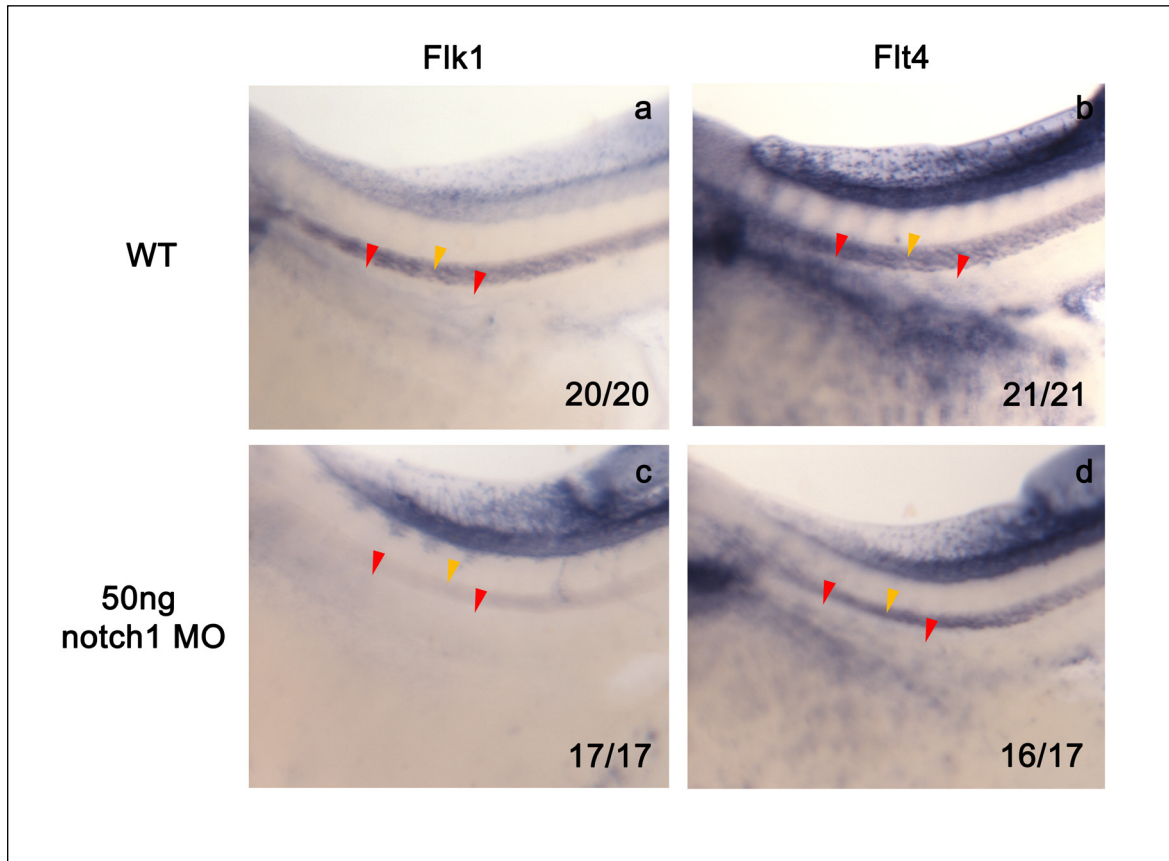


Figure 5.7 Expression of endothelial genes *flk1* and *flt4* is not maintained in *notch1* morphants

Notch1 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of *flk1* and *flt4* in the DA of *notch1* morphants is not maintained. Red arrowheads mark the location of the DA and orange arrowheads mark the notochord.

5.2.6. Primitive haematopoiesis is normal in *notch1* morphant embryos, and primitive erythrocytes circulate normally in the embryo

Expression of *notch1* has not been observed in the Xenopus VBI, and thus it was not expected to be involved in primitive haematopoiesis. Primitive haematopoiesis in *notch1*^{-/-} mice was found to be relatively normal, as analysed by colony forming cell (CFC) activity in the yolk sac (Kumano et al., 2003). Similarly, zebrafish embryos treated with notch inhibitor DAPT, a γ -secretase inhibitor which prevents cleavage of the notch receptor, or *mindbomb* mutant zebrafish, mutant for a protein essential for notch ligand maturation, showed no effect on primitive haematopoiesis (Burns et al., 2005; Gering and Patient, 2005). However, notch signalling has been implicated as a factor controlling apoptosis of primitive erythrocytes in the mouse yolk sac (Robert-Moreno et al., 2007).

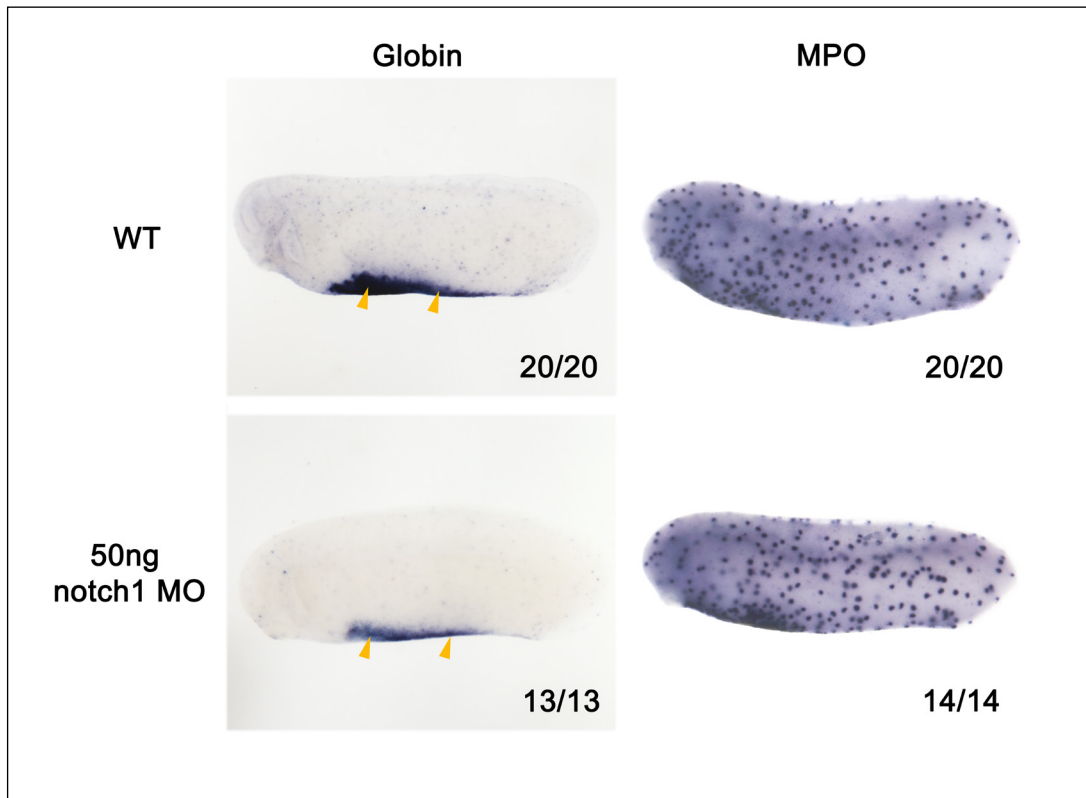
Expression analysis of endothelial markers, *flk1* and *egfl7*, in the VBI of *notch1* morphants has already been discussed (chapter 5.2.4), being found to be quite normal. Similarly, expression analysis of *scl* in primitive erythroid cells in the VBI demonstrates relatively normal expression in *notch1* morphants (Figure 5.5, panel d, orange arrowheads). To further analyse programming of the VBI in *notch1* morphants, expression analysis of *globin* was carried out in stage 28 embryos, and was found to be marginally reduced (Figure 5.8A, panel c, orange arrowheads). As expression of *scl* is relatively normal, but *globin* is reduced in *notch1* morphants, this suggests that the primitive erythroid cells are present in the VBI but are either delayed in their differentiation, or do not undergo full differentiation. In addition to primitive erythrocytes and vasculature, early primitive myeloid cell specification is unaffected, as demonstrated by the normal expression of *mpo* (Figure 5.8A, panel d). Therefore, *notch1* can be regarded as not essential for endothelial or myeloid development in the VBI, but is required for full, or timely, maturation of primitive erythroid cells.

Analysing the expression of primitive haematopoietic markers later in development can provide essential information about circulation in embryos. At stage 39, primitive erythrocytes can be seen circulating throughout wild type embryos, as analysed by the expression of *globin* and *gata-1* (Figure 5.8B, panels a and b, respectively). Erythrocytes can be seen in the dorsal aorta (red arrowheads), cardinal vein (green arrowheads), and can be seen within the heart (blue arrowheads). Similarly, in *notch1* morphants, erythrocytes are seen circulating in the embryo (Figure 5.8B, panels c and d), thus *notch1* is not required for the circulation of primitive erythrocytes. The similar expression patterns revealed for both *globin* and *gata1* in wild type and *notch1* morphant embryos at stage 39 suggests normal numbers of primitive erythrocytes in *notch1* morphant embryos. Thus, specification of primitive erythroid cells must occur in the VBI, and the reduced *globin* expression seen at stage 28 may be due to a delay in differentiation.

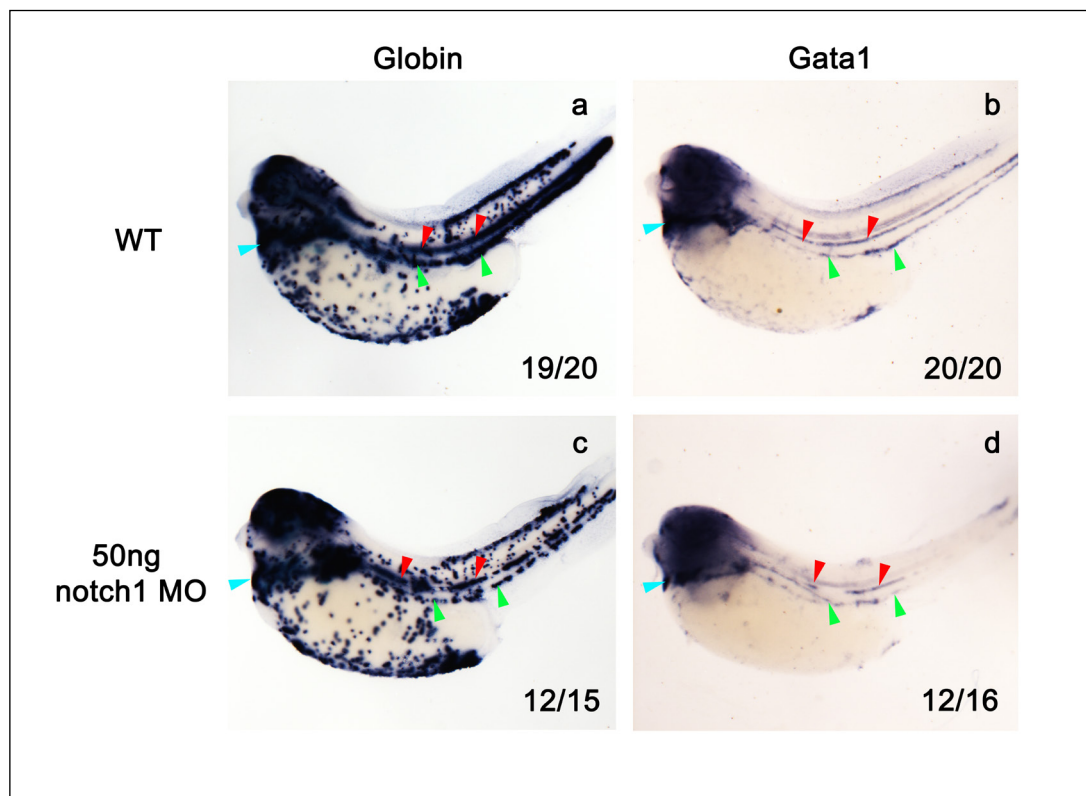
Figure 5.8 Primitive haematopoiesis is not affected by depletion of *notch1*, and primitive erythrocytes circulate relatively normally in the embryo

Notch1 MO was injected into both cells of two-cell embryos which were collected at stage 28 or 38-39 and analysed by *in situ* hybridisation. **A.** At stage 28, expression of *globin*, a marker of primitive erythrocytes, is reduced in *notch1* morphants, whilst expression of *mpo*, a marker of primitive myeloid cells, is normal. **B.** At stage 39, erythrocytes can be seen circulating throughout embryos. Red arrowheads mark the DA, green arrowheads mark the cardinal vein, orange arrowheads mark primitive erythrocytes in the VBI and blue arrowheads mark primitive erythrocytes in the heart.

A.



B.



5.2.7. *Notch1* is required for the expression of downstream hairy and enhancer of split genes

Genes from the hairy and enhancer of split related family are well documented as direct targets of notch signalling (see chapter 1.8.2). In the *Xenopus* DA, expression of five hairy and enhancer of split related genes has been identified. These five genes are activated in two waves, corresponding to the initiation of expression of two notch receptors (see chapter 3.5). Whilst *hesr2* is not dependent on notch signalling, expression of *esr7* and *esr10* is thought to be directly regulated by notch4 signalling (see chapter 4.6.2). In addition, *esr1* and *hesr1* have been demonstrated to be downstream of notch4 signalling. However, expression of both *esr1* and *hesr1* initiates immediately after *notch1* expression initiates, and are therefore candidate target genes of notch1 signalling.

In *notch1* morphant embryos, expression of *hesr1* is lost, and expression of *esr1* is downregulated in the *Xenopus* DA (Figure 5.9, panels d and e, respectively, red arrowheads), suggesting they are targets of *notch1*. In addition, expression of *notch4* is normal in *notch1* morphants (Figure 5.9, panel f, red arrowheads): as *notch1* expression is normal in *notch4* morphant embryos also (Figure 4.8), this suggests the two notch receptors act in parallel to programme HSCs.

5.3. *Esr1*

Like *esr7* and *esr10*, *esr1* is a WRPW-containing enhancer of split related gene expressed in the *Xenopus* DA (Figure 3.7), and a paralogue of mouse *hes5*. Expression of *esr1* initiates at stage 35, immediately after *notch1* expression initiates. As *hes5* is a direct target of notch signalling (Ohtsuka et al., 1999), and *esr1* is downregulated by in *notch1* morphants, it is expected that *esr1* is a direct notch target gene in the *Xenopus* DA.

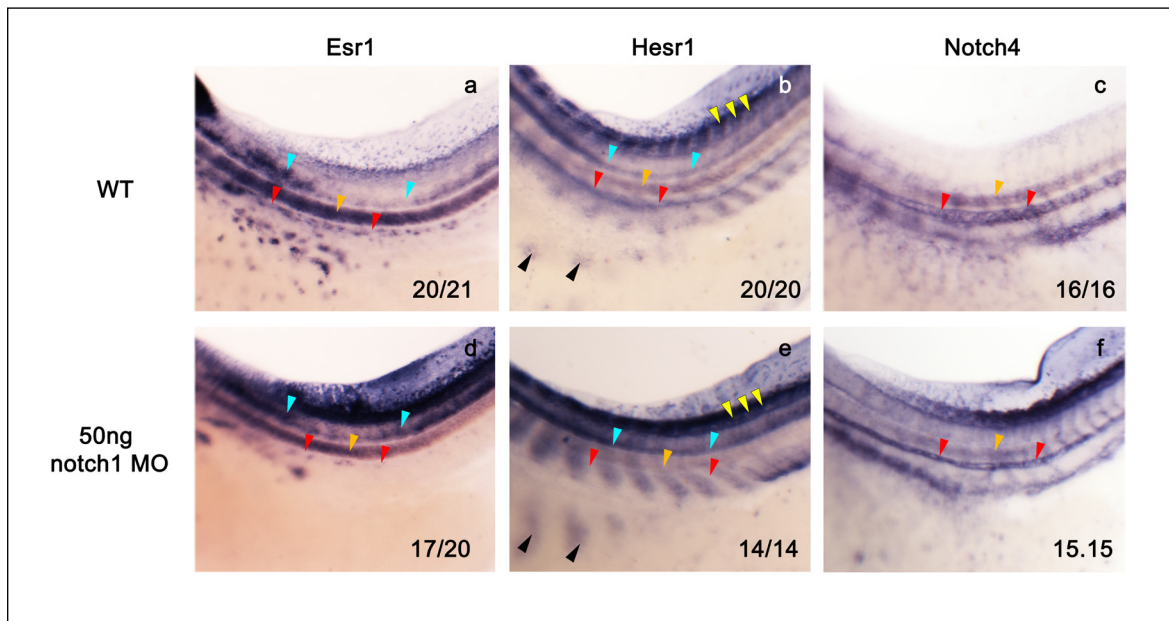


Figure 5.9 *Notch1* regulates the expression of hairy and enhancer of split genes in the DA

Notch1 MO was injected into both cells of a two-cell stage embryo, collected at stage 38-39, and analysed by *in situ* hybridisation. Expression of both *esr1* and *hes1* is significantly reduced in the DA of *notch1* morphants. However, expression of *notch4* is normal. Red arrowheads mark the DA, blue arrowheads mark the neural tube, orange arrowheads mark the notochord, black arrowheads mark migrating hypaxial muscle and yellow arrowheads mark the somites.

5.3.1. Defects in *esr1* morphant embryos

A morpholino targeting both *esr1* (unigene Xl.8440) and *esr1b* (unigene Xl.48575) has been published (Sakano et al., 2010) (Figure 5.10A, sequence can be found in Table 2.3). The morpholino is designed to bind to all 25 nucleotides of corresponding *esr1* sequence. When aligned against the *esr1b* sequence, two mismatches are found, but this is predicted by morpholino designers at Gene Tools to work with normal efficiency against both pseudoalleles. The *esr1* MO sequence was blasted against the *X.laevis* nucleotide collection to look for potential off-target effects, and no predicted targets other than *esr1/1b* were detected. To assess the effect of *esr1* depletion on *Xenopus*, embryos were injected at the 2-cell stage with 20-50ng *esr1* morpholino. At 50ng, embryos looked unhealthy, showing signs of non-specific toxicity during early development, including developmental delay, necrosis, and death from stage 20. Embryos injected with 20ng, 30ng, and 40ng looked healthy, showing no gross morphological defects (40ng shown in Figure 5.10B). Observation of *esr1* MO injected embryos at stage 39 revealed normally beating hearts but no circulation in the tail of the embryos. In wild type siblings, circulation was evident from stage 37. As no gross morphological defects were observed in embryos injected with 40ng *esr1* MO, this was chosen as the optimal dose for analysing *esr1* depletion in *Xenopus* embryos.

A.



B.

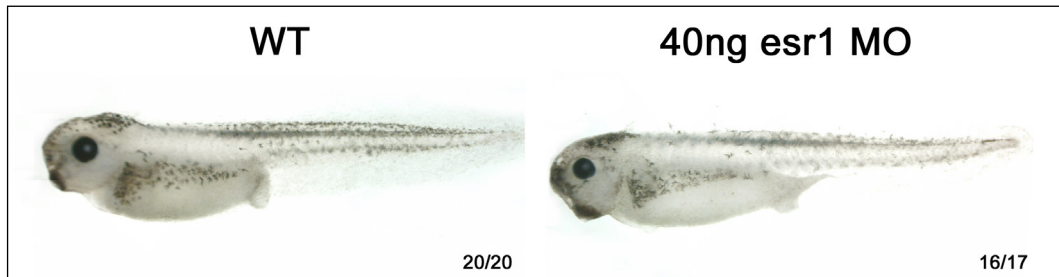


Figure 5.10 *Esr1* MO does not cause gross morphological defects

A. *esr1* MO (red) targets *esr1* and *esr1b* translation start site. Green highlights translation start site whilst blue highlights mismatches. **B.** Stage 39 *notch1* morphant embryos (right) do not show gross morphological defects when compared to wild type sibling controls (left).

5.3.2. *Esr1* morpholino inhibits translation of *esr1*

In order to confirm the ability of the *esr1* morpholino to bind to its target sequence, Gateway technology was employed to create an *esr1-gfp* construct containing the *esr1* morpholino binding site tagged with *gfp* (see section 2.1.4.11). Injection of 200pg *esr1-gfp* mRNA into the animal pole of 1-cell stage embryos generated a GFP signal, as visualised under a fluorescence microscope (Figure 5.2Biii), whilst co-injection of 200pg *esr1-gfp* mRNA with 40ng *esr1* MO produced no visible GFP signal (Figure 5.2Biv), suggesting *esr1* MO is capable of binding to the *esr1* MO target sequence and preventing translation. As a control, co-injection of 40ng *esr1* MO with 200pg *gfp* (Figure 5.2ii) elicited a strong GFP signal, comparable to that of 200pg *gfp* alone (Figure 5.2Bi), suggesting *esr1* MO does not bind non-specifically to *gfp*.

5.3.3. *Esr1* is required for haematopoietic stem cell programming

A role for *esr1* in embryonic haematopoietic stem cell programming has not been reported. Whilst a role for murine *hes5*, the orthologue of *esr1*, in B- and T-cell development has been reported (Varnum-Finney et al., 2008), earlier investigations of *hes5* in haematopoietic development have not been described. To investigate if there is a role for *esr1* in *Xenopus* haematopoietic stem cell development, stage 39 embryos injected with 40ng *esr1* MO were analysed by *in situ* hybridisation for the expression of HSC markers. Expression of putative HSC marker, *runx1*, was significantly downregulated in morphant embryos such that expression in the DA cannot be detected (Figure 5.12, panel d, red arrowheads). Expression of both *spiB* and *gfi1*, HSC markers downstream of *runx1*, is absent in *esr1* morphant embryos, as expected because of the loss of *runx1* expression (Figure 5.12, panels e and f, respectively, red arrowheads). Thus, *esr1* is required for haematopoietic stem cell programming in the *Xenopus* DA.

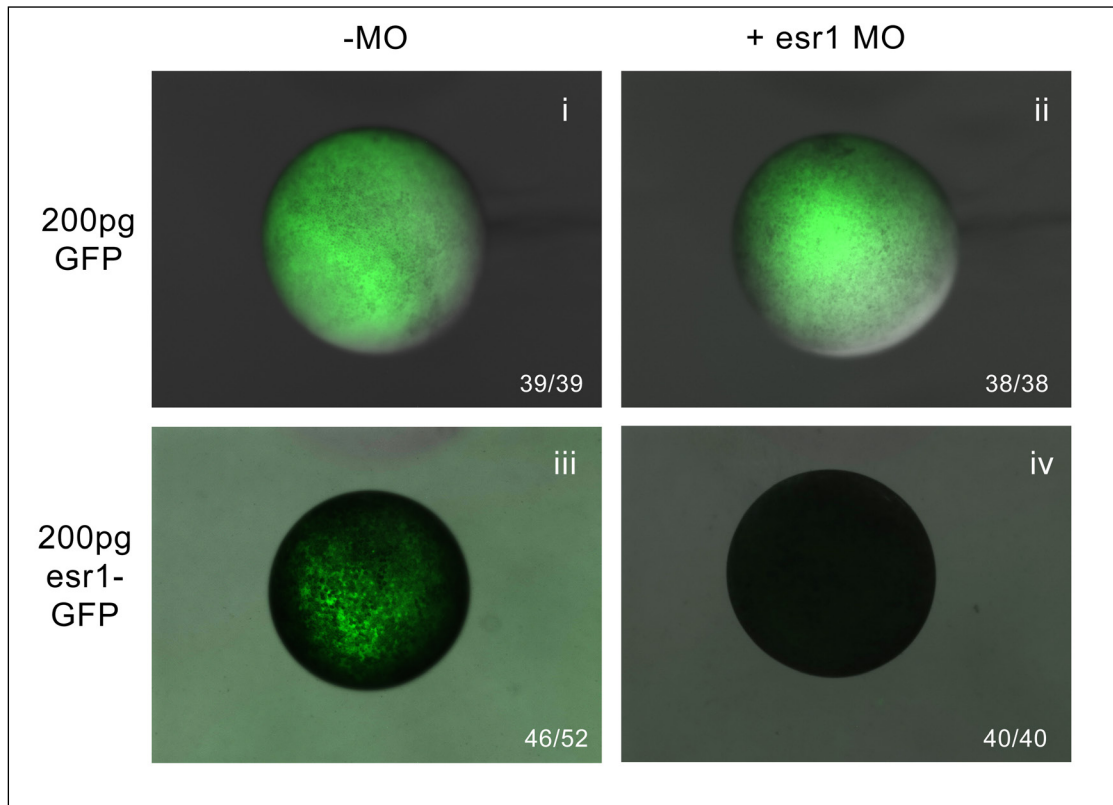
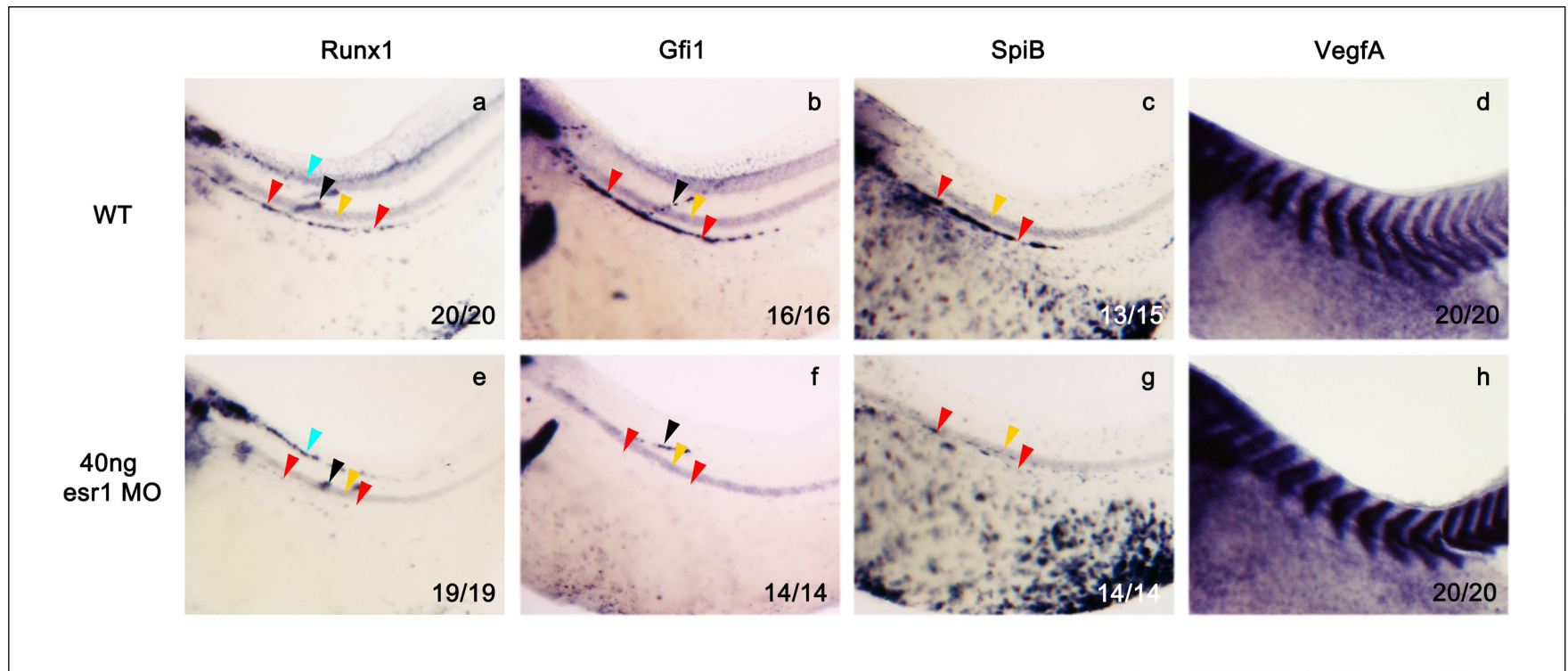


Figure 5.11 *esr1* MO is capable of preventing translation from *esr1* MO binding site

i) 200ng *gfp* mRNA generates a strong GFP signal. **ii)** 200ng *gfp* mRNA co-injected with 40ng *esr1* MO generates a strong signal. **iii)** 200ng *esr1-gfp* generates a weak signal. **iv)** 200ng *esr1-gfp* co-injected with 40ng *esr1* MO elicits no GFP signal.

Figure 5.12 *Esr1* is required for the expression of haematopoietic stem cell markers.

Esr1 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of HSC markers, *runx1*, *gfi1* and *spiB*, is lost in *esr1* morphant embryos when compared to WT sibling controls. Expression of *vegfa* in the somites is normal. Red arrowheads mark the dorsal aorta, blue arrowheads mark neural expression, black arrowheads mark the lateral line and orange arrowheads mark the notochord.



VEGF signalling from the somites to the DA is known to regulate the expression of *runx1* and haematopoietic genes downstream of *runx1* (Leung, 2010). As *esr1* is expressed in the presomitic mesoderm (Figure 3.8), it may have a role in somitogenesis. Expression analysis of *vegfa* in *esr1* morphant embryos reveals normal expression of *vegfa* in the somites, and relatively normal somitic patterning (Figure 5.12, panel h). Therefore, the requirement for *esr1* in HSC programming is restricted to the DA, and is not due to a somitic defect perturbing *vegfa* expression.

Similarly to the expression analysis of *gata2* in *notch4*, *esr10* and *notch1* morphants, *gata2* is not disrupted in *esr1* morphants at stage 32, but is disrupted at stage 39 (Figure 5.13, panel c and d, respectively, red arrowheads). This again points to two independent methods of *gata2* regulation in the *Xenopus* DA, dependent on developmental stage. Loss of haematopoietic markers was used as a positive control for all subsequent *esr1* MO morphant phenotypes.

5.3.4. Haemangioblast programming is unaffected by loss of *esr1*

Whilst *esr1* is not expressed in lateral mesoderm populations during early development and haemangioblast programming (Figure 3.8), injection of *esr1* MO from the 2-cell stage provides the possibility that early programming of the haemangioblast cells is compromised in *esr1* morphants. In order to investigate if *esr1* has a role in haemangioblast programming, stage 28 embryos injected with 40ng *esr1* MO were analysed for haematopoietic and endothelial markers in the DLP. Expression of haematopoietic marker *scf* in *esr1* morphant embryos is relatively normal in the DLP (Figure 5.14, panel d, red arrowheads). Expression of endothelial markers is also normal in the DLP: *flk1*, a VEGF receptor required for haemangioblast cell migration to the midline, is expressed normally in *esr1* morphant embryos (Figure 5.14, panel e, red arrowheads).

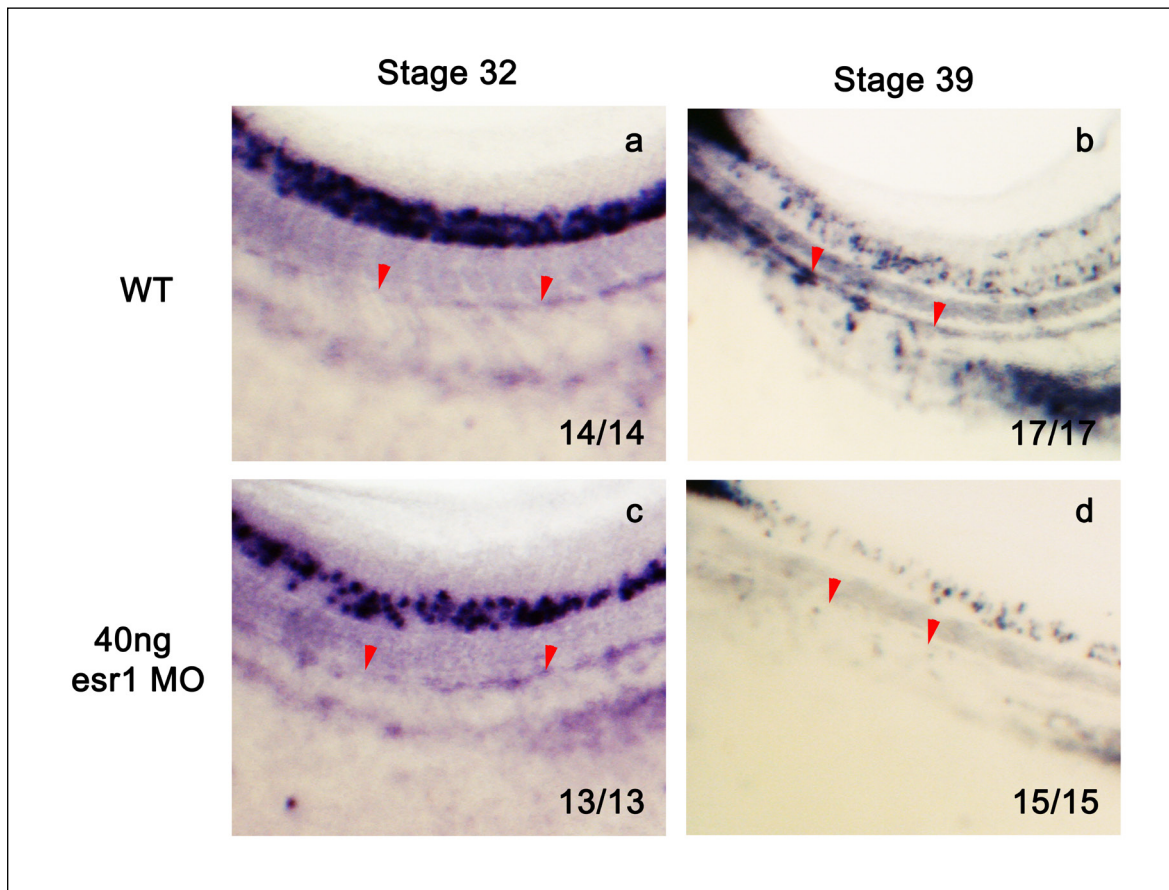


Figure 5.13 Depletion of *esr1* reduces *gata2* expression at stage 39

Esr1 MO was injected into both cells of two-cell embryos which were collected at stage 32 or 38-39 and analysed by *in situ* hybridisation. *Gata2* expression initiates normally in *esr1* morphant embryos, as evident by expression in the DA at stage 32, but is not maintained, as evident by the loss of expression at stage 39. Red arrowheads mark the DA.

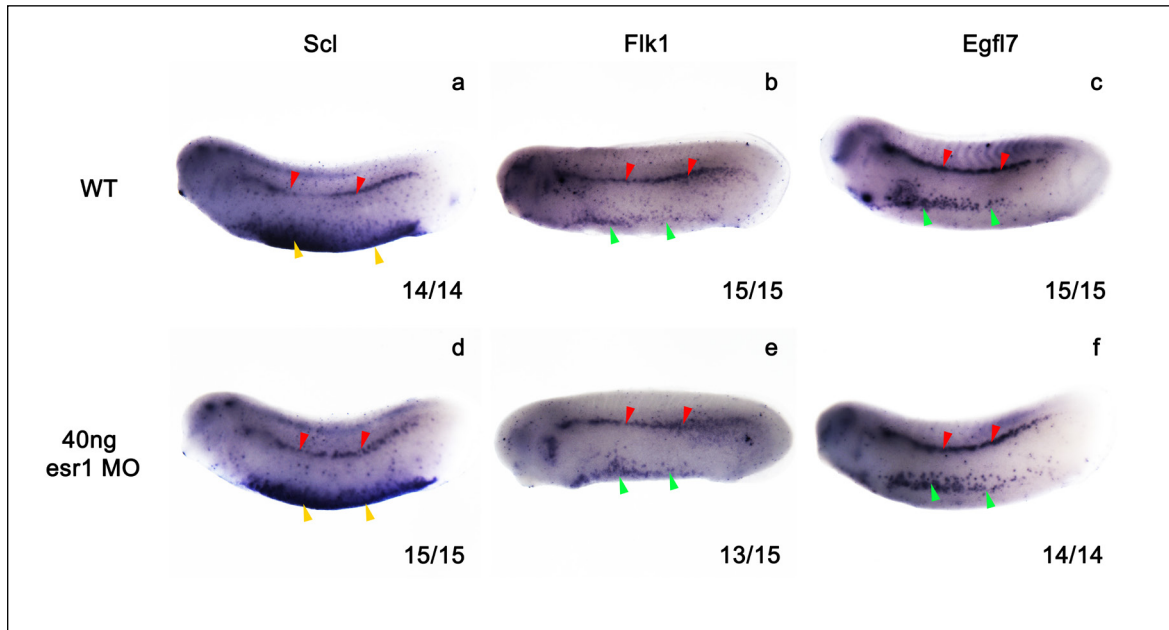


Figure 5.14 Programming of the haemangioblast is normal in *esr1* morphants

Esr1 MO was injected into both cells of two-cell embryos which were collected at stage 27-28 and analysed by *in situ* hybridisation. Expression of haematopoietic marker *scl*, and endothelial markers *flk1* and *egfl7*, is unaffected in *esr1* morphants. Expression can be seen in the DLP (red arrowheads), developing primitive erythrocytes in the VBI (orange arrowheads) and in the developing vitelline vessels (green arrowheads).

Similarly, endothelial marker *egfl7* is also expressed normally in the DLP of *esr1* morphant embryos (Figure 5.14, panel f, red arrowheads), suggesting the genetic regulatory network upstream of *egfl7* is also programmed normally.

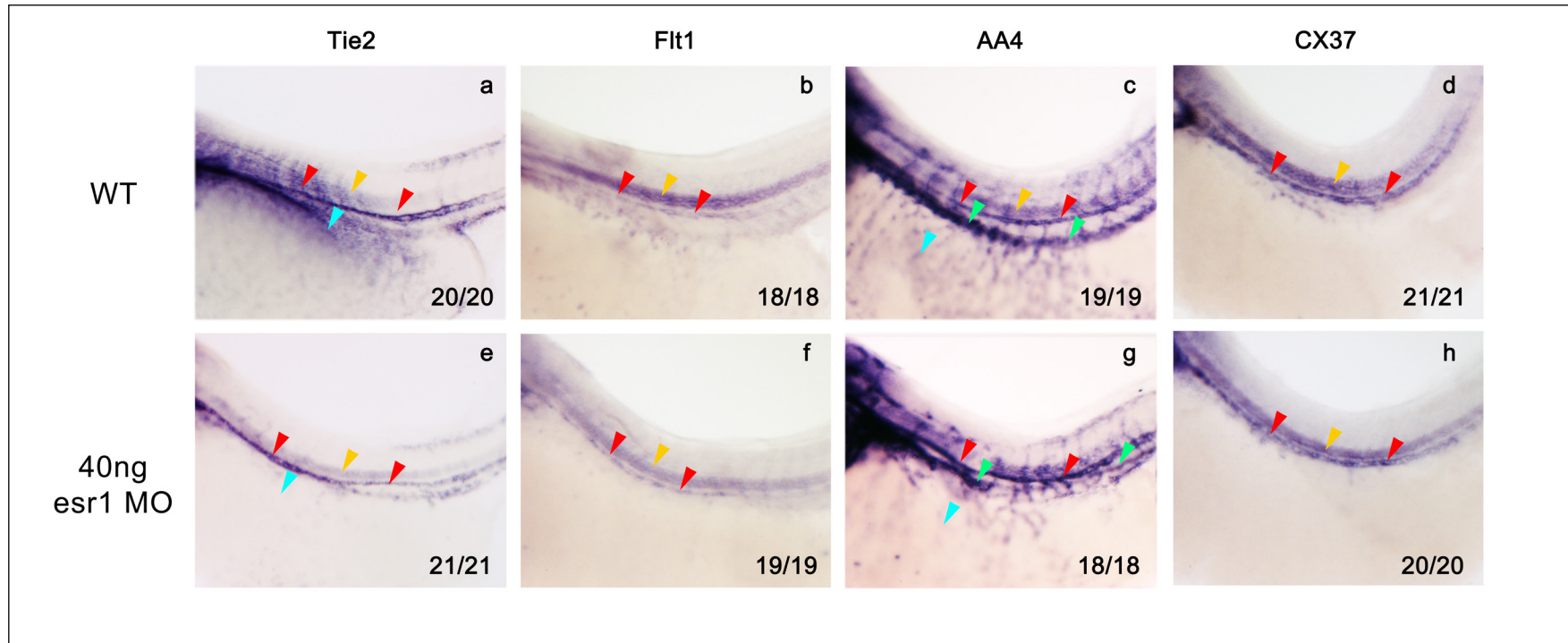
Expression of *scl*, *flk1* and *egfl7* can also be seen in the VBI at this stage. *Scl* marks haematopoietic cells, and is expressed in the developing primitive erythrocytes of the VBI. Expression of *scl* is normal in the VBI of *esr1* morphants when compared to wild type sibling controls (Figure 5.14, panels d and a respectively, orange arrowheads), suggesting that primitive haematopoiesis is unaffected by a loss of *esr1*. Similarly, expression of endothelial markers, *flk1* and *egfl7*, in the VBI is relatively unaffected in *esr1* morphants (Figure 5.14, panels e and f, respectively, green arrowheads).

5.3.5. Endothelial and dorsal aorta specification is unaffected by loss of *esr1*, but *flk1* and *flt4* expression is maintained

A role for *esr1*, or its orthologue *hes5*, in vascular programming has not been reported. In order to understand if the haematopoietic defect found in *esr1* *Xenopus* morphants is the result of an *esr1* effect on HSC programming, it is essential to see if there is an earlier vascular programming defect. To investigate the role of *esr1* in *Xenopus* vascular and arterial development, stage 39 embryos injected with 40ng *esr1* MO were analysed by *in situ* hybridisation for the expression of endothelial and arterial markers. Expression of endothelial markers, *tie2*, *flt1* and *AA4*, revealed normal expression in the DA of *esr1* morphants (Figure 5.15, panels e, f and g, respectively, red arrowheads). Similarly, expression of arterial marker, *cx37*, is also normal in the DA of *esr1* morphant embryos (Figure 5.15, panel h, red arrowheads).

Figure 5.15 Expression of endothelial and arterial genes is normal in *esr1* morphants

Esr1 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of endothelial markers, *tie2*, *flt1*, and *AA4*, and of arterial marker, *cx37*, is normal in the DA of *esr1* morphants. Formation of the vitelline vasculature appears slightly affected by depletion of *esr1*. Red arrowheads mark dorsal aorta, green arrowheads mark the vein, orange arrowheads mark the notochord, and blue arrowheads mark the vitelline vasculature.



However, in the *esr1* morphants, expression of *tie2* and *aa4* in the vitelline vessels is slightly reduced, suggesting *esr1* has a role in the development of the vitelline vasculature (Figure 5.15, panels e and g, respectively, blue arrowheads).

Esr1 contains both a bHLH and an orange domain, and thus could potentially mediate the repression of *flk1* and *flt4* in the DA. To analyse this, expression analysis of *flk1* and *flt4* was carried out in stage 39 *esr1* morphants. At stage 39, expression of *flk1* and *flt4* has been downregulated, and cannot be seen in the DA of wild type embryos (Figure 5.16, panel a and b, respectively, red arrowheads). In *esr1* morphant embryos, expression of *flk1* and *flt4* can be seen in the DA at stage 39, and thus expression is not being repressed in the DA (Figure 5.16, panel c and d, respectively, red arrowheads). This suggests that *esr1* has a role in the repression of initiator-containing, TATA-less, endothelial genes in the DA

5.3.6. Primitive haematopoiesis is normal in *esr1* morphant embryos, but primitive erythrocytes do not circulate normally

Expression of *esr1* has not been observed in the VBI, and therefore should not be involved in primitive haematopoiesis (Figure 3.8). Furthermore, a role for *esr1*, or its orthologue *hes5*, in primitive haematopoiesis has not been reported. Expression analysis of endothelial markers, *flk1* and *egfl7*, in the VBI of stage 28 *esr1* morphants has shown normal expression of vitelline vasculature markers (Figure 5.14, panels e and f, respectively, green arrowheads). Similarly, expression of *scl* in the VBI of stage 28 *esr1* morphant embryos reveals development of normal primitive erythroid cells (Figure 5.14, panel d, orange arrowheads). To further analyse primitive haematopoiesis programming in *Xenopus esr1* morphants, expression analysis of *globin* was carried out in stage 28 embryos, when primitive erythroid cells are being specified.

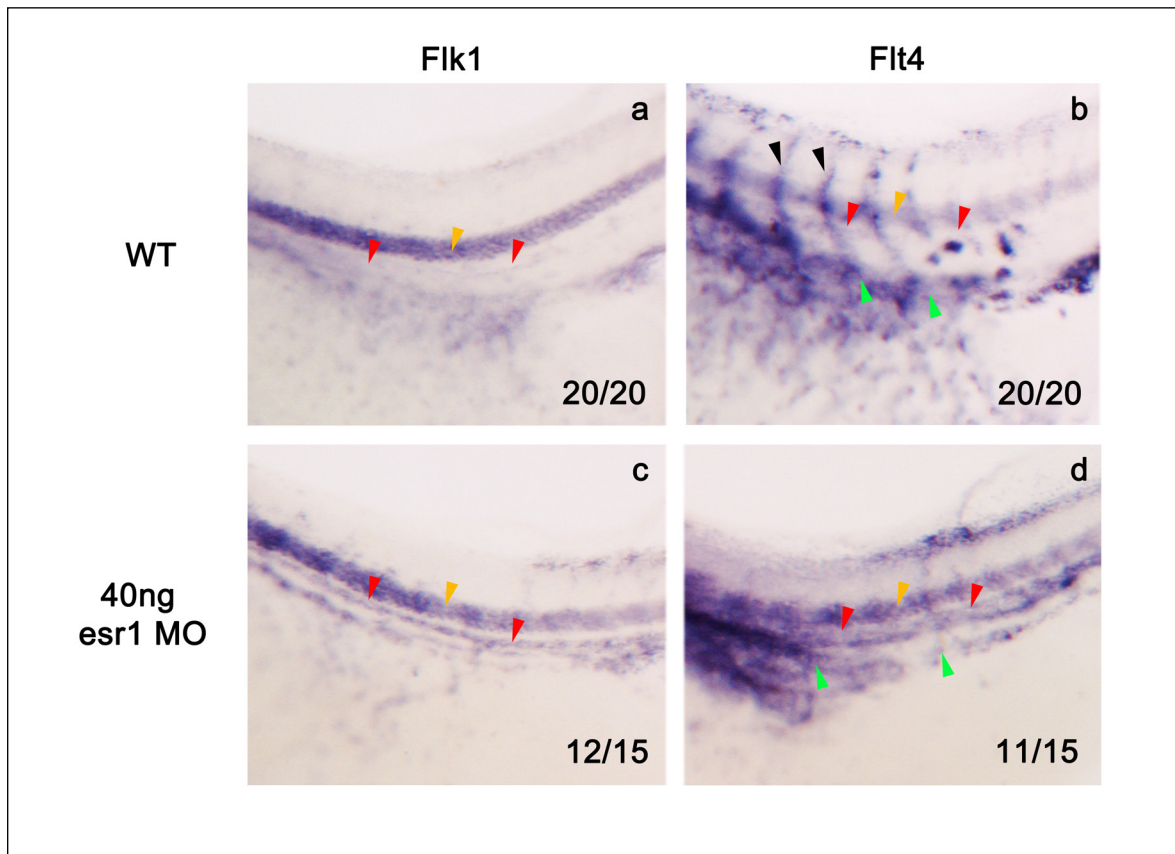


Figure 5.16 Expression of endothelial genes *flk1* and *flt4* is maintained in the DA of *esr1* morphant embryos

Esr1 MO was injected into both cells of a two-cell embryo which were collected at stage 39, and analysed by *in situ* hybridisation. Expression of both *flk1* and *flt4* in the DA of *esr1* morphants is maintained, whilst it is repressed in the DA of wild type embryos. Red arrowheads mark the DA, green arrowheads mark the vein, orange arrowheads mark the notochord and black arrowheads mark the intersomitic vessels.

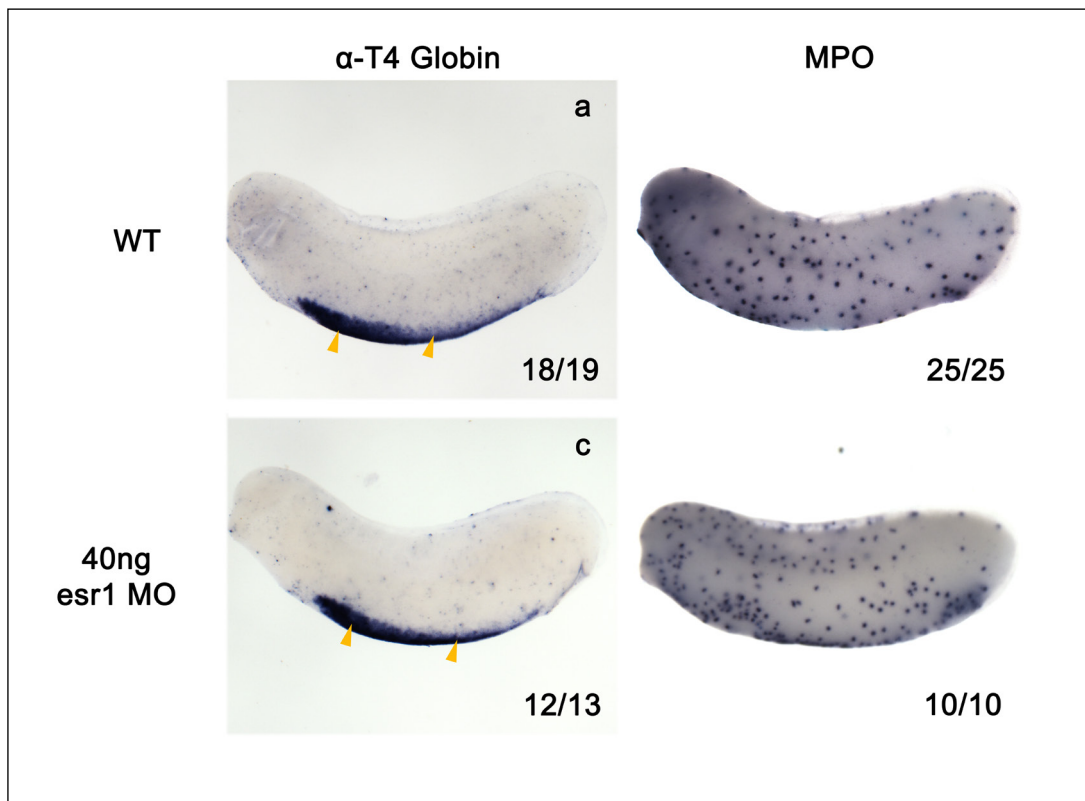
Expression of *globin* was found to be normal in *esr1* morphant embryos when compared to WT siblings, and thus primitive haematopoiesis appears to be unaffected by a loss of *esr1* (Figure 5.17A, panels a and c, respectively). In addition to the primitive erythrocytes, early primitive myeloid cells are also specified normally in the VBI, as analysed by expression of *mpo* (Figure 5.17A, panel d). Thus, *esr1* is not essential for primitive haematopoiesis at stage 28.

Analysing the expression of primitive haematopoietic markers later in development can provide essential information about circulation in embryos. At stage 39, primitive erythrocytes can be seen circulating throughout wild type embryos, as analysed by the expression of *globin* and *gata-1* (Figure 5.17B, panels a and b, respectively). Primitive erythrocytes can be seen in the dorsal aorta (red arrowheads), cardinal vein (green arrowheads), and within the heart (blue arrowheads). However, in *esr1* morphants, primitive erythrocytes are seen pooling in the VBI (Figure 5.17B, panels c and d, orange arrowheads). This is consistent with the observation that blood cells are not seen circulating through the tail in living stage 39 *esr1* morphant embryos (chapter 5.3.1). As the heart is beating normally in these embryos, the lack of circulation is likely due to a vascular defect. A reduced number of vitelline vessels are seen in *esr1* morphants stained for endothelial markers, *tie2* and *aa4*, at stage 39 (Figure 5.15, panels e and g, blue arrowheads). Connection of the VBI to the rest of the circulatory network through the vitelline vasculature is essential to allow primitive erythrocytes to circulate throughout the embryo, and thus this reduced number of vitelline vessels may account for the lack of circulation. Alternatively, it is possible that *esr1* is required for the lumenisation of vessels. If lumens are not formed in *esr1* morphants, primitive erythrocytes would not be able to circulate through the vessels. In order to determine if *esr1* is required for lumenisation of the *Xenopus* DA, *in situ* hybridisation on sections would need to be carried out, whereby the vessel diameter can be studied.

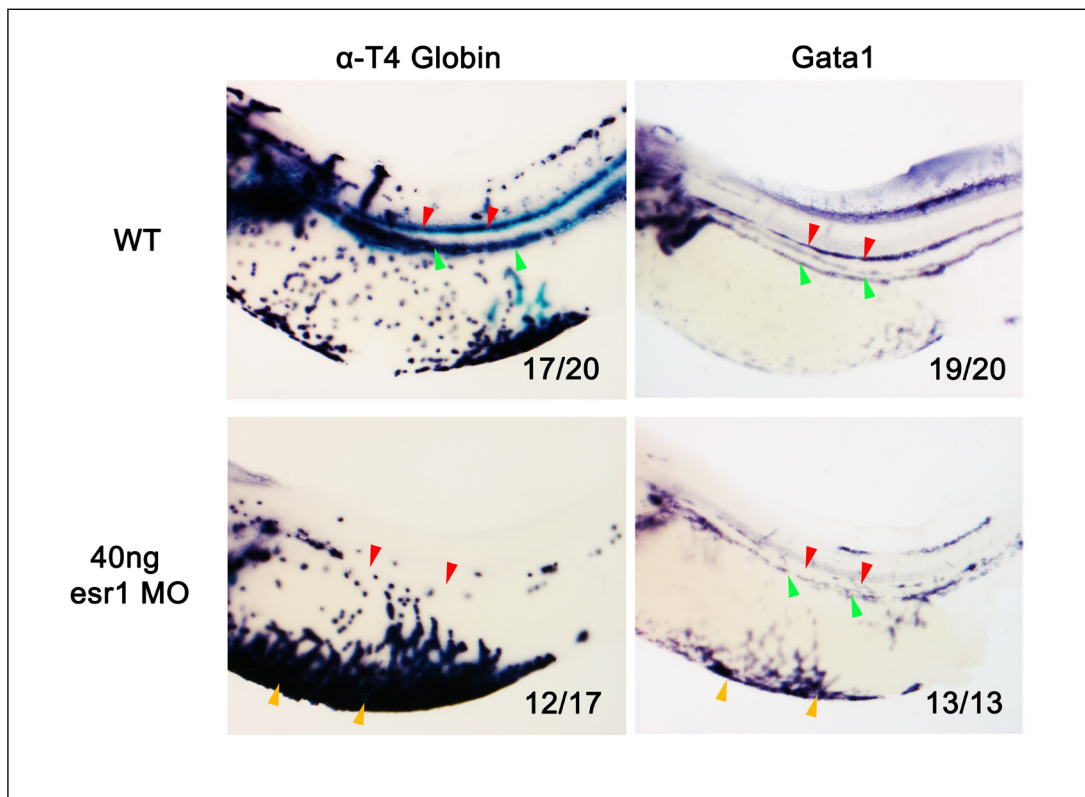
Figure 5.17 Primitive haematopoiesis is not affected by depletion of *esr1*, but primitive blood cells do not circulate in an *esr1* deficient embryo.

Esr1 MO was injected into both cells of two-cell embryos which were collected at stage 28 or 38-39 and analysed by *in situ* hybridisation. **A.** At stage 28, expression of α -T4 globin, a marker of primitive erythrocytes, is normal in *esr1* morphants, as is *mpo*, a marker of primitive myeloid cells. **B.** At stage 39, erythrocytes can be seen circulating throughout wild type embryos, but circulating cells are not evident in *esr1* morphants. Red arrowheads mark the DA, green arrowheads mark the cardinal vein, orange arrowheads mark primitive erythrocytes in the VBI and blue arrowheads mark the heart.

A.



B.



Blood flow is known to be required for the maintenance of HSC markers in the zebrafish DA from 36hpf, after initial cluster emergence, without affecting initiation of *runx1* in the DA (North et al., 2009; Wang et al., 2011). The absence of blood flow in *esr1* morphant embryos is not thought to be a contributing factor to the loss of HSC markers at stage 39, for the same reasoning loss of HSC markers in *notch4* morphants is not thought to be due to loss of circulation (see chapter 4.2.5).

5.3.7. *Esr1* is required for the expression of downstream hairy and enhancer of split gene, *hesr1*

Five genes from the hairy and enhancer of split related family are expressed in the DA, with four of these seeming to be notch target genes, as analysed by depletion of *notch4* and *notch1* signalling (chapters 4.2.6, 4.3.1 and 5.2.7). Expression analysis in *notch1* morphants has revealed *esr1* and *hesr1* to be downstream of *notch1* signalling. To determine if these genes interact at a genetic level, expression analysis of *hesr1* was carried out in embryos injected with 40ng *esr1* MO. Expression of *hesr1* is lost in *esr1* morphant embryos, suggesting *esr1* acts to regulate *hesr1* expression in the DA (Figure 5.18, panel c, red arrowheads). In addition, expression of *notch4* is normal in *esr1* morphant embryos, further demonstrating that the earlier programming of the DA in *esr1* morphants is normal (Figure 5.18, panel d, red arrowheads).

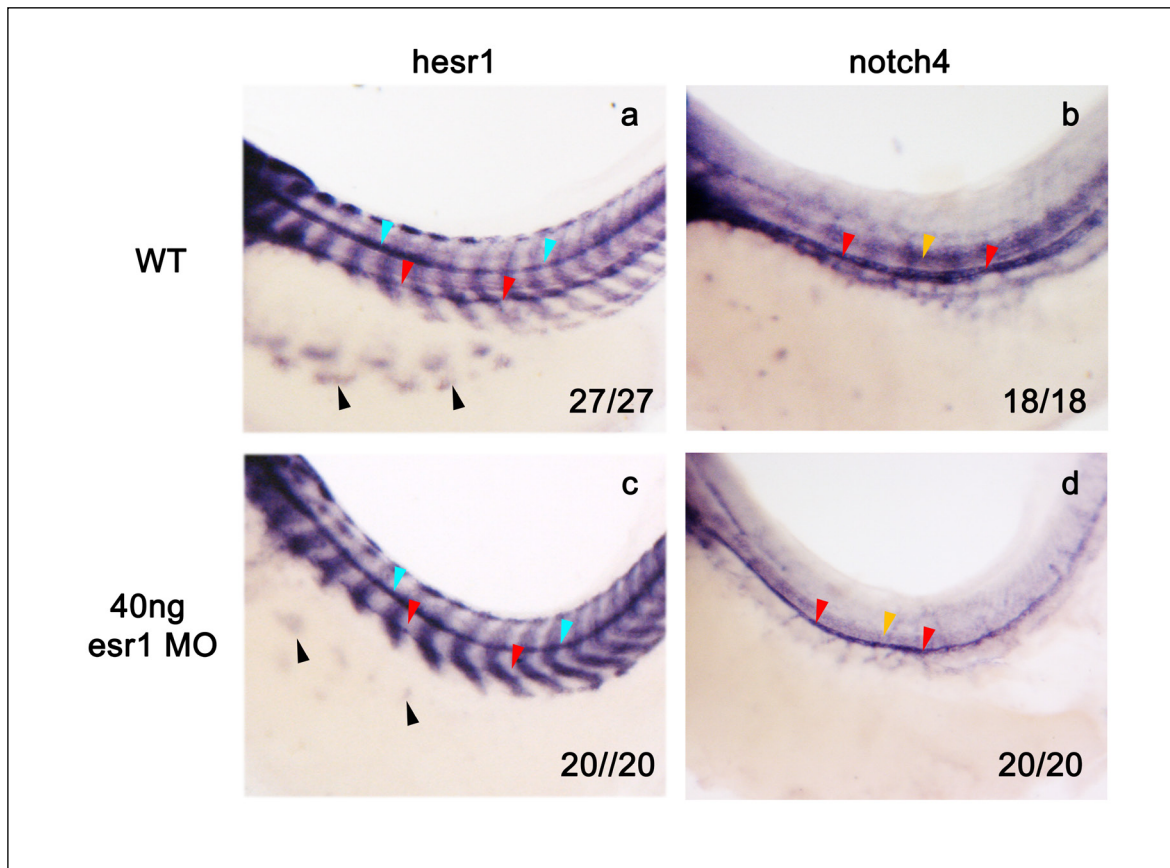


Figure 5.18 *Esr1* regulates the expression of *hesr1* in the DA, but does not regulate *notch4*

Esr1 MO was injected into both cells of two-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of *hesr1* is significantly reduced in the DA of *esr1* morphants, suggesting that *esr1* acts upstream of *hesr1* to regulate its expression. However, expression of *notch4* is normal. Red arrowheads mark the DA, blue arrowheads mark the neural tube, orange arrowheads mark the notochord and black arrowheads mark migrating hypaxial muscle.

5.4. *Hesr1*

Hesr1 is a YRPW-containing hairy and enhancer of split related gene and is expressed in the Xenopus DA immediately after *notch1* expression initiates (Figure 3.11). As *hesr1* is a member of a large family of genes mostly thought to act downstream of notch signalling, *hesr1* was expected to be a notch target gene in the Xenopus DA also. Expression analysis in *notch4* and *notch1* morphant embryos reveals *hesr1* is downstream of notch, in agreement with a recent publication demonstrating *hesr1* is a notch target gene in haematopoietic cells (Hamidi et al., 2011).

5.4.1. Defects in *hesr1* morphant embryos

A morpholino targeting *hesr1* and *hesr1b* has been tested and published (Taelman et al., 2006), and so this was used for *hesr1* studies in this thesis (see Table 2.3 and Figure 5.19A for sequence). To assess the effect of *hesr1* depletion in Xenopus, embryos were injected at the 2-cell stage with 10-40ng *hesr1* morpholino. At all concentrations injected, embryos looked unhealthy, showing signs of toxicity during tailbud stage, including developmental delay, and oedema.

All embryos began to die at stage 30-32. As *hesr1* is strongly expressed in other areas of the embryo in addition to the DA, including strong somitic expression, this toxicity of the morpholino was thought to be due to patterning defects in non-DA tissues. Injection of 15ng *hesr1* MO into 1 cell of the two-cell embryo was able to replicate published studies (Taelman et al., 2006; and Figure 5.19B), and embryos were able to survive past stage 32, suggesting that the morpholino used was acting as published. Injection into the two ventral blastomeres of the four-cell stage embryo (ventral marginal zone: VMZ) is known to target the DLP, and subsequently the DA (Ciau-Uitz et al., 2000). Injection of *hesr1* into the VMZ was carried out at 15ng, 20ng and 25ng. Embryos injected with 25ng *hesr1* MO developed a shortened anteroposterior axis and developmental delay. Embryos injected with 15ng and 20ng looked healthy, showing no gross morphological defects (20ng shown in Figure 5.19C). Observation of *hesr1* MO injected embryos

at stage 39 revealed normally beating hearts and normal circulation of erythrocytes in the tail. As no gross morphological defects were observed in embryos injected with 20ng *hesr1* MO, this was chosen as the optimal dose for analysing *hesr1* depletion in *Xenopus* embryos.

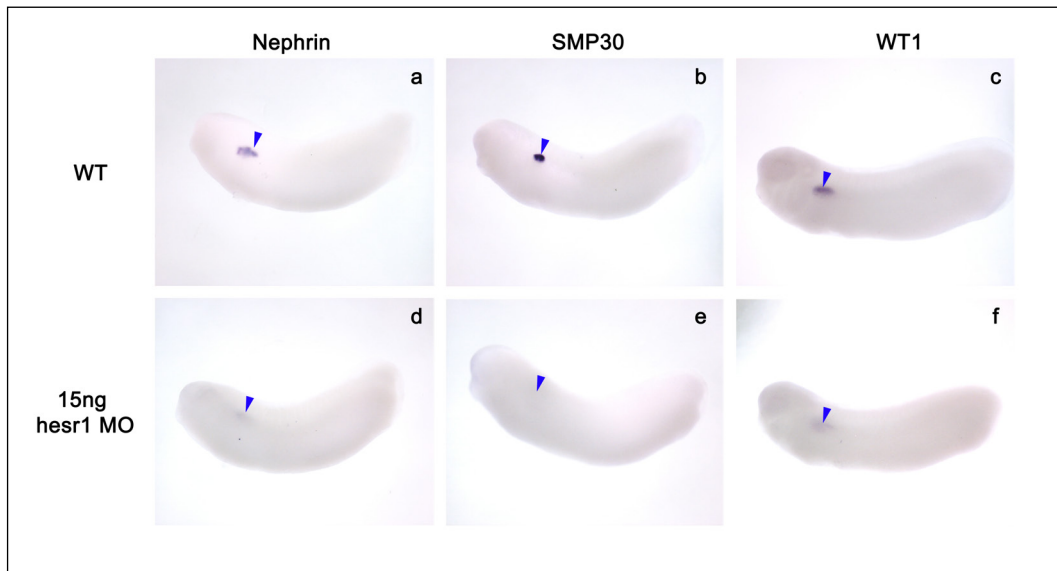
5.4.2. *Hesr1* morpholino inhibits the translation of *hesr1*

In order to confirm the ability of the *hesr1* morpholino to bind to its target sequence, Gateway technology was employed to create a *hesr1-gfp* construct containing the *hesr1* morpholino binding site tagged with *gfp* (see section 2.1.4.11). Injection of 200pg *hesr1-gfp* mRNA into the embryo generated a GFP signal, as visualised under a fluorescence microscope (Figure 5.20iii), whilst co-injection of 200pg *hesr1-gfp* mRNA with 20ng *hesr1* MO produced no visible GFP signal (Figure 5.20iv), suggesting *hesr1* MO is capable of binding to the *hesr1* MO target sequence and preventing translation. As a control, co-injection of 20ng *hesr1* MO with 200pg *gfp* (Figure 5.20ii) elicited a strong GFP signal, comparable to that of 200pg *gfp* alone (Figure 5.20i), suggesting *hesr1* MO does not bind non-specifically to *gfp*.

A.



B.



C.

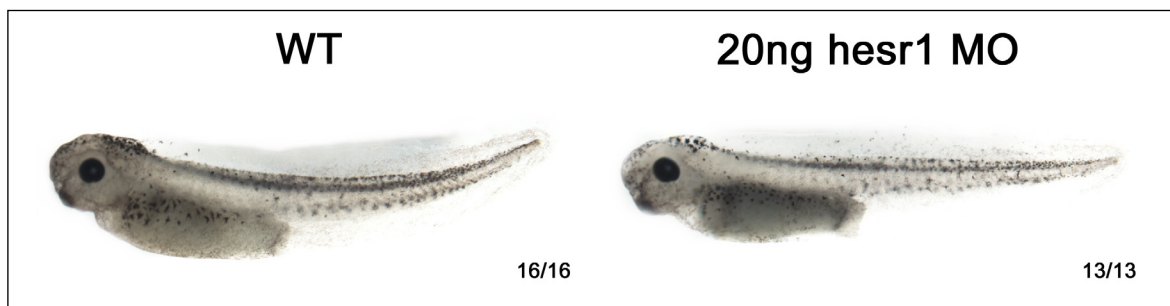


Figure 5.19 *Hesr1* MO does not cause gross morphological defects

A. Schematic diagram of *hesr1* MO (red) next to *hesr1a* and *hesr1b* mRNA sequence (black). The morpholino binds in the 5' UTR and the translation start site is found 3' to this sequence. **B.** Injection of 15ng *hesr1* MO into one cell of a two-cell embryo results in loss of pronephric markers on the corresponding side, in agreement with published reports (Taelman et al., 2006). Blue arrowheads mark the pronephros **C.** Stage 39 *hesr1* morphant embryos (right) show no gross morphological defects when compared to wild type sibling controls (left).

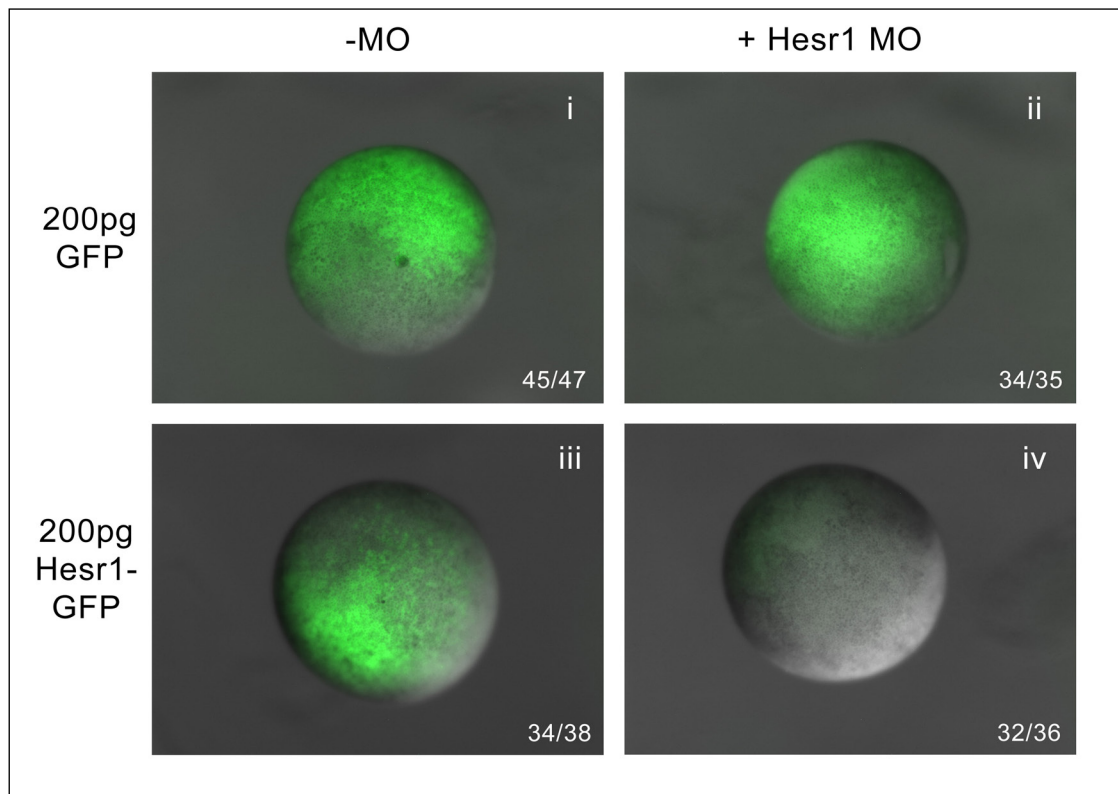


Figure 5.20 *hesr1* MO is capable of preventing translation from *hesr1* MO binding site

i) 200ng *gfp* mRNA generates a strong GFP signal. ii) 200ng *gfp* mRNA co-injected with 20ng *hesr1* MO generates a strong signal. iii) 200ng *hesr1-gfp* generates a strong signal. iv) 200ng *hesr1-gfp* co-injected with 25ng *hesr1* MO generates little to no GFP signal.

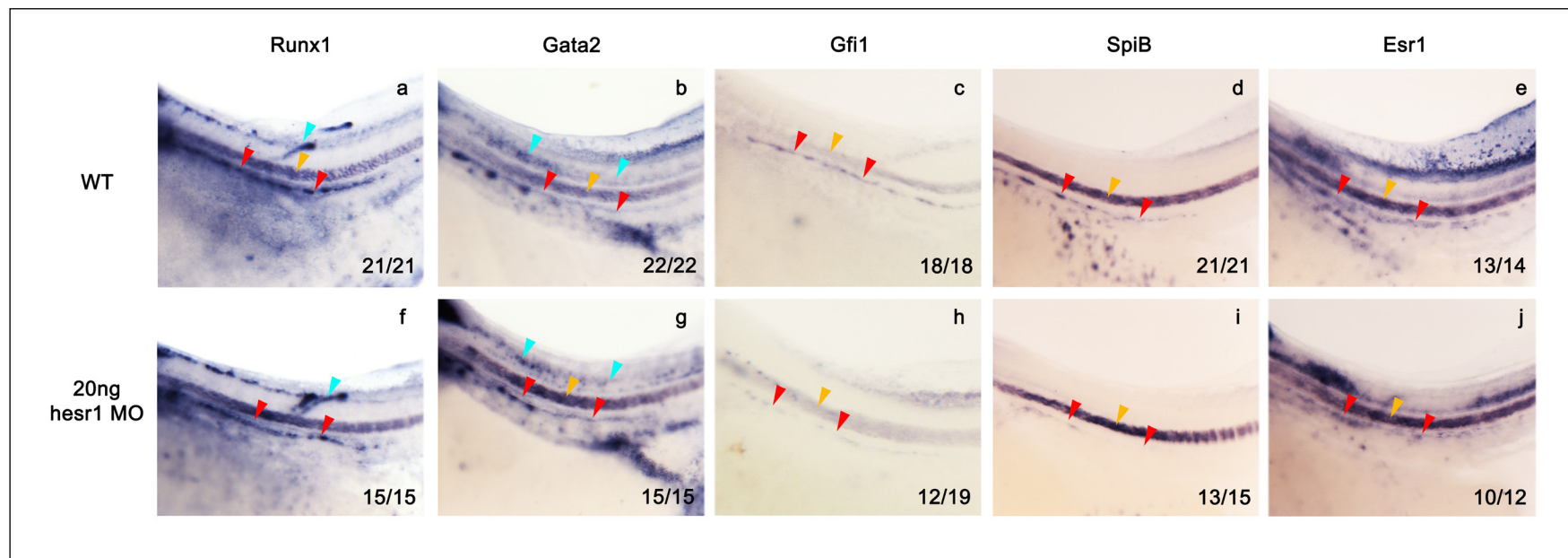
5.4.3. *Hesr1* is required for haematopoietic stem cell programming downstream of *runx1*

A role for *hesr1* in HSC specification has not been described. As *hesr1* is expressed in the DA downstream of *notch4*, *esr7*, *esr10*, *notch1* and *esr1*, which are all required for HSC programming, it is possible that *hesr1* is also required for HSC development. In order to investigate if *hesr1* has a role in HSC specification, stage 39 embryos injected with 20ng *hesr1* MO were analysed by *in situ* hybridisation for the expression of HSC markers. Expression of HSC marker, *runx1*, and its upstream regulator, *gata2*, was found to be normal in the DA of *hesr1* morphant embryos (Figure 5.21, panels f and g, respectively, red arrowheads). However, expression of HSC markers, *gfi1* and *spiB*, which are known to be downstream of *runx1*, was severely reduced (Figure 5.21, panels h and i, respectively, red arrowheads). This suggests that *hesr1* acts to specify the HSC programme downstream of, or in parallel to, *runx1*.

As previously discussed, VEGFA signalling from the somites is known to be essential for both DLP and DA specification (Ciau-Uitz et al., 2010b; Leung, 2010). As *hesr1* is expressed strongly in the somites, it is possible that a somitic defect caused by downregulation of *hesr1* could abrogate VEGF signalling from the somites to the DLP and/or DA. In cases where VEGFA signalling has been compromised in the somites and has therefore affected DLP or DA programming, expression of *runx1* in the DA has been absent (Ciau-Uitz et al., 2010b; Leung, 2010). As *runx1* expression is normal in the DA in *hesr1* morphants, this provides evidence of normal VEGFA signalling to both the DLP and the DA. However, expression analysis of VEGFA in *hesr1* morphants should be carried out in order to definitely say that somitic *vegfa* expression is normal.

Figure 5.21 *Hesr1* is required for the expression of haematopoietic stem cell markers downstream of *runx1*

Hesr1 MO was injected into the two VMZ cells of four-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of *runx1* and *gata2* is relatively normal in *hesr1* morphants, whilst expression of *gfi1* and *spiB*, HSC markers downstream of *runx1*, is severely reduced. Expression of *esr1* is normal. Red arrowheads mark the DA, orange arrowheads mark the notochord and blue arrowheads mark neural expression.



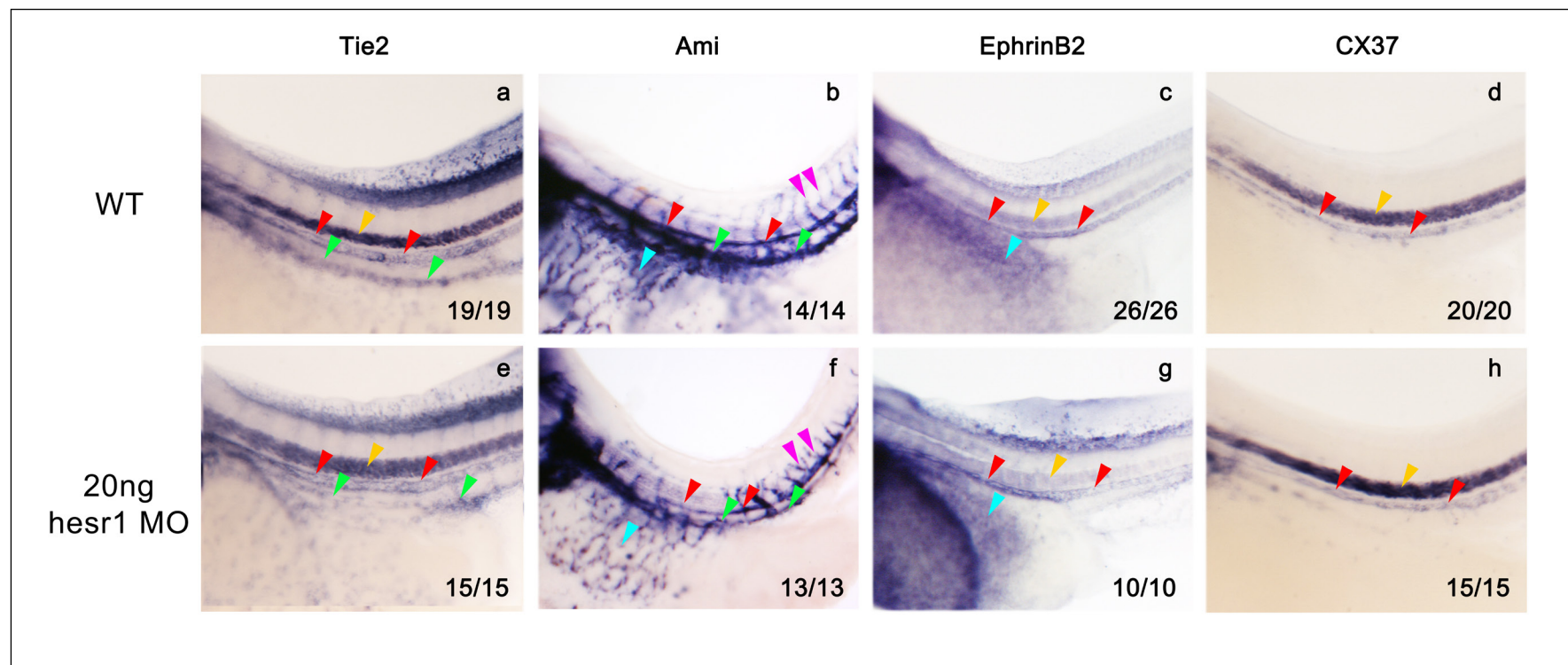
Both *esr1* and *hesr1* are downstream of *notch1* (Figure 5.9), and *esr1* regulates the expression of *hesr1* (Figure 5.18). In order to see if there is reciprocal regulation of *esr1* by *hesr1*, expression analysis was carried out for *esr1* in *hesr1* morphant embryos. This expression was found to be relatively normal, if slightly reduced (Figure 5.21, panel j, red arrowheads), suggesting that *hesr1* acts downstream of *esr1*.

5.4.4. Endothelial and dorsal aorta specification is unaffected by loss of *hesr1*

Hesr1 is known to have a role in yolk sac remodelling and embryonic vascular angiogenesis, although *hesr1* is redundant to *hesr2* in this role (Fischer et al., 2004). Whilst *hesr1*^{-/-} embryos were found to be viable with no discernible phenotype, *hesr1/hesr2* double knockout mice were found to have a greater phenotype than *hesr2*^{-/-} mice alone, and similar to that of *notch1*^{-/-} embryos. Initial vasculogenesis was found to be quite normal in the mutant embryos as endothelial cells were found in the normal location of the paired DA and PCVs. However, the vessels were much smaller, arterial programming was not initiated (analysed by expression of *ephrinB2*) and recruitment of smooth muscle cells was reduced, leading to vascular haemorrhage (Fischer et al., 2004). To investigate if *hesr1* is required for DA programming in *Xenopus*, stage 39 embryos injected with 20ng *hesr1* MO were analysed by *in situ* hybridisation for the expression of endothelial and arterial markers. Endothelial markers, *tie2* and *ami*, are expressed fairly normally in the DA of *hesr1* morphant embryos (Figure 5.22, panels e and f, red arrowheads). Expression of *ami* seems marginally weaker in the DA of morphant embryos when compared to wild type (Figure 5.22, panels f and b, respectively, red arrowheads). This could suggest an endothelial programming defect, or may be artifactual. The observation that *tie2* is expressed normally in the DA would suggest that the endothelial programme is intact at this stage.

Figure 5.22 Expression of endothelial and arterial markers is normal in *hesr1* morphant embryos

Hesr1 MO was injected into the two VMZ cells of four-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of endothelial markers, *tie2* and *ami*, and of arterial markers, *ephrinB2* and *cx37*, is relatively normal in the DA of *hesr1* morphants. *Ami* expression appears slightly weaker than in wild types, however as other endothelial and arterial markers are normal, this may be artifactual. Intersomitic vessel and vitelline vasculature defects are apparent. Red arrowheads mark the dorsal aorta, green arrowheads mark the vein, orange arrowheads mark the notochord, blue arrowheads mark vitelline vasculature, and purple arrowheads mark intersomitic vessels.



Expression of arterial markers, *ephrinB2* and *cx37*, is also normal in the DA of *hesr1* morphant embryos (Figure 5.22, panels g and h, respectively, red arrowheads), suggesting arterial programming may also be normal.

Expression of *ami* and *ephrinB2* in the vitelline vasculature is also relatively normal in morphant embryos, suggesting *hesr1* is dispensable for vitelline vessel development (Figure 5.22, panels f and g, respectively, blue arrowheads). However, there is an obvious defect in ISV development: expression of *ami* in ISVs reveals that the ISVs have sprouted but have not fully developed to reach the most dorsal part of the embryo, as in wild type embryos (Figure 5.22, panels f and b, respectively, purple arrowheads). Thus, whilst *hesr1* is dispensable for endothelial or arterial programming, it is required for the angiogenic development of ISVs.

Hesr1 has been reported to downregulate the expression of *flk1* in endothelial cells (Henderson et al., 2001; Holderfield et al., 2006; Taylor et al., 2002). Whilst *esr1* has been shown to repress *flk1* and *flt4* in the DA, *hesr1* is downstream of *esr1*, and thus may confer this repression. However, at stage 39, expression of *flk1* and *flt4* is not maintained in *hesr1* morphant embryos (Figure 5.23, panels c and d, respectively, red arrowheads). Thus, *hesr1* is not responsible for repression of initiator-containing, TATA-less, endothelial genes in the *Xenopus* DA.

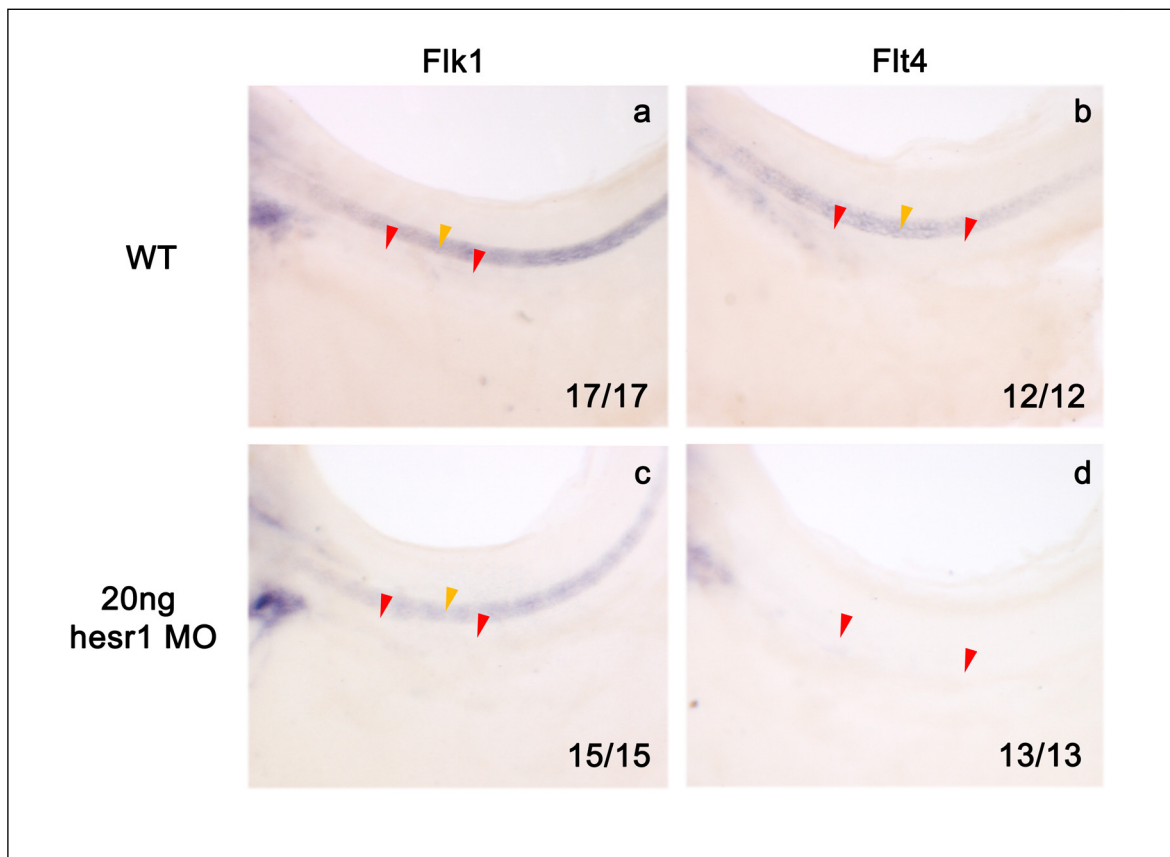


Figure 5.23 Expression of endothelial genes *flk1* and *flt4* is not maintained in *hesr1* morphant embryos

Hesr1 MO was injected into the two VMZ cells of four-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Expression of *flk1* and *flt4* is not maintained in the DA of *hesr1* morphants. Red arrowheads mark the location of the DA, orange arrowheads mark the notochord.

Concurrent with the observation that endothelial and arterial programming is normal in *hesr1* morphant embryos, primitive erythrocytes can be seen circulating normally around the embryo (Figure 5.24). This suggests that the vitelline vasculature is correctly specified to allow primitive erythrocytes to enter circulation, and that the DA and PCVs have formed lumens through which the blood can flow. However, there is an apparent loss of *globin*-expressing primitive erythrocytes in *hesr1* morphant embryos, suggesting that *hesr1* plays a role in primitive erythrocyte development or specification during development.

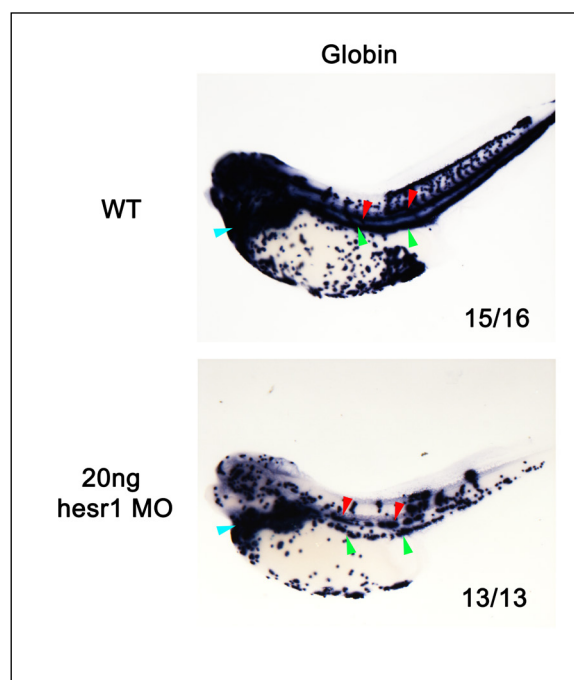


Figure 5.24 Primitive erythrocytes circulate normally in *hesr1* morphant embryos

Hesr1 MO was injected into the two VMZ cells of four-cell embryos which were collected at stage 38-39 and analysed by *in situ* hybridisation. Primitive erythrocytes can be seen circulating in the DA (red arrowheads), cardinal veins (green arrowheads) and in the heart (blue arrowheads) in both wild type sibling controls and in *hesr1* morphant embryos. However, there seems to be a reduction of primitive erythrocytes expressing *globin* in *hesr1* morphant embryos.

5.5. Discussion

5.5.1. Overview

Notch1 is known to be essential for vascular programming and remodelling in mouse embryos (Kim et al., 2008; Krebs et al., 2000; Limbourg et al., 2005). In addition, evidence has been presented for a role of *notch1* in HSC programming, although in the absence of an arterial analysis, this HSC phenotype could be due to a precluding arterial defect (Kumano et al., 2003; Nakagawa et al., 2006).

This study clearly demonstrates, for the first time, a role for *notch1* in haematopoietic stem cell programming without affecting prior haemangioblast, endothelial or arterial programming. Two *notch1* target genes have also been discovered: *esr1* and *hesr1* are expressed directly after *notch1* expression initiates, and are controlled by *notch1* signalling. *Esr1* morphants are found to phenocopy *notch1* morphants in terms of haematopoietic and arterial programming, although they also demonstrate a role for *esr1* in repression of endothelial genes. *Hesr1*, on the other hand, is responsible for the HSC programme downstream of *runx1*.

5.5.2. Notch1 signalling directly regulates expression of *esr1* and *hesr1*

The *notch1* expression pattern in *Xenopus laevis* reveals neural and somitic expression, and arterial-specific vascular expression can be seen from stage 34 (Figure 3.1 and Figure 3.11). Regulation of *notch1* expression in the DA is controlled by paracrine VEGFA signalling of long and medium isoforms from the somites to endothelial cells during migration (stages 28-31), and expression of *vegfa* has been shown to require *eto2* (Leung, 2010). At stage 35, immediately after *notch1* expression initiates, expression of two hairy and enhancer of split related genes, *esr1* and *hesr1*, is initiated (Figure 3.11). Expression of both *esr1* and *hesr1* was found to be *notch1*-dependent as expression was absent following injection of *notch1* MO (Figure 5.9). Unfortunately,

due to the lack of available antibodies in *Xenopus*, it has not yet been possible to carry out ChIP-seq experiments to investigate whether this is a direct regulation. However, the evidence presented here suggests the probability that *notch1* directly regulates *esr1*, and possibly *hesr1*: expression data shows *esr1* and *hesr1* expression is initiated immediately after *notch1* (Figure 3.11), morpholino data shows that they are regulated by *notch1* signalling (Figure 5.9), and hairy and enhancer of split genes are widely regarded as direct notch target genes (comprehensively reviewed in chapter 1.8.2). However, as *esr1* regulates the expression of *hesr1*, *notch1* may have no direct input into *hesr1* expression. From this data, a genetic regulatory network can be proposed (Figure 5.25).

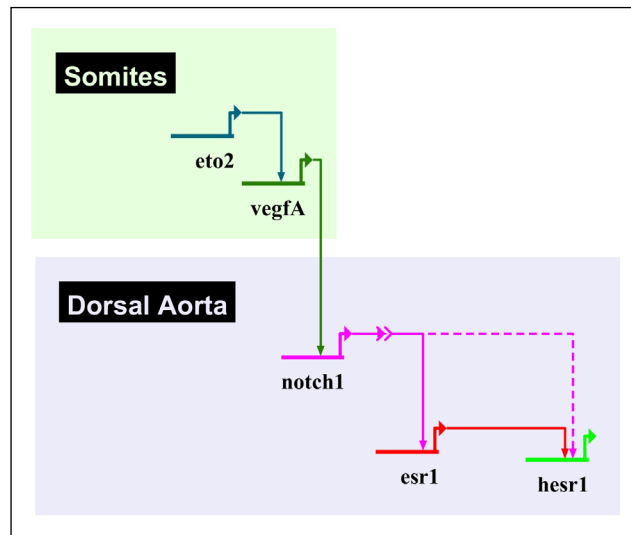


Figure 5.25 Schematic diagram showing the proposed network of gene regulation upstream and downstream of *notch1*

Notch1 expression in the DA is controlled by VEGFA medium and long isoforms signalling from the somites to the endothelial cells during migration (st 28-31), expression of which is regulated by *eto2* (Leung, 2010). *Notch1* regulates expression of both *esr1* and *hesr1*. However, as *esr1* also regulates expression of *hesr1*, the *notch1* input to *hesr1* expression may not be direct (shown as a dashed line).

5.5.3. Notch1 and its target genes *esr1* and *hesr1* are required for HSC programming

Whilst a role for *notch1* in haematopoietic stem cell development has been suggested (Burns et al., 2005; Kumano et al., 2003; Lawson et al., 2001; Nakagawa et al., 2006), precluding vascular or arterial defects has hindered the ability to determine if *notch1* is more directly required for HSC programming. Here, a role for *notch1* and its target genes, *esr1* and *hesr1*, in programming of the haemogenic endothelium has been demonstrated, with no associated arterial defects.

Runx1 has been well-defined as an essential regulator of definitive haematopoiesis and haematopoietic cluster formation, and is therefore a commonly used marker for HSC programming (Kissa and Herbomel, 2010; North et al., 1999; North et al., 2002). In *Xenopus*, *runx1* expression in the DA is initiated at stage 35, and is restricted ventrally, before being expressed in emerging clusters (Ciau-Uitz et al., 2000; Fernandez, 2007). It has also been found to be toward the top of the genetic regulatory network controlling haematopoietic programming, being upstream of *scl*, *lmo2*, *spiB*, *gfi1* and *ikaros* (Fernandez, 2007). In *notch1* and *esr1* morphants, expression of *runx1*, *spiB* and *gfi1* is lost, suggesting that *notch1* and *esr1* are essential for HSC programming. As *runx1* is essential for HSC cluster emergence, it is predicted that clusters would not form in *notch1* or *esr1* morphants. Carrying out *in situ* hybridisation on stage 43 sectioned embryos is needed to confirm that this is as predicted, and analysis of B-cell development using B-cell q-PCR techniques would enable the analysis of long-term definitive haematopoietic defects in *notch* pathway morphants.

Runx1 does not regulate *gata2* expression, and therefore must act downstream of, or in parallel to, *gata2* (Fernandez, 2007). The presence of a GATA binding site in the promoter of murine *runx1* suggests that GATA factors are able to directly regulate transcription of *runx1* (Nottingham et al., 2007), and, as the only known GATA factor expressed in the *Xenopus* DA, *gata2* is a strong

candidate for *runx1* transcriptional regulation. In the *Xenopus* DA, *gata2* expression initiates in the DA as soon as progenitors reach the midline, at stage 31. In both *notch1* and *esr1* morphants, expression of *gata2* is normal at stage 32, but disrupted at stage 39. This suggests that *notch1*, probably via *esr1*, is required for *gata2* maintenance. Conflicting reports on the mechanism of *gata2* regulation have been published: in 2001, it was reported that *notch1* activated *gata2* in a *hes1*-dependent manner (Kumano et al., 2001), whilst a later report claimed a *hes/hey*-independent mechanism for *gata2* regulation by showing CSL/NICD binding directly to the *gata2* promoter (Robert-Moreno et al., 2005). More recent in depth analysis of CSL/NICD binding at the *gata2* promoter has demonstrated that whilst CSL is able to bind to the promoter, it does not alter transcription of *gata2* (Hamidi et al., 2011). Thus, it is likely that *gata2* transcription is mediated by *hes1*. In *Xenopus*, *hes1* is not expressed in the DA, and thus cannot be controlling expression of *gata2* here. Data presented in this chapter suggests that *esr1* (*hes5*) is the mediating factor in *gata2* regulation in *Xenopus*.

Hesr1 acts differently to the other notch signalling components in HSC programming. Analysis of HSC markers in *hesr1* morphants reveals normal expression of both *runx1* and *gata2*, but downregulation of *spiB* and *gfi1*, genes downstream of *runx1*. This suggests that *hesr1* acts in parallel to, or downstream of, *runx1* in the regulation of downstream HSC markers. In osteoblastic cell cultures, *hesr1* has been found to interact with *runx2*, affecting the transcriptional regulation capacity of *runx2* (Zamurovic et al., 2004). As *runx* factors are closely related and share many domains, including the DNA-binding runt domain, it is possible that *hesr1* is interacting with *runx1* in the *Xenopus* DA to alter transcription downstream of *runx1*.

From the data discussed above, a genetic regulatory network showing notch regulation of the HSC programme has been compiled (Figure 5.26).

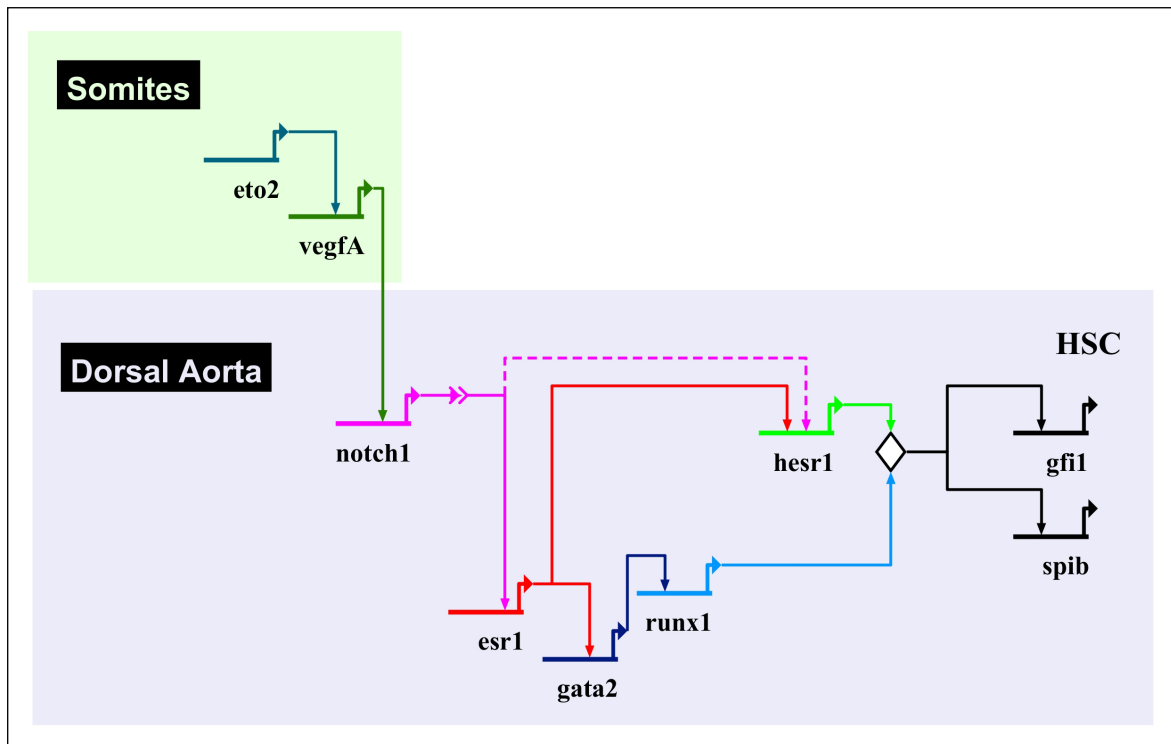


Figure 5.26 Schematic diagram showing the proposed network of HSC gene regulation controlled by *notch1*

Eto2-VEGF-notch1-esr1-hesr1 interaction has been described and shown above (figure 5.25). Hesr1 regulates HSC genes in parallel to runx1. Esr1 regulates gata2, which is proposed to regulate *runx1*: because notch ICD binding sites have not been identified in the *runx1* promoter. In turn, runx1 has been shown to regulate downstream HSC genes *spib* and *gfi1* (Fernandez, 2007).

Haematopoietic stem cells are believed to arise from the ventral wall of the DA, and endothelial cells have been imaged rounding up and emerging from the DA (Kissa and Herbomel, 2010, and reviewed in chapter 1.5). When studying the programming of haemogenic endothelium and the subsequent emergence of HSCs, it is important to consider that a defect in the earlier vascular or arterial programming is likely to influence the programming of haemogenic endothelium and HSCs. Importantly, endothelial and arterial programming appear unaffected in *notch1*, *esr1* and *hesr1* morphant embryos, as analysed by endothelial and arterial markers. As such, the

haematopoietic defects observed can be regarded as a primary effect of *notch1*, *esr1* or *hesr1* depletion.

5.5.4. Esr1 is responsible for repression of endothelial genes in the DA

Hesr1 has been reported to downregulate expression of initiator-containing TATA-less genes, such as *flk1* and *flt4*, in endothelial cells (Henderson et al., 2001; Holderfield et al., 2006; Taylor et al., 2002). However, analysis of *hesr1* morphants revealed no expression of *flk1* or *flt4* in the DA, mirroring expression seen in wild type siblings. Thus, *hesr1* is not responsible for repression of endothelial genes in the *Xenopus* DA. The repression of these promoters by *hesr1* was found to be due to the bHLH and orange domains, and independent of the YRPW domain (Holderfield et al., 2006). As *esr1* contains both a bHLH and an orange domain, it could potentially be mediating the repression of *flk1* and *flt4* in the DA, instead of *hesr1*. In *esr1* morphant embryos, expression of both *flk1* and *flt4* is maintained in the DA, suggesting *esr1* is responsible for repression of the endothelial genes. As endothelial gene expression is maintained in the DA of *esr1* morphants, this leads to the possibility that the role for *esr1* in HSC programming could be due to earlier vascular programming defects. However, in morphant embryos for all upstream regulators of *esr1*, vascular and arterial programming is shown to be normal whilst the HSC programme is still downregulated. Thus, the HSC and vascular programmes can be uncoupled.

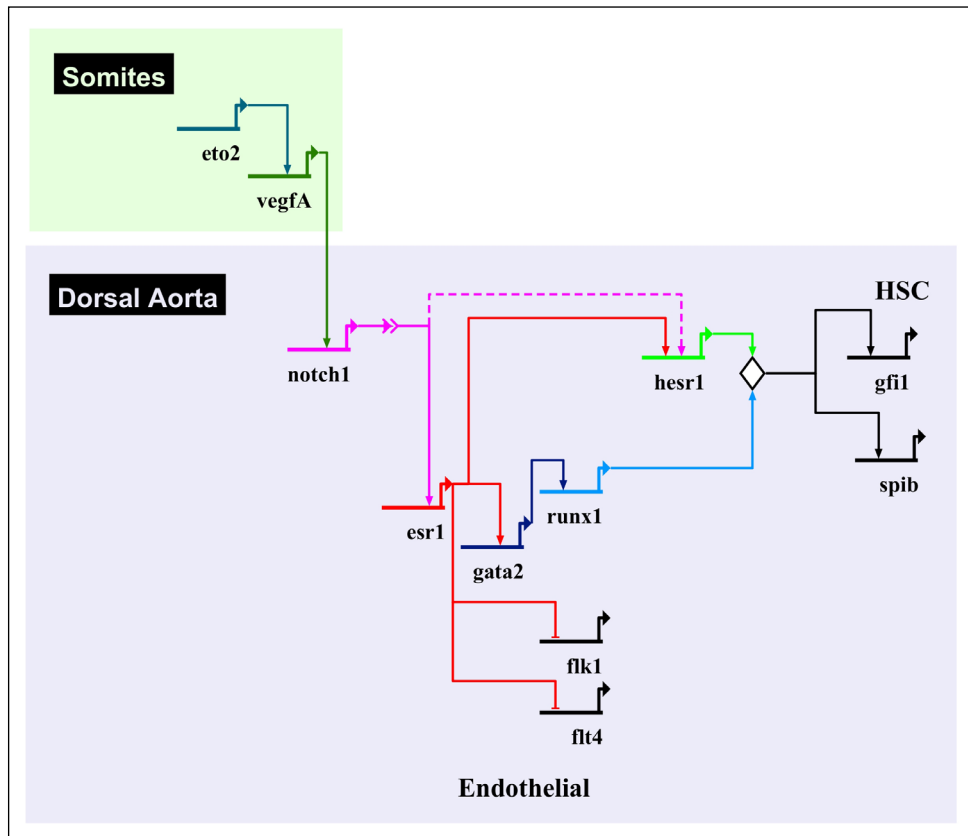


Figure 5.27 Schematic diagram showing the proposed network of HSC and endothelial gene regulation in the DA

Eto2-VEGF-notch1-esr1-hesr1 interaction has been described and shown above (figure 5.25), as has HSC gene regulation (Figure 5.27). Esr1 is responsible for repressing endothelial genes, *flk1* and *flt4*, in the DA.

5.6. Conclusions

In conclusion, the data presented in this chapter reveals that notch1 signalling is specifically required for HSC programming in the *Xenopus* DA, whilst not affecting earlier vascular programming. Downstream of notch1, *esr1* is required for the HSC programme and for repression of endothelial genes, whilst *hesr1* is required downstream of, or parallel to, *runx1* for HSC programming. A gene regulatory network governing HSC programming and endothelial gene repression has been generated from data presented here, and from published data, although it should be noted that the nature of these interactions have not been investigated, so it is not known whether they are direct or indirect.

Chapter 6. Characterising the dual roles of *notch1* and *notch4* in arterial development

6.1. Introduction

Relevant to dorsal aorta and HSC programming, more than one notch receptor is expressed: in *Xenopus*, *notch1*, *notch3* and *notch4* are all expressed in the DA (see chapter 3.2), in mouse, *notch1* and *notch4* expression has been observed (Robert-Moreno et al., 2005; Villa et al., 2001), and in zebrafish, *notch1b* and *notch3/5* are expressed (Bertrand et al., 2010b; Lawson et al., 2001). Many of the approaches undertaken to abrogate notch signalling in mouse and fish embryos have blocked all notch signalling, rather than a specific receptor.

Using a zebrafish mutant of mindbomb (*mib*), a protein essential for notch ligand maturation, to disrupt notch signalling, notch has been implicated in arterial specification (Lawson et al., 2001; Lawson et al., 2002). Together with vascular malformations, loss of notch signalling in zebrafish embryos results in the loss of artery-specific marker, *ephrinB2*, expression and the misexpression of venous-associated *EphB4* in arteries. Conversely, ectopic activation of notch signalling in veins prevents *EphB4* expression (Lawson et al., 2001; Lawson et al., 2002). In addition to these vascular defects, loss of HSC markers, *runx1* and *c-myb*, was found in *mib* mutants, and in embryos treated with notch signalling inhibitor, DAPT (Burns et al., 2005; Gering and Patient, 2005). However, primitive haematopoiesis was found to be normal in both DAPT-treated and *mib* mutant embryos (Burns et al., 2005; Gering and Patient, 2005).

Mutation of *Rbpsuh* (encoding CSL) in mouse, or double knockout of *Notch1* and *Notch4*, leads to substantial vascular defects, including vascular remodelling defects and loss of *ephrinB2* in arterial cells, as well as embryonic death (Krebs et al., 2004; Krebs et al., 2000; Oka et al., 1995). Further analysis of *Rbpsuh* mutant mouse embryos revealed that E9.5 P-Sp cultures cannot form CFCs,

thus the haematopoietic potential of the P-Sp region is lost (Robert-Moreno et al., 2005). However, whilst vascular morphology was reported to be abnormal in *Rbpsuh* mutant mice in this study, analysis of arterial programming was not carried out. Previous studies using *Rbpsuh* mutant mice have reported a loss of arterial marker, *ephrinB2*, in the DA, and thus the haematopoietic defect reported by Robert-Moreno is likely due in part to arterial programming deficiencies (Krebs et al., 2004; Robert-Moreno et al., 2005). Similarly to zebrafish, knockout of *mindbomb* in mice leads to a loss of haematopoietic markers in the DA, whilst primitive haematopoiesis is normal (Yoon et al., 2008). However, again, analysis of arterial specification was absent from this report. In addition, knockouts of other notch signalling components have demonstrated vascular defects, including ADAM metalloprotease (Hartmann et al., 2002) and γ -secretase (Herreman et al., 1999). Thus, uncoupling the notch receptor contributions to arterial specification and haematopoietic stem cell programming has not yet been reported.

The individual roles of both notch1 and notch4 in *Xenopus* HSC programming has already been presented in this thesis. However, a role for notch signalling in *Xenopus* arterial development has not been found. A functional redundancy of notch receptors may be responsible for the normal specification of the artery in individual notch receptor knockdowns. Here, analysis of total notch signalling inhibition on development of the DA and HSCs is analysed, through use of a specific γ -secretase inhibitor, DAPM. The DAPM-treated embryos have a slightly more severe phenotype than *notch4* or *notch1* single knockdowns, and phenocopy *esr1* morphant embryos perfectly, suggesting that the separate notch inputs converge at *esr1* regulation. Notch signalling was found to be essential for HSC programming, and for repression of endothelial genes *flk1* and *flt4*. However, notch is dispensable for arterial specification in *Xenopus*.

6.2. Identifying suitable small molecular chemical inhibitors of notch signalling

A popular technique for the inhibition of notch signalling *in vitro* or *ex utero* is the application of specific γ -secretase inhibitors. *Xenopus* embryos are particularly amenable to the application of small molecule chemical inhibitors (Wheeler and Liu, 2012), and so this approach was undertaken in order to analyse notch input to DA and HSC programming. Specific γ -secretase inhibitors are commonly employed to abrogate notch signalling: γ -secretase is an enzyme responsible for the cleavage of the NICD from the transmembrane portion of the receptor at the S3 cleavage site, thus allowing NICD to travel to the nucleus. By blocking γ -secretase activity, the NICD remains tethered to the cell membrane, and thus notch signalling is not activated.

Xenopus laevis embryos are grown at 14-23°C, considerably lower than the temperature at which zebrafish are grown, or *in vitro* cell culture takes place. In light of this, it is necessary to consider solubility of chemicals across the range of temperatures at which *X.laevis* is grown. DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), a very commonly used γ -secretase inhibitor for cell culture and zebrafish work (Bertrand et al., 2010b; Geling et al., 2002; Gering and Patient, 2005; Hadland et al., 2004; Lee et al., 2008; Rowlinson and Gering, 2010), was observed precipitating out of solution when used to treat *X.laevis* embryos (Aldo Ciau-Uitz, personal communication). DAPM (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine methyl ester), a notch inhibitor derived from DAPT (Dang et al., 2009), was found not to precipitate at 23°C, and was therefore a good candidate for notch inhibition in *X.laevis*. Having a molecular weight of 390.4, it is smaller than DAPT (molecular weight 432.46), and should therefore have no problems penetrating the embryo. DAPM was dissolved at a concentration of 100mM/ μ L in dimethyl sulfoxide (DMSO), and stored at -20°C.

As no notch receptors are expressed in the DLP (Figure 3.2), application from stage 20 would allow plenty of time for the chemical to fully penetrate the embryo before initiation of *notch4* expression at stage 31, without causing prior haemangioblast defects. Titration of DAPM revealed a loss of *runx1* but normal *ephrinB2* expression at concentrations from 40µM up to 200µM (the highest dose tested). A dose of 100µM was chosen, providing a reliable knockdown of notch signalling through analysis of *runx1* expression. At 100µM, embryos were found to have a smaller, and less developed, eye (Figure 6.1, red arrowheads), reduced pigmentation (blue arrowheads) and an obvious kink in the tail (green arrowheads). Perturbed eye and pigment development has been observed for *notch1*, *notch4* and *esr10* morphant embryos, and so it is not surprising to find the same defects in DAPM-treated embryos. However, a kink in the tail, suggestive of a somitic patterning problem, has not been observed in morphant embryos, even though *notch1* is heavily expressed in the somites. Redundancy between notch receptors in somitogenesis may be the cause of this, and by blocking all notch signalling, a fuller phenotype is observed. DAPM-treated embryos reveal a normally beating heart, but no circulation in the tail, whilst in control embryos, circulation was evident from stage 37.

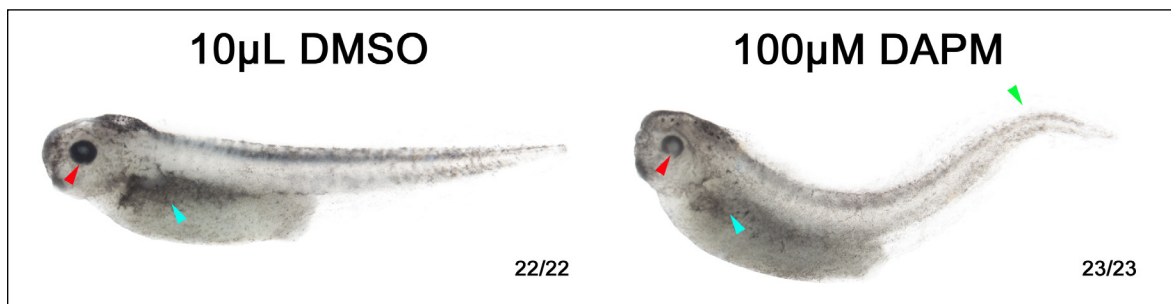


Figure 6.1 DAPM-treated embryos show eye and pigment development problems, and a kink in the tail

Morphology of stage 39 DAPM-treated embryos (right) compared to sibling embryos treated with DMSO – the solvent used to dissolve DAPM (left). Red arrowheads mark the eye, blue arrowheads mark pigment cells, and the green arrowhead marks the kink in the tail.

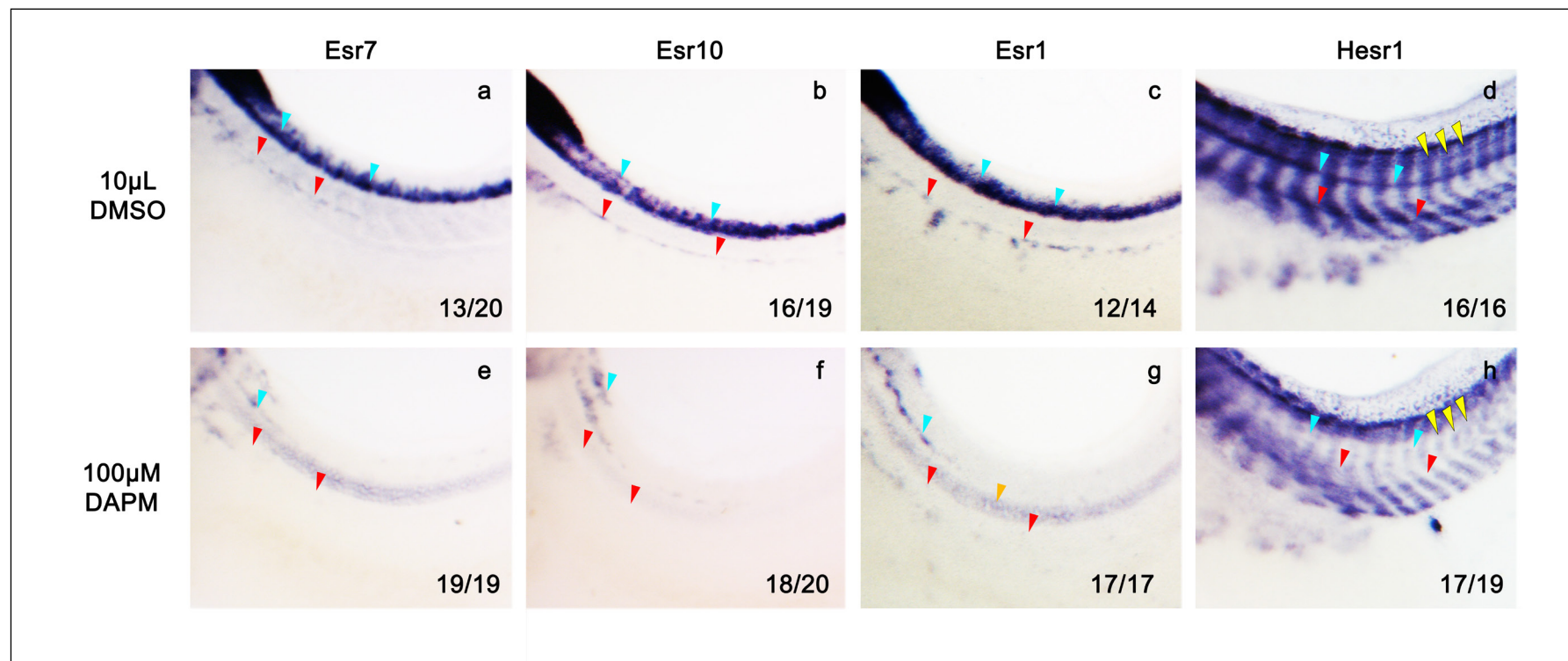
6.3. DAPM effectively reduces expression of downstream hairy and enhancer of split genes

To confirm inhibition of notch signalling in the *Xenopus* DA, expression analysis of the four proposed notch target genes was undertaken in embryos treated with 100µM DAPM (hereafter referred to as 'DAPM-treated embryos'), and compared to control siblings treated with 10µL DMSO (hereafter referred to as 'control embryos'). Expression of *esr7*, *esr10*, *esr1* and *hesr1* is lost in the DA in DAPM-treated embryos (Figure 6.2, panels e, f, g and h, respectively, red arrowheads), confirming chemical inhibition of notch signalling in the DA. Furthermore, neural expression of *esr7*, *esr10* and *esr1* is lost in DAPM-treated embryos (Figure 6.2, panels e, f and g, blue arrowheads). Notch signalling is widely reported as an essential signalling pathway in neural development (reviewed in Pierfelice et al., 2011), thus this is an expected phenotype after embryo-wide inhibition of notch signalling. That neural expression is not lost in *notch4* morphants (Figure 4.8) is expected as *notch4* is not expressed in the neural tissues. Neural expression of hairy and enhancer of split genes is also normal in *notch1* morphants (Figure 5.9), which is slightly more surprising considering *notch1* is expressed in the neural tube (Figure 3.1). This may suggest a functional redundancy of notch receptors in neural tube programming: both *notch2* and *notch3* are expressed here in addition to *notch1* (Figure 3.1).

Analysis of notch receptor expression in the DA of DAPM-treated embryos reveals normal expression (Figure 6.3, panels c and d, red arrowheads), suggesting that the notch receptors do not auto-regulate their own expression, or regulate the expression of each other in the DA. This is consistent with the observation that *notch1* expression is normal in the DA of *notch4* morphant embryos, and vice versa (Figure 4.8 and Figure 5.9, respectively).

Figure 6.2 Notch signalling regulates the expression of hairy and enhancer of split genes in the DA and the neural tube

Embryos were treated with either 10 μ L DMSO (control) or 100 μ M DAPM from stage 20, collected at stage 33 or 38-39, and analysed by *in situ* hybridisation. Expression of *esr7*, *esr10*, *esr1* and *hes1* is lost from the DA in DAPM-treated embryos. In addition, expression is lost from the neural tube. Red arrowheads mark the DA, blue arrowheads mark the neural tube, orange arrowheads mark the notochord, and yellow arrowheads mark the somites.



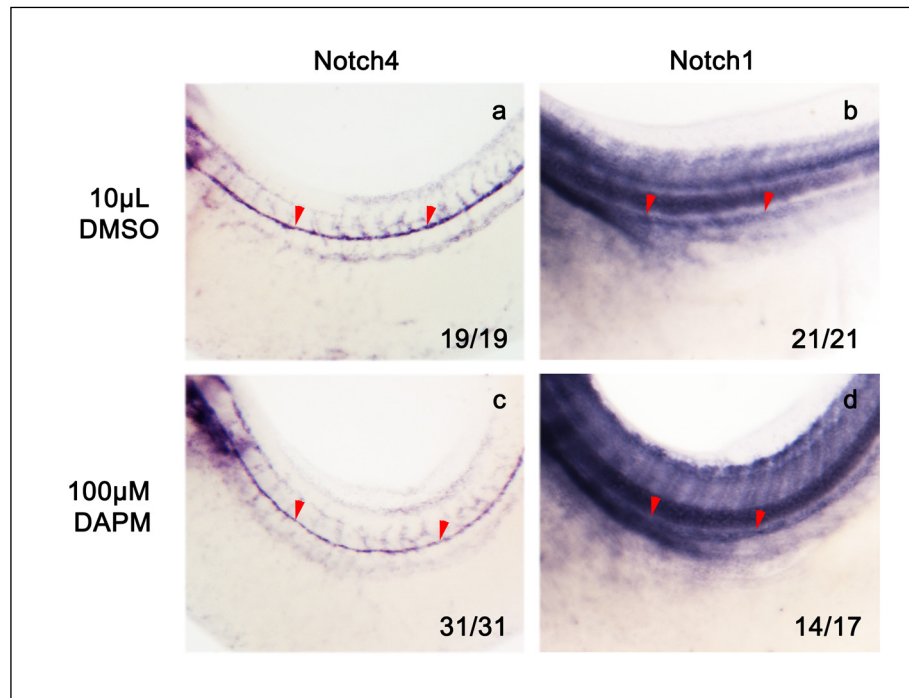


Figure 6.3 Notch signalling does not auto-regulate in the DA

Embryos were treated with either 10µL DMSO (control) or 100µM DAPM from stage 20, collected at stage 33 or 38-39, and analysed by *in situ* hybridisation. Expression of *notch4* and *notch1* is normal in the DA in DAPM-treated embryos, suggesting notch does not auto-regulate. Red arrowheads mark the DA.

6.4. Application of DAPM reduces haematopoietic stem cell marker expression

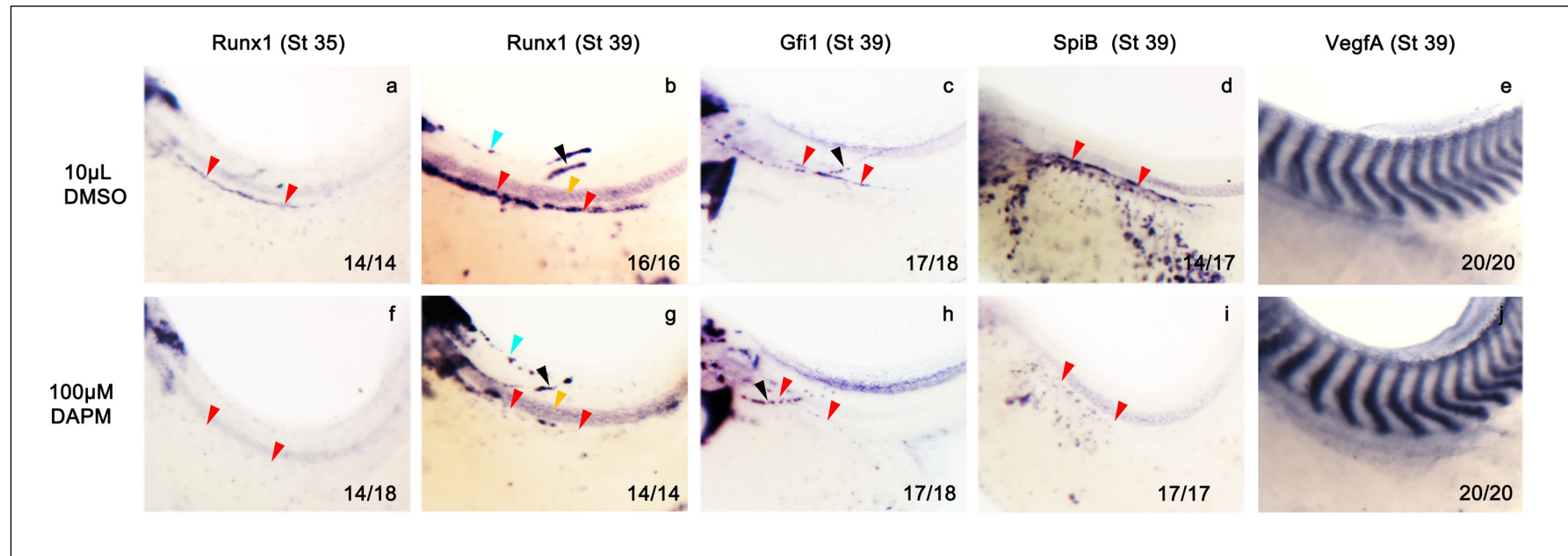
Notch1 and notch4 have both been shown to be essential for correct haematopoietic stem cell programming via morpholino knockdown experiments (chapters 5.2.3 and 4.2.3, respectively). Therefore, application of DAPM is expected to abrogate HSC marker expression also. To investigate the dual contribution of notch signalling to the HSC programme, expression analysis of HSC markers was carried out in stage 35 and stage 39 embryos treated with DAPM. Expression of HSC marker, *runx1*, was lost in the DA of stage 39 embryos (Figure 6.4, panel g, red arrowheads). Analysis of *runx1* in the DA of stage 35 embryos, when expression first initiates in the DA, also reveals a loss of expression, suggesting *runx1* expression never initiates in notch signalling deficient embryos (Figure 6.4, panel f, red arrowheads). Expression of both *spiB* and *gfi1* is also lost in DAPM-treated embryos, as expected from the loss of *runx1* expression (Figure 6.4, panels h and i, respectively, red arrowheads).

VEGF signalling from the somites to the DA is known to regulate expression of *runx1* (Leung, 2010). As notch signalling is well known to be involved in somitogenesis, and DAPM-treated embryos exhibit a bent tail, suggestive of a somitic defect, it is possible that incorrect somitic development could cause loss of somitic *vegfa* expression, and thus abrogate HSC programming. However, expression analysis of *vegfa* in DAPM-treated embryos reveals normal expression of *vegfa* in the somites, and normal somitic patterning (Figure 6.4, panel j). Therefore, as the requirement for notch signalling in HSC programming is not due to a loss of somitic VEGFA signalling, it is likely to be a direct requirement for notch in the DA.

In all notch pathway morphants studied in this thesis, a role for notch signalling in the maintenance of *gata2* has been identified, whilst initiation of expression is normal.

Figure 6.4 Notch signalling is required for the expression of haematopoietic stem cell markers

Embryos were treated with either 10 μ L DMSO (control) or 100 μ M DAPM from stage 20, collected at stage 35 or 39, and analysed by *in situ* hybridisation. Expression of HSC markers is lost in DAPM-treated embryos: *runx1* fails to initiate at stage 35, and expression is still absent at stage 39. Expression of *spiB* and *gfi1* is also lost in the DA. Expression of *vegfa* in the somites is normal. Red arrowheads mark the DA, blue arrowheads mark neural expression, black arrowheads mark the lateral line nerves, and orange arrowheads mark the notochord.



DAPM-treated embryos also show a loss of *gata2* expression at stage 39, whilst expression at stage 32 is normal (Figure 6.5, panels f and d, respectively, red arrowheads), suggesting a role of notch in the maintenance of *gata2*. Expression analysis of *gata2* at stage 35 in DAPM-treated morphants reveals loss of expression also (Figure 6.5, panel e, red arrowheads). As *esr1* has been shown to regulate *gata2*, downstream of notch1, and *esr1* expression initiates at stage 35, this suggests that *gata2* maintenance is dependent on notch signalling once *esr1* expression initiates. Loss of haematopoietic markers was used as a positive control for all subsequent DAPM experiments.

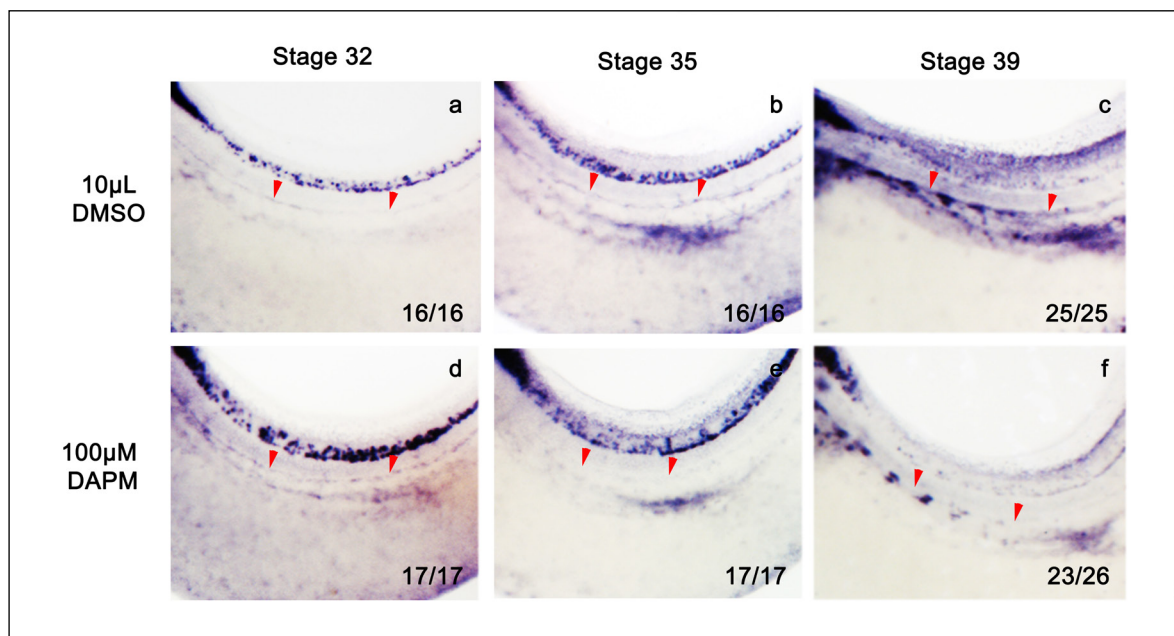


Figure 6.5 Notch signalling is required for the maintenance of *gata2*

Embryos were treated with either 10μL DMSO (control) or 100μM DAPM from stage 20, collected at stage 32, 35, or 39, and analysed by *in situ* hybridisation. Expression of *gata2* is normal at stage 32, but is lost at stages 35 and 39, suggesting notch is required for the maintenance of *gata2*. Red arrowheads mark the DA.

6.5. Haemangioblast programming is not affected by application of DAPM

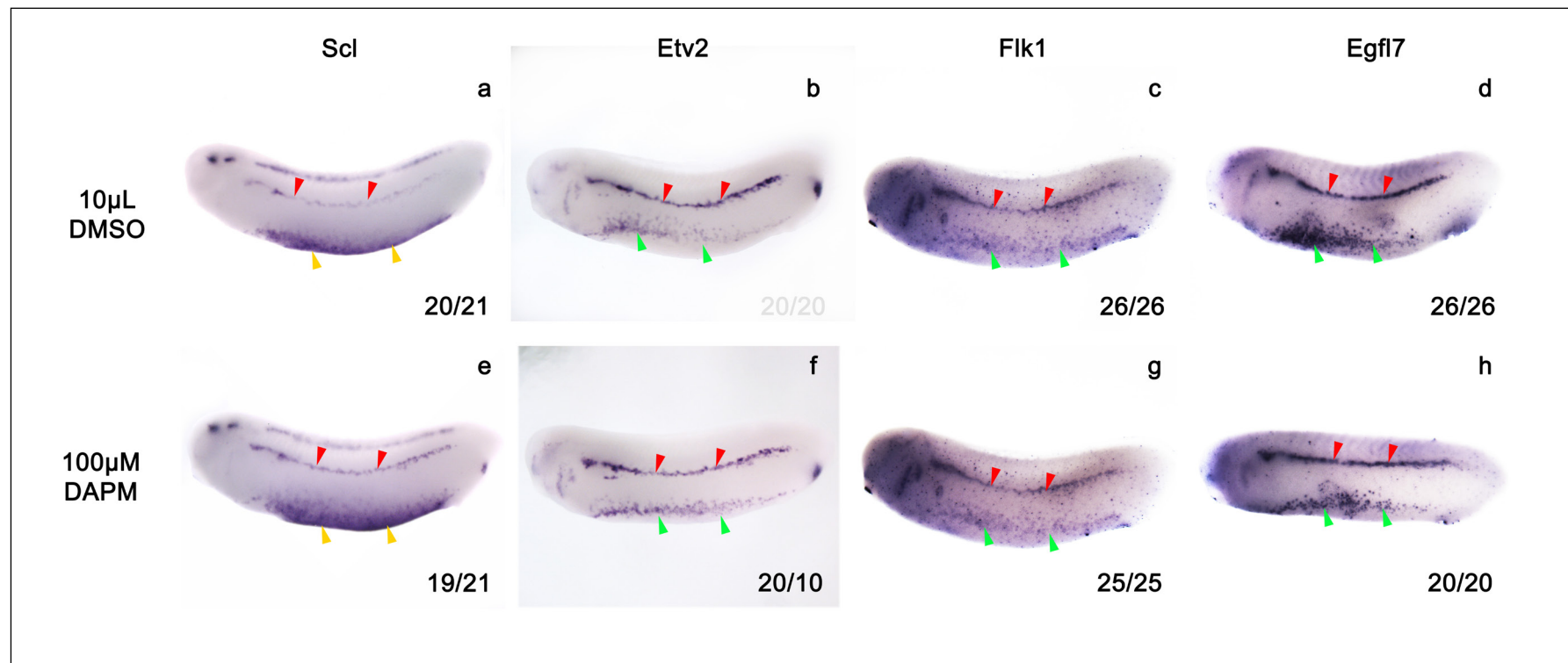
Whilst notch receptors are not expressed in lateral mesoderm populations during haemangioblast programming (Figure 3.2), treatment of embryos with DAPM from stage 20 means there is some possibility of an early programming effect. In order to investigate if DAPM treatment perturbs haemangioblast specification, stage 28 DAPM-treated embryos were analysed for haematopoietic and endothelial markers in the DLP. Expression of the haematopoietic marker, *scl*, in DAPM-treated embryos is relatively normal in the DLP (Figure 6.6, panel e, red arrowheads). Furthermore, expression of the endothelial markers, *etv2*, *flk1* and *egfl7*, is normal in DAPM-treated embryos (Figure 6.6, panels f, g and h, respectively, red arrowheads).

Expression of *vegfa* in the somites is crucial for correct haemangioblast specification. VEGFA signals from the somites to the lateral mesoderm, controlling expression of *scl* and genes downstream of *scl* (figure 1.11, and Ciau-Uitz et al., 2010b). As notch receptors are expressed in the somites during haemangioblast specification, it is essential to determine if DAPM-treated embryos have a somitic VEGFA deficiency due to loss of notch signalling, which could impact on haemangioblast programming. As *scl* expression is normal in the DLP of DAPM-treated embryos, it argues that VEGFA signalling from the somites to the DLP is normal at this stage.

Expression of *scl*, *etv2*, *flk1* and *egfl7* is also seen in the VBI, marking primitive erythrocytes (*scl*), and endothelial cells which will give rise to the vitelline vessels (*etv2*, *flk1* and *egfl7*).

Figure 6.6 Notch signalling is dispensable for haemangioblast programming

Embryos were treated with either 10µL DMSO (control) or 100µM DAPM from stage 20, collected at stage 28, and analysed by *in situ* hybridisation. Expression of haematopoietic marker *scl*, and endothelial markers *etv3*, *flk1* and *egfl7*, is unaffected in DAPM-treated embryos. Expression can be seen in the DLP (red arrowheads), developing primitive erythrocytes (orange arrowheads), and in the developing vitelline vessels



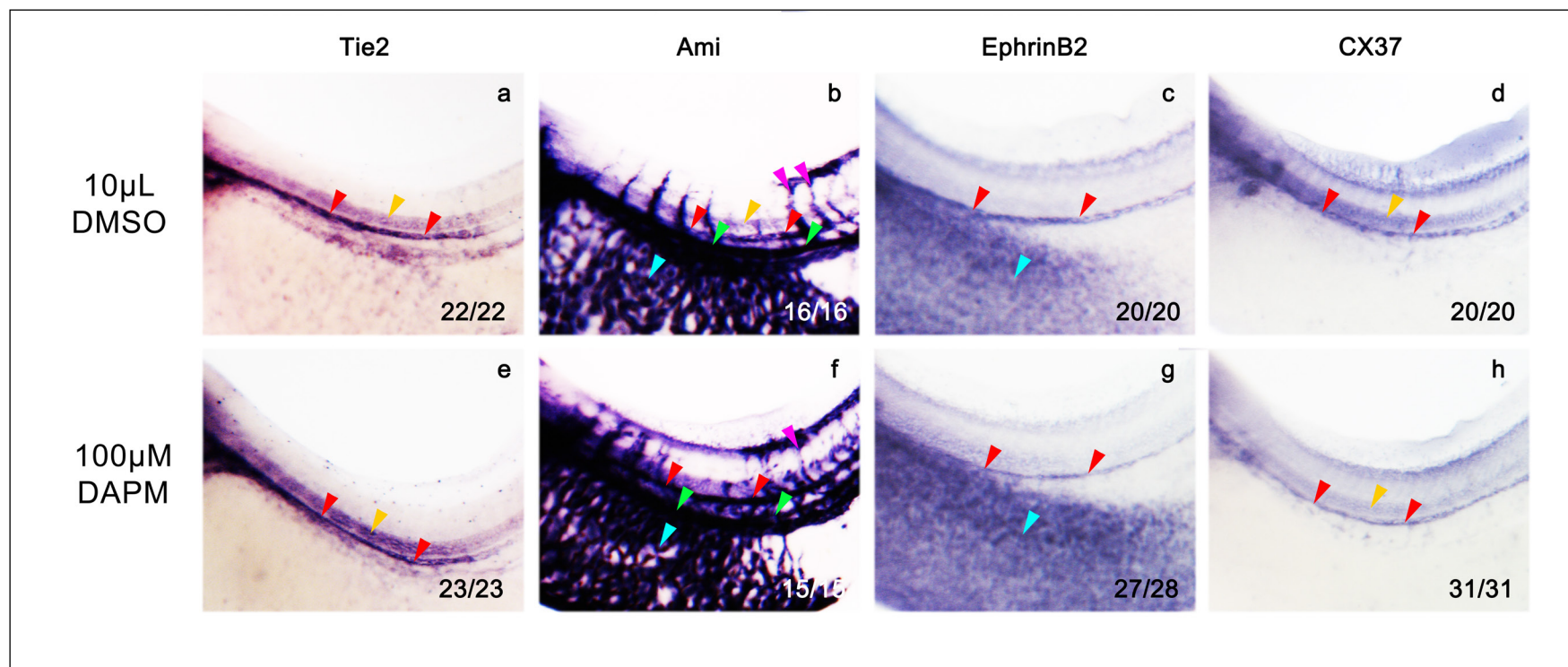
Expression of all four markers is normal in the VBI (Figure 6.6, panels e-h, orange and green arrowheads), suggesting that primitive erythrocyte and vitelline vessel programming is unaffected by DAPM treatment.

6.6. Endothelial and dorsal aorta specification is not affected by application of DAPM, but expression of endothelial genes *flk1*, *flt4* and *ephB4* is maintained

Notch signalling has strongly been associated with vascular development, and a perturbation of notch signalling in both mouse and zebrafish embryos has been shown to cause substantial vascular and arterial defects (Hartmann et al., 2002; Herreman et al., 1999; Krebs et al., 2004; Krebs et al., 2000; Lawson et al., 2001; Lawson et al., 2002). However, individual knockdown of *notch1* and *notch4* using specific antisense morpholinos in *Xenopus* embryos has shown that notch is dispensable for vascular and arterial programming in *Xenopus*. A functional redundancy of notch receptors may be responsible for the normal specification of the artery in individual notch receptor knockouts. For this reason, analysis of endothelial and arterial markers in DAPM-treated embryos was undertaken to look for a role of notch signalling in vascular development. DAPM-treated embryos were analysed for expression of the endothelial markers, *tie2* and *ami*, and normal expression was found in the DA and vitelline vessels (Figure 6.7, panels e and f, red arrowheads and blue arrowheads, respectively). In addition, the arterial markers, *ephrinB2* and *cx37*, were expressed normally in the DA, suggesting that arterial programming is normal in the absence of notch signalling (Figure 6.7, panels g and h, respectively, red arrowheads). However, expression of *ami* revealed an intersomitic vessel defect, suggesting that notch signalling is required for angiogenic remodelling (Figure 6.7, panel f, pink arrowheads). This is in agreement with notch morphant embryos in this thesis also showing an ISV defect.

Figure 6.7 Notch signalling is dispensable for endothelial and arterial programming

Embryos were treated with either 10 μ L DMSO (control) or 100 μ M DAPM from stage 20, collected at stage 33 or 38-39, and analysed by *in situ* hybridisation. Expression of endothelial markers, *tie2* and *ami*, is normal in DAPM-treated embryos, as is expression of arterial markers, *ephrinB2* and *cx37*. Red arrowheads mark the DA, blue arrowheads mark vitelline vasculature, green arrowheads mark the vein, pink arrowheads mark intersomitic vessels, and orange arrowheads mark the notochord.



Esr1 has been shown to be required for repression of the initiator-containing, TATA-less, endothelial genes, *flk1* and *flt4*, in the DA (chapter 5.3.5). As *esr1* expression is completely lost in DAPM-treated embryos (Figure 6.2), it was believed that DAPM-treated embryos would also exhibit a maintenance of *flk1* and *flt4*. In addition, *ephB4* expression is repressed at the same time as *flk1* and *flt4* expression is repressed (Figure 3.12), and *ephB4* has been found to be repressed by *hesr2* in zebrafish (Zhong et al., 2001), thus expression of *ephB4* may also be maintained in DAPM-treated embryos. Expression analysis confirmed this theory as DAPM embryos revealed expression of *flk1*, *flt4* and *ephB4* in the DA, whilst expression was repressed in control embryos (Figure 6.8, red arrowheads).

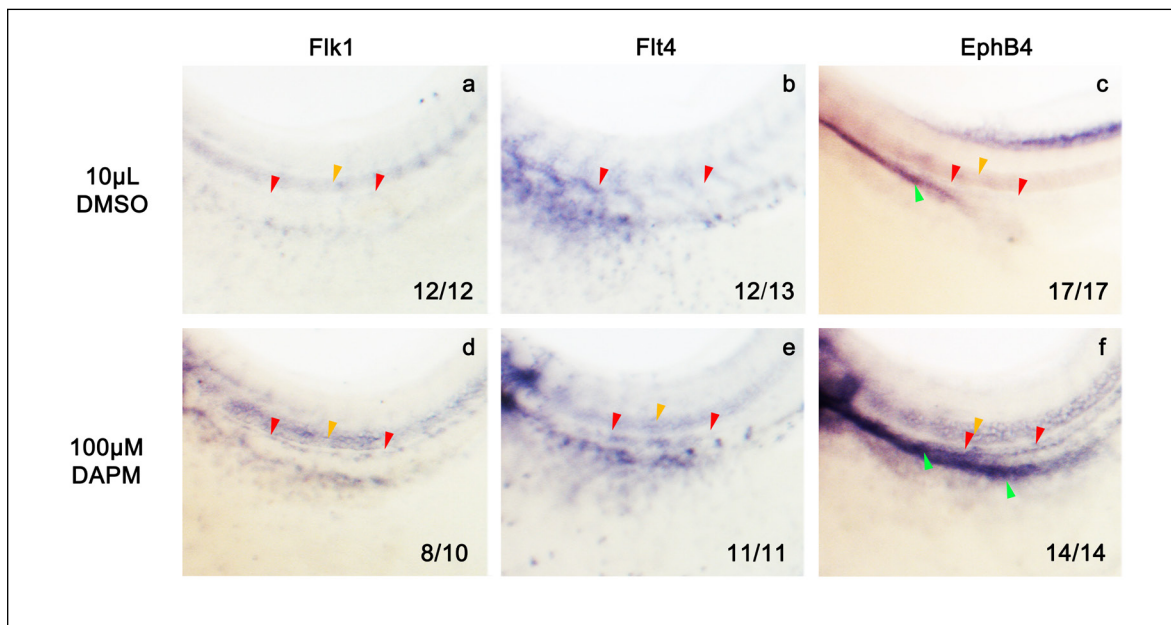


Figure 6.8 Expression of endothelial genes *flk1*, *flt4* and *ephB4* is maintained in DAPM-treated embryos

Embryos were treated with either 10µL DMSO (control) or 100µM DAPM from stage 20, collected at stage 38-39, and analysed by *in situ* hybridisation. Expression of *flk1* and *flt4* is maintained in the DA of DAPM-treated embryos, suggesting that notch signalling is required for repression of the endothelial programme in the DA. Red arrowheads mark the DA, orange arrowheads mark the notochord, and green arrowheads mark the vein.

6.7. Primitive haematopoiesis is not affected by application of DAPM, but primitive erythrocytes do not circulate

Expression of notch receptors is not seen in the VBI, and therefore notch signalling should not be involved in primitive haematopoiesis. Supporting this notion, *notch1* and *notch4* morphants show relatively normal primitive haematopoiesis (chapters 5.2.6 and 4.5.6). Furthermore, notch signalling mutants, and notch inhibitor treated zebrafish embryos have been shown to exhibit normal primitive haematopoiesis (Burns et al., 2005; Gering and Patient, 2005).

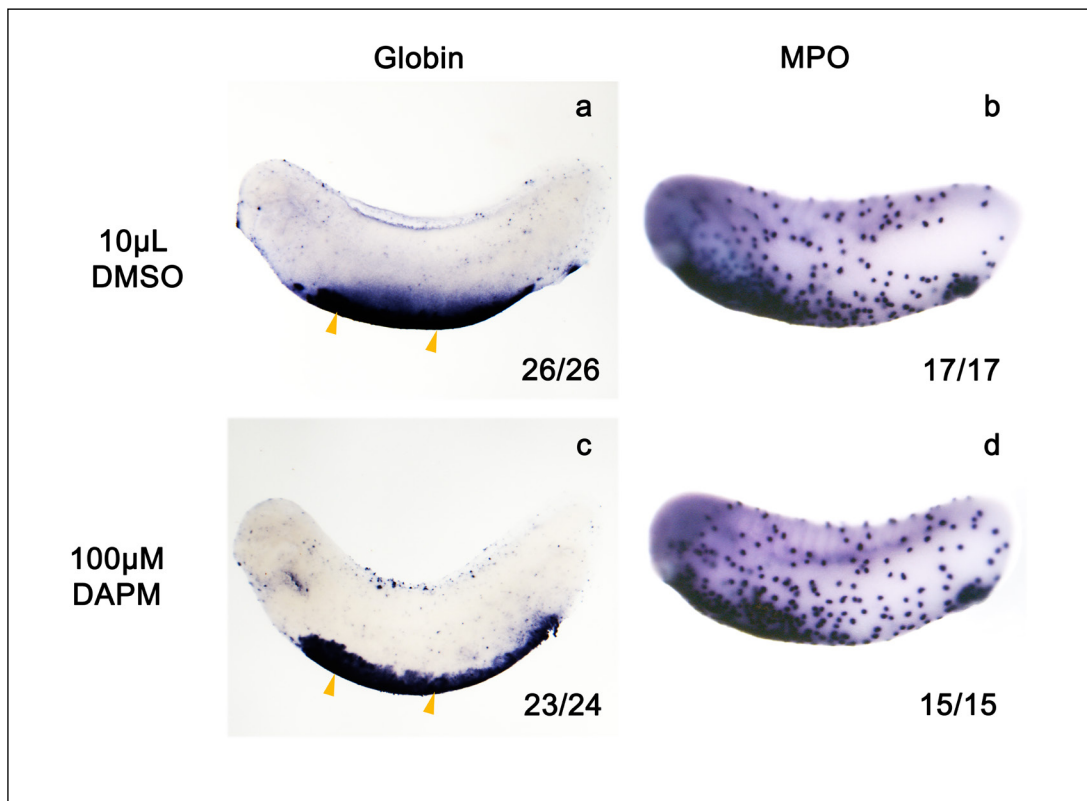
Expression of the endothelial markers, *etv2*, *flk1* and *egfl7*, in the VBI of DAPM-treated embryos revealed normal endothelial programming of the vitelline vasculature at this stage (Figure 6.6). Similarly, expression of the primitive erythroid marker, *scl*, in the VBI demonstrates normal primitive erythroid programming in DAPM-treated embryos (Figure 6.6). To further analyse programming of the VBI in DAPM-treated embryos, expression analysis of *globin* was carried out at stage 28, and was found to be normal (Figure 6.9A, panel c, orange arrowheads). As both *scl* and *globin* are expressed normally in the VBI, this suggests that primitive erythrocytes are being normally programmed in DAPM-treated embryos. In addition to primitive erythrocytes and vitelline vessels, early primitive myeloid cell specification is unaffected in DAPM-treated embryos, as analysed by expression of *mpo* (Figure 6.9A, panel d). Therefore, notch signalling is not required for primitive haematopoiesis.

Analysing the expression of primitive haematopoietic markers later in development can provide essential information about circulation in embryos.

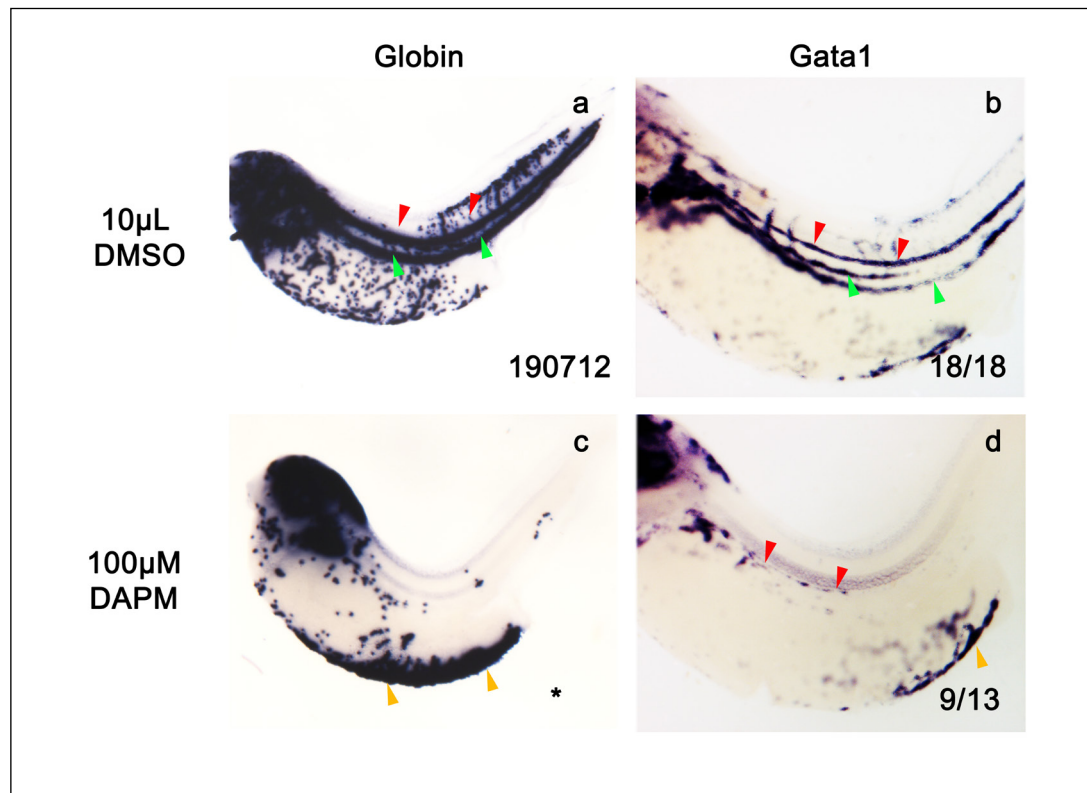
Figure 6.9 Primitive haematopoiesis is not affected in DAPM-treated embryos, but primitive erythrocytes do not circulate throughout treated embryos

Embryos were treated with either 10 μ L DMSO (control) or 100 μ M DAPM from stage 20, collected at stage 38-39, and analysed by *in situ* hybridisation. **A.** At stage 28, expression of the primitive erythrocyte marker, globin, is normal, as is expression of the primitive myeloid marker, *mpo*. **B.** At stage 39 erythrocytes can be seen circulating throughout control embryos, but they are seen pooling in the VBI of DAPM-treated embryos. Red arrowheads mark the DA, green arrowheads mark the vein, and orange arrowheads mark primitive erythrocytes in the VBI.

A.



B.



At stage 39, primitive erythrocytes can be seen circulating throughout the DA (red arrowheads) and vein (green arrowheads) in control embryos, as analysed by expression of *globin* and *gata1* (Figure 6.9B, panels a and b, respectively). In DAPM-treated embryos, however, erythrocytes are seen pooling in the VBI (Figure 6.9B, panels c and d, orange arrowheads). This is consistent with the observation that blood cells are not seen circulating in the tails of living DAPM-treated embryos (chapter 6.2). As the heart is beating normally in DAPM-treated embryos, the lack of circulation is likely due to a vascular defect. As the vitelline vasculature appears normally programmed (Figure 6.7, blue arrowheads), the primitive erythrocytes should be able to leave the VBI and enter circulation. As notch signalling is known to regulate lumen size in murine embryos (Carlson et al., 2005; Kim et al., 2008; Krebs et al., 2000; Miniati et al., 2010; Uyttendaele et al., 2001), DAPM-treated embryos may exhibit a collapsed lumen, thus preventing circulation of primitive erythrocytes throughout the embryo. Analysis of arterial gene expression in DAPM-treated embryos reveals a thinner looking aorta, supporting the idea of a collapsed lumen (Figure 6.7, panels g and h, red arrowheads). *In situ* hybridisation on sections would enable analysis of vessel diameter, and thus reveal whether notch signalling is required for lumenisation in *Xenopus*.

Blood flow is known to be required for the maintenance of HSC markers in the zebrafish DA from 36hpf, after initial cluster emergence, without affecting initiation of *runx1* in the DA (North et al., 2009; Wang et al., 2011). The absence of blood flow in DAPM-treated embryos is not thought to be a contributing factor to the loss of HSC markers at stage 39, for the same reasoning loss of HSC markers in *notch4* morphants is not thought to be due to loss of circulation (see chapter 4.2.5). Furthermore, loss of *runx1* at stage 35 in DAPM-treated embryos, prior to the onset of circulation, suggests that initiation of *runx1* is dependent on notch signalling, and not on circulation of blood cells.

6.8. Discussion

6.8.1. Overview

Notch signalling is widely regarded as essential for vascular and arterial programming, and angiogenic remodelling in mouse and zebrafish embryos, with consequential loss of HSC markers (Burns et al., 2005; Carlson et al., 2005; Gering and Patient, 2005; Hartmann et al., 2002; Herreman et al., 1999; Kim et al., 2008; Krebs et al., 2004; Krebs et al., 2000; Lawson et al., 2001; Lawson et al., 2002; Limbourg et al., 2005; Miniati et al., 2010; Uyttendaele et al., 2001; Yoon et al., 2008). Individual morpholino knockdown of *notch1* and *notch4*, and of target genes *esr7*, *esr10*, *esr1* and *hesr1* has demonstrated an essential role for notch signalling in HSC programming, but not for arterial programming (Chapter 4 and Chapter 5). Whilst it was apparent that the two notch receptors were both absolutely essential for correct HSC programming, it was possible that loss of both notch1 and notch4 signalling would elicit a stronger effect due to redundancy. For example, the notch receptors could have had redundant roles in arterial programming. However, this study clearly demonstrates that a global loss of notch signalling in *Xenopus* embryos from stage 20 results in the loss of HSC markers, whilst arterial programming is essentially normal, thus, there is no difference in these aspects when comparing individual notch receptor knockdown experiments to DAPM treatment experiments. However, DAPM treatment was found to cause a maintenance of endothelial genes in the DA, reflecting the *esr1* morphant phenotype.

6.8.2. A dual contribution of notch receptors for repression of endothelial genes

DAPM-treated embryos generate the same phenotype as *notch1* and *notch4* morphant embryos with regards to loss of HSC markers and normal arterial marker expression. However, in contrast to *notch1* and *notch4* morphant embryos, a maintenance of initiator-containing, TATA-less

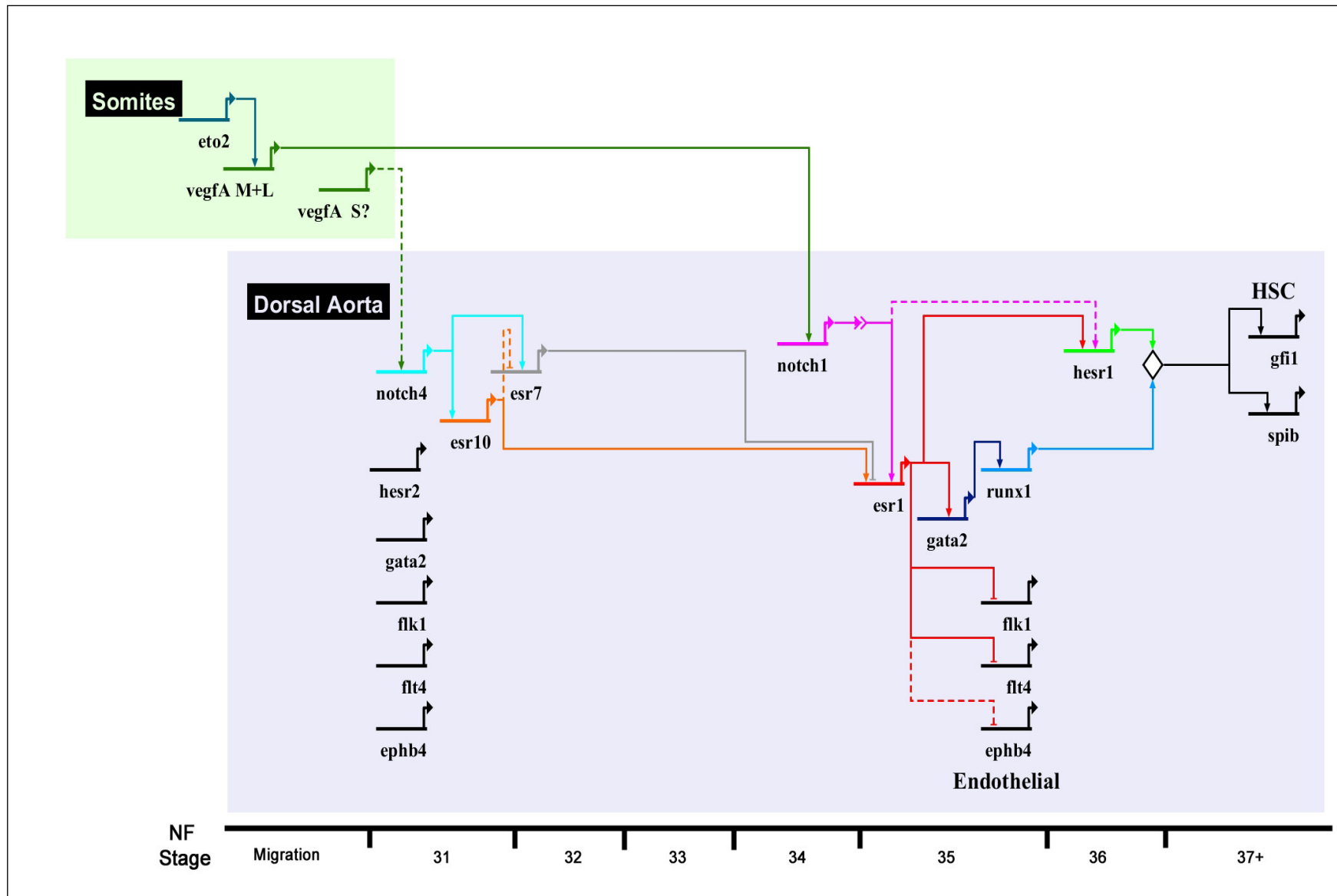
endothelial genes is observed in DAPM-treated embryos. The only other morphant to show a maintenance of these genes is *esr1* (chapter 5.3.5). Thus, DAPM is more closely phenocopying *esr1* morphant embryos, suggesting that the two notch signalling pathways may converge at this point. Notch1 and notch4 signalling pathways both regulate expression of *esr1* (Figure 5.9 and Figure 4.8, respectively). When knocking out *notch4*, or its target gene *esr7*, an increase in *esr1* expression is seen, and thus in turn, *esr1* would still be available to repress the endothelial genes. When knocking out the notch4 target gene, *esr10*, or *notch1*, a downregulation of *esr1* expression can be seen. However, *esr1* expression is not completely lost in either *esr10* or *notch1* morphants: weak expression of *esr1* can be seen in the DA for both morphants (Figure 4.28 and Figure 5.9, respectively), and thus residual *esr1* appears capable of repressing endothelial genes. However, when inhibiting both notch pathways using DAPM, a complete loss of *esr1* expression is seen (Figure 6.2), thus, repression of endothelial genes is not possible, similar to loss of *esr1* through morpholino injection. *Flk1* and *flt4* are known to be repressed by interactions of *hesr1* with the promoter (Henderson et al., 2001; Holderfield et al., 2006; Taylor et al., 2002), but *ephB4* expression has also been reported to be repressed by *hesr1* (Zhong et al., 2001). However, the mechanism of *ephB4* repression is unknown: it may result from *hesr1* interaction with the promoter, as is the case for *flk1* and *flt4*, or it may be a secondary effect.

From this data, and bringing together data from chapters 4 and 5, a complete genetic regulatory network of notch input to HSC and arterial programming is proposed (Figure 6.10). In this model, the two notch signalling pathways converge on *esr1* as a pivotal transcription factor regulating the expression of downstream HSC and endothelial genes. Interestingly, in all morphants and in DAPM-treated embryos, expression of HSC genes is lost, regardless of the level of *esr1* expression. In *notch4* and *esr7* morphants, *esr1* expression is substantially elevated, in *notch1* and *esr10* morphants, *esr1* expression is downregulated, and in DAPM-treated embryos, *esr1* expression is

lost, but expression of *runx1*, and subsequent downstream HSC genes, is always lost. In *hesr1* morphant embryos, expression of *esr1* is normal in the DA, and *runx1* expression is also normal. This suggests that the correct level of *esr1* activity is required for HSC programming, and any alteration, up or down, of this critical level, results in the mis-specification of the haematopoietic programme. Hairy and enhancer of split genes are known to bind both transcriptional repressors and transcriptional activators, to either enhance or repress transcription depending on the situation (comprehensively reviewed in chapter 1.8.2). An increase or decrease in *esr1* may cause an alteration in the levels of free/bound transcriptional activators and repressors. For example, an increase in *esr1* may cause the sequestration of activating transcription factors, preventing them from binding to haematopoietic gene promoters to increase transcription. Alternatively, a loss of *esr1* may reduce sequestration of other repressors, thus allowing these other transcriptional repressors to bind at HSC gene promoters and in turn prevent transcription. These are just two possible ideas: the abundance of hairy and enhancer of split interactions documented to date present dozens of possibilities which could lead to the repression of HSC genes. Furthermore, it has been demonstrated that overexpression and knockdown of *hesr1* in endothelial cells elicits the same effect (Henderson et al., 2001). Whilst altering the levels of *esr1* expression can result in the loss of haematopoietic gene expression, it is clear that only a complete loss of *esr1* can result in the maintenance of endothelial genes. Thus, whether there is a high (*notch4* and *esr7* morphants), normal (wild type and *hesr1* morphants), or low (*esr10* and *notch1* morphants) abundance of *esr1*, repression of initiator-containing, TATA-less genes is still possible. Only in the complete absence of *esr1* expression (*esr1* MO and DAPM treatment) is repression of *flk1* and *flt4* compromised.

Figure 6.10 Schematic diagram showing the proposed genetic regulatory network for notch1 and notch4 controlling HSC specification and endothelial gene repression

Notch4 signalling to *esr7*, *esr10* and *esr1* has been modelled previously (Figure 4.29), as has the notch1 contribution to HSC and endothelial genes (Figure 5.27). The VEGFA contribution to *notch1* expression (Leung, 2010) and to *notch4* expression (proposed, see chapter 4.6.5) is also depicted. Genes without connections at stage 31 (*hesr2*, *gata2*, *flk1*, *flt4*, *ephB4*) are representing expression at this stage unaffected by notch signalling. Dotted line represents proposed interactions. These interactions are proposed to be direct interactions based on discussed evidence, but is not yet proven. Abbreviations: vegfA M+L = vegfA medium and long isoforms, vegfA S = vegfA short isoform.



6.8.3. Chemical inhibition may not provide a complete block of notch signalling

Application of DAPM to zebrafish embryos does not alter expression of *ephrinB2*, whilst in notch morphant or *mib* mutant embryos *ephrinB2* expression is significantly reduced (Martin Gering, personal communication). This may represent a functional disadvantage of chemical inhibition, suggesting only a partial knockdown of notch signalling. In this scenario, low levels of notch signalling may be enough to activate and maintain arterial specification, but not enough to activate the HSC programme, therefore pointing to a gradient of notch signalling controlling arterial and HSC programming. This is similar to the proposition that low levels of *esr1* are enough to repress *flk1* and *flt4* in the DA, but not enough to activate expression of HSC markers.

In *Xenopus*, both *notch1* and *notch4* morphant embryos show normal arterial specification, replicating DAPM experiments, and therefore suggest the lack of an arterial phenotype is a true readout of the requirement for notch signalling in arterial specification. However, in *notch4* morphant embryos, some wild type *notch4* mRNA is retained (Figure 4.2B), and therefore may represent only a partial knockdown of *notch4* in *Xenopus*, and this residual wild type *notch4* mRNA may be enough to activate the arterial programme. Alternatively, as only one allele of *notch1* and *notch4* has thus far been identified in *Xenopus*, it is possible that an unidentified notch allele expressed in the DA is not targeted by the *notch1/notch4* morpholinos, and is able to activate the arterial programme. A further attempt to knockout notch signalling was undertaken using a CSL morpholino to prevent notch transcriptional activation in order to verify if there is a role of notch signalling in arterial programming, but unfortunately at the concentrations of morpholino tested (20-40ng), no affect on haematopoiesis was observed (data not shown).

6.9. Conclusions

In conclusion, the data presented in this chapter reveals that notch signalling is required for HSC programming in the *Xenopus* DA. Combined loss of notch1 and notch4 signalling by DAPM treatment does not affect arterial specification, but causes maintenance of endothelial gene expression. This phenocopies the *esr1* morphant, suggesting that notch1 and notch4 act to regulate *esr1* expression, and that the two notch pathways converge at this point. A gene regulatory network showing the combined notch1 and notch4 signalling pathways for HSC specification and endothelial gene repression has been presented.

Chapter 7. Conclusions

7.1. Summary

Notch signalling has been proposed to drive haematopoietic stem cell programming, and dysregulation of notch signalling is known to cause haematological disorders. Understanding the role of notch signalling in haematopoiesis potentially allows us to find new treatments for haematological malignancies. Furthermore, understanding the signals governing HSC programming is necessary in order to generate HSCs from ES or iPS cells *in vitro*, which could benefit the treatment of haematopoietic diseases. The aim of this thesis has been to understand the role of notch signalling in haematopoietic stem cell formation *in vivo*, and to uncouple it from an earlier arterial requirement for notch signalling.

In this thesis, a specific role for notch signalling in haematopoietic stem cell development is demonstrated. Two notch receptors are expressed in the DA, and both initiate the expression of target genes from the hairy and enhancer of split family. Knockout of *notch4*, or its target genes, *esr7* and *esr10*, or knockdown of *notch1* or one of its target genes, *esr1*, or inhibition of both notch signalling pathways through application of a chemical inhibitor, results in the loss of the haematopoietic stem cell marker, *runx1*, in addition to others, with no loss of arterial programming. Thus, the two notch receptors have non-redundant roles in the programming of HSCs. *Hesr1*, a second target gene of *notch1*, is found to control expression of HSC genes downstream of *runx1*, and is proposed to act in parallel with *runx1* to control HSC specification. The two notch signalling pathways, initiated by *notch1* and *notch4*, converge at the regulation of *esr1* expression. Knockdown of *esr1*, or of both notch signalling pathways via chemical inhibitor application, leads to the maintenance of the endothelial genes, *flk1* and *flt4*, in the DA. Notch signalling appears dispensable for programming of the haemangioblast, migration of cells to the midline, and specification of the aorta. In addition, it is also dispensable for primitive

haematopoiesis, although in some cases the primitive erythrocytes are unable to circulate throughout the embryo, suggesting a possible defect in lumenisation of the vessels. This data shows, for the first time, a specific role for notch signalling in haematopoietic stem cell programming, independent of arterial programming.

7.2. Future work

Throughout this thesis, future work has been discussed. Where morphant and treated embryos do not show circulating erythrocytes, it will be important to carry out *in situ* hybridisation on sections to determine if notch signalling is affecting the lumenisation of the vessels, and therefore preventing erythrocytes from circulating. In addition, identification of the *esr7b* 5'UTR sequence will establish if both pseudoalleles are being targeted by *esr7* MO. As *esr7* morphant embryos demonstrate a loss of HSC marker expression, similar to that of other notch signalling morphant embryos, this suggests the morpholino is targeting both pseudoalleles. However, sequence analysis is essential to confirm this. Expression of *runx1* is absent in all morphant embryos, except *hesr1* morphant embryos, and is also absent in DAPM-treated embryos. Runx1 is essential for haematopoietic cluster formation in mice and *Xenopus* (Fernandez, 2007; Kissa and Herbomel, 2010; North et al., 1999), and thus loss of *runx1* expression in haemogenic endothelium in notch pathway morphants should inhibit haematopoietic cluster formation in the DA. Analysis of cluster formation in stage 43 sectioned morphant embryos is needed to reveal if this is as predicted, and analysis of B-cell development (definitive derivatives of HSCs) using B-cell q-PCR techniques would enable the analysis of long-term haematopoietic defects. Unfortunately, functional assays for HSC activity, such as the long-term reconstitution assay, are currently not technically possible in *Xenopus*. In addition, whilst thymocytes are the first definitive lineage in *Xenopus*, present at day 4, chimeric studies have demonstrated that the primitive precursors in the VBI can contribute

to the T-cell population in *Xenopus* larvae (Bechtold et al., 1992). Thus, whilst analysis of the thymus in zebrafish is indicative of the definitive lineage, in *Xenopus* both primitive and definitive lineages are found here. B-cells are derived exclusively from the definitive lineage (Chen and Turpen, 1995), and q-PCR for immunoglobulin variable heavy chain genes during B-cell development can be used to investigate definitive haematopoietic lineage development (Mussmann et al., 1998).

Notch signalling is a well-studied juxtacrine signalling pathway, and neighbouring cells are thought to express only notch receptors or ligands, never both, except during the initial notch signalling setup, due to lateral inhibition. *In situ* hybridisation is fairly sensitive for detection of gene expression, but unfortunately does not offer exceptional spatial resolution. It would be interesting to use a higher resolution technique to look more closely at the spatial separation of notch ligands and receptors, and to see if expression of either is associated with the selection of haemogenic endothelial cells to form intraluminal haematopoietic clusters. For example, it could be the case that the selection of ventral DA cells expressing notch receptors may form haematopoietic clusters, whilst ligand expressing cells do not. A newly published technique offers fluorescent *in situ* hybridisation at a higher resolution through hybridisation chain reactions (HCR), amplifying the fluorescent signal of expressed genes (Choi et al., 2010). This gives a higher spatial resolution when used in conjunction with confocal microscopy, and allows expression analysis of several genes in one embryo using different fluorophores. This technique has not yet been published in *Xenopus*, but it is believed it should be able to cross easily to *Xenopus* (Tatjana Sauka-Spengler, personal communication). Furthermore, it would be interesting, using a proximal ligation assay, to detect which notch receptor binds to which notch ligand in development of the *Xenopus* DA (Soderberg et al., 2006).

Whilst experiments using NO agonists and antagonists, similar to the experiments carried out in zebrafish, would definitively answer whether or not blood flow is required for *runx1* initiation in *Xenopus*, these experiments have unfortunately not been possible. Application of both SNAP (NO agonist) and L-NAME (NO antagonist) at early stages on *Xenopus* embryos has been carried out, to look at the effect of NO signalling on *runx1* initiation (data not shown). Whilst no effect on *runx1* initiation was observed with either L-NAME or SNAP, with no positive control for the activity of the chemical the experiments were of little use. It is currently not possible to look at the role of NO signalling on *runx1* maintenance in *Xenopus*; to look at stages equivalent to zebrafish 36hpf, where *runx1* expression is dependent on sheer stress/NO, this would be past the recommended home office time point for use of embryos (stage 43/5 days). Furthermore, the *Xenopus* epidermis is becoming extremely thick by this point, and application of chemical inhibitors may not have an effect due to poor penetration of the embryo. An alternative approach that may be beneficial to understanding whether blood flow controls *runx1* initiation is to block the heart beat, thereby preventing blood flow and sheer stress in the embryo. Morpholino depletion of troponin in *Xenopus* may prevent heart beat, as does the silentheart mutation in zebrafish (Sehnert et al., 2002). Similarly, application of BDM has been shown to block heart beat in zebrafish embryos, and may be a useful tool to prevent heart beat in *Xenopus* (Bartman et al., 2004).

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