# The role of BMP signalling in HSC ontogeny

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J.B.S Haldane was once approached by a lady who said:

"*Even given the billions of years that you say were available for evolution, I simply cannot believe it is possible to go from a single cell to a complicated human body*."

He replied:

"*But madam you did it yourself, and it only took you nine months*."

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# Abbreviations

aa: amino acid

AGM: aorta-gonad-mesonephros

ATG: translation start site

aVBI: anterior ventral blood island

BL-CFC: blast colony forming cells

BLAST: basic local alignment tool

Bp: base pair

cDNA: complementary deoxyribonucleic acid

ChIP: chromatin immunoprecipitation

CHT: caudal haematopoietic tissue

DNA: deoxyribonucleic acid

DIG: digoxigenin

DLP: dorsal lateral plate mesoderm

DMSO: dimethyl sulfoxide

EDTA: ethylenediamine tetraacetate

EHT: endothelial haematopoietic transition

ES cell: embryonic stem cell

EST: expressed sequence tag

EtOH: ethanol

FACS: fluorescence activated cell sorting

GFP: green fluorescent protein

GRN: gene regulatory network

HCG: human chorionic gonadotropin

HIV: human immunodeficiency virus

hpf: hours post-fertilisation

HSC: haematopoietic stem cell

ICM: intermediate cell mass

IPS cell: induced pluripotent stem cell

ISS: *in situ* hybridisation on sections

ISV: intersomitic vessels

MeOH: methanol

mRNA: messenger ribonucleic acid

MO: morpholino oligonucleotide

Nt: nucleotides

PCR: polymerase chain reaction

PCV: posterior cardinal vein

PLM: posterior lateral mesoderm

pVBI: posterior ventral blood island

Q-PCR: quantitative polymerase chain reaction

RNA: ribonucleic acid

tBR: truncated BMP receptor

UTR: untranscribed region

VBI: ventral blood island

VEGF: vascular endothelial growth factor

VMZ: ventral marginal zone

WISH: whole-mount *in situ* hybridisation

WT: wild-type

# Abstract

The haematopoietic stem cell (HSC) is found in the adult human bone marrow, where it gives rise to all the circulating blood cells throughout adulthood. Understanding the signalling events that programme these cells during development will improve HSC *in vitro* culture, their generation from embryonic stem cells or induced pluripotent stem cells, and their potential therapeutic application. HSCs bud from the floor of the dorsal aorta and seed the bone marrow via circulation. The precursors to the dorsal aorta and HSCs are called haemangioblasts, which are found in the dorsal lateral plate mesoderm in *Xenopus*. The knowledge of the location of these precursors allows their programming to be studied in detail during embryonic development. A key pathway implicated in the programming of HSCs is the BMP signalling pathway.

Here, using both a small molecule inhibitor and a transgenic *Xenopus* line, BMP signalling has been inhibited post-gastrulation without perturbing the gross morphology of the embryo. This has shown that BMP signalling is required for HSC programming in the dorsal lateral plate mesoderm via the expression of a critical haematopoietic transcription factor, *gata2*. Morpholino knockdown of *evi3* has revealed it to be essential for HSC programming in the dorsal lateral plate mesoderm, where it is required for the expression of *gata2*. Furthermore, as *evi3* is known to bind to the active BMP signalling complex, and as *evi3* knockdown phenocopies post-gastrulation BMP inhibition, *evi3* appears to be required for BMP signalling to initiate *gata2* expression in the DLP.

Taken together, the findings presented here demonstrate an essential post-gastrulation role of BMP signalling and Evi3 for programming HSCs in *Xenopus*.

# Chapter 1
# Introduction

# Chapter 1 : Introduction

## 1.1 Haematopoiesis

The word haematopoiesis comes from the ancient Greek, ‘haima’ meaning ‘blood’ and ‘poiesis’ meaning ‘to make’. The ancient origin of this word reflects our longstanding fascination with the study of blood and its production by the body. The haematopoietic system is responsible for generating a number of different circulating cell types, each of which performs a specialised function in the body. Red blood cells transport oxygen to all cells of the body. Platelets prevent excessive bleeding following injury and are derived from megakaryocytes. Granulocytes (neutrophils, basophils and eosinophils) and macrophages fight infections. B and T lymphocytes are the circulating cells of the adaptive immune system. B-lymphocytes produce antibodies, while T-lymphocytes can directly kill foreign, infected or cancerous cells. Without these circulating cells most organisms die very early in embryogenesis.

As the lifespan of these haematopoietic cells is limited (human erythrocytes last for between 110 and 120 days (Shemin and Rittenberg, 1946) whilst some human leukocytes live for only approximately 9 days (Kline and Cliffton, 1952)), the haematopoietic system must be able to continually replace these cells. To fulfil the requirement of being able to produce a number of different cell types and to continue to do so for the life of an organism, the cellular origin of these haematopoietic cells must have two key features. These cells must be able to differentiate into multiple different cell types and these cells must have the ability to self-renew. A cell with both of these characteristics is called a haematopoietic stem cell (HSC).

## 1.2 The haematopoietic stem cell (HSC)

The bone marrow was identified as the site of an "unknown component vital for blood production" through high dose radiation experiments; this unknown component is now known to be the HSC. When the femur of a mouse, and the bone marrow within, was protected from radiation, the whole organism appeared to be protected and was able to survive an otherwise lethal dose of radiation (Jacobson et al., 1949). The later intravenous injection of bone marrow cells from a healthy mouse into a lethally irradiated mouse proved that recovery was in fact due to a specialised cell, which resides in the bone marrow and is able to rescue the full haematopoietic system of a mouse (Lorenz et al., 1951; Lorenz et al., 1952; Nowell et al., 1956). To this day the serial transplantation of cells into lethally irradiated mice remains the gold standard test for HSCs (Muller et al., 1994; Medvinsky and Dzierzak, 1998).

The first successful bone marrow transplantation was reported in 1968 and was used to save the life of a child with X-linked lymphopenic immune syndrome (Gatti et al., 1968). This confirmed that the human bone marrow also contained HSCs capable of replenishing the haematopoietic compartment of a recipient. Today, bone marrow transplantation is used to treat a number of different blood disorders, including leukaemia, severe aplastic anaemia (Ades et al., 2004), and, more recently, HIV (Hutter et al., 2009), although the latter is still an observation rather than a viable treatment plan. The HSC resides in the adult human bone marrow, where it self-renews and produces different blood cells via more lineage-restricted intermediate progenitors (Figure 1.1).

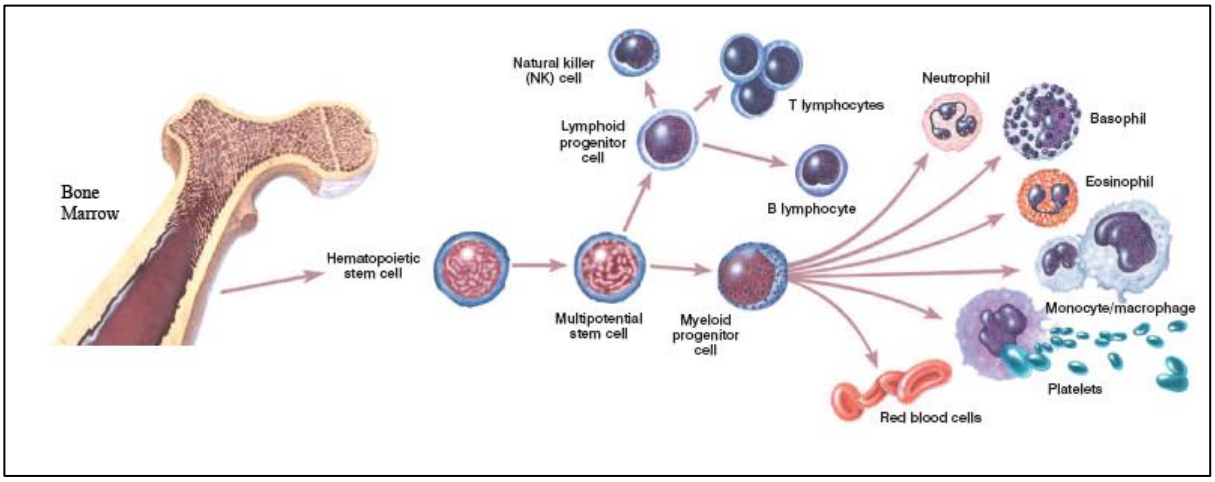



**Figure 1-1: The adult haematopoietic stem cell niche and progeny**

The haematopoietic stem cell resides in the adult bone marrow. This cell is able to produce all the circulating blood cells through more lineage-committed haematopoietic progenitors. Image taken from (Jos Domen, 2011).

The adult bone marrow is not, however, the site of origin for the HSC, which is first detected in the midgestation embryo. The embryonic programming of the HSC is an excellent paradigm for studying the signalling events and transcription factors required for stem cells and the importance of these events in early embryogenesis (Orkin and Zon, 2008). Understanding the programming events involved in HSC ontogeny will prove important in the *in vitro* expansion of these cells for therapeutic purposes, including stem cell transplantation, and potentially *in vitro* production of blood cells for transfusions (Sauvageau et al., 2004). It has also been noted that the transcription factors and signalling events involved in programming the HSC during embryonic development are often deregulated during leukaemogenesis and may prove to be useful therapeutic targets (Wong et al., 2008).

### 1.3 Two waves of vertebrate haematopoiesis

During embryogenesis, two distinct waves of haematopoiesis occur. The first is transient and occurs in the human, mouse and chicken yolk sac (Dieterlenlievre, 1975; Silver and Palis, 1997; Godin and Cumano, 2002), the zebrafish intermediate cell mass (ICM) (Willett et al., 1999a), and the *Xenopus* ventral blood island (VBI) (Ciau-Uitz et al., 2000a). The main function of this first wave of haematopoiesis is to produce erythrocytes to supply the embryo with oxygen. The large nucleated appearance of red blood cells produced in this initial wave led to the term ‘primitive haematopoiesis’, as these erythrocytes resemble those of non-mammalian ‘primitive’ vertebrates (Kovach et al., 1967). It has subsequently been shown that, once in circulation, human embryonic erythrocytes do eventually enucleate, however, the term has stuck and is now widely used (Kingsley et al., 2004; Fraser et al., 2007; McGrath et al., 2008). Primitive macrophages are also produced in this initial wave, and are

required to prevent infection and to clear apoptosing cells during tissue remodelling (Cline and Moore, 1972; Hopkinson-Woolley et al., 1994; Naito et al., 1996).

As the embryo develops, a second, or definitive, wave of haematopoiesis produces the HSC and all the blood cell types required by the adult organism. As the HSCs produced in this definitive wave will generate all the haematopoietic progenitors and blood cells for the whole life of the organism, the ontogeny of HSCs is of great interest to the haematopoietic field. Understanding the events involved in making an HSC, how it makes cell fate decisions and what happens when these events are perturbed, will greatly advance our ability to expand these cells *in vitro* and to use them for therapy.

The adult HSC emerges in close association with the endothelial lineage and was first found in the midgestation aorta-gonad-mesonephros (AGM) region in mouse (Muller et al., 1994), specifically in the dorsal aorta (Medvinsky et al., 1996) and the vitelline and umbilical arteries (de Bruijn et al., 2000; de Bruijn et al., 2002; Ivanovs et al., 2011; Gordon-Keylock et al., 2013). Clusters of haematopoietic cells can clearly be seen budding from the floor of the dorsal aorta and this process is well conserved across vertebrate species including human, pig, chick, quail, zebrafish and *Xenopus* (Dieterlen-Lievre and Martin, 1981; Garcia-Porrero et al., 1995; Godin et al., 1999; Ciau-Uitz et al., 2000a; Ciau-Uitz et al., 2000b; Gering and Patient, 2005) (Figure 1.2).

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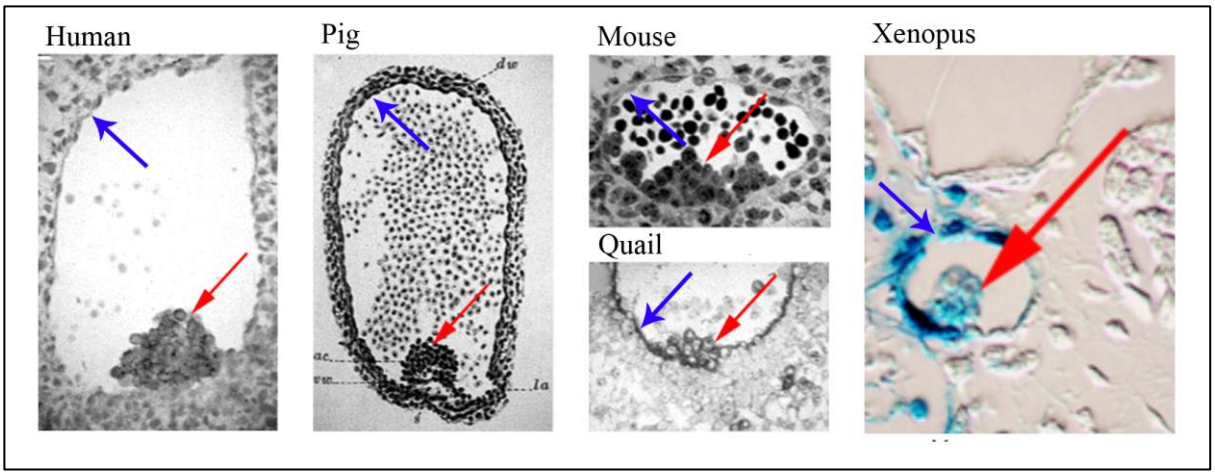



**Figure 1-2: Haematopoietic clusters budding from the floor of the dorsal aorta**

Haematopoietic budding from the floor of the dorsal aorta is well conserved across vertebrate species. This image shows transverse sections through the dorsal aortae of human, pig, mouse, quail and *Xenopus* embryos with dorsal at the top and ventral at the bottom. Haematopoietic clusters are labelled with a red arrow whilst the dorsal aorta is labelled with a blue arrow. Image modified from (Dieterlen-Lievre et al., 2002)

In addition to the primary sites of haematopoiesis, some of the haematopoietic progenitors and their derivatives colonise secondary haematopoietic tissues such as the thymus and bone marrow, where the adult blood stem cell will remain for the adult life of the organism. These secondary haematopoietic sites provide additional signals required for the maturation of the HSC and its progenitors and provide a niche, which supports these cells. In human and mouse development, secondary sites of haematopoiesis include the foetal liver and ultimately the thymus and bone marrow. The foetal liver is colonised at approximately 10.5 weeks post-fertilisation in humans and at E11 in mice (Kumaravelu et al., 2002; Dzierzak et al., 2005; Gekas et al., 2005; Ottersbach and Dzierzak, 2005), whilst the bone marrow is colonised prior to birth.

The seeding of secondary haematopoietic tissues is also seen in other models of vertebrate development. In zebrafish the intermediate cell mass (ICM) is the initial site of haematopoietic activity, producing blood cells that begin to circulate after only 26 hr post-fertilisation (hpf) (Willett et al., 1999b). Adult haematopoietic cells bud from the floor of the dorsal aorta at 30 hpf where they migrate to the caudal haematopoietic tissue (CHT) (Bertrand et al., 2010). Lymphoid development is seen in the thymus from day 4 and shortly afterwards the kidney marrow becomes the adult niche of the HSC (Kissa and Herbomel, 2010b). The *Xenopus* embryo initiates primitive haematopoiesis from the ventral blood island (VBI). This tissue can be separated into the anterior VBI (aVBI) and the posterior VBI (pVBI) (see section 1.4.3). The aVBI makes a small number of erythrocytes and a large number of myeloid cells (Tashiro et al., 2006; Costa et al., 2008a; Walmsley et al., 2008). The pVBI makes erythrocytes and a small number of T cells (Maeno et al., 1985b; Turpen and Smith, 1985; Rollins-Smith and Blair, 1990; Turpen et al., 1997a). At stage 43 in *Xenopus,* adult HSCs bud from the dorsal aorta and move to secondary sites. In post-morphogenesis the liver is the main haematopoietic organ, whilst the spleen, thymus and

bone marrow play a lesser role. The liver is where almost all of the red blood cells and thrombocytes are made in the adult along with eosinophils and monocytes. The bone marrow plays a small role in adult haematopoiesis, as it is the site where neutrophils are made, mainly in the femur. The thymus and spleen are the sites of lymphoid production and together these four sites maintain the haematopoietic system of the adult. (Kau and Turpen, 1983; Maeno et al., 1985a; Hadji-Azimi et al., 1987; Turpen and Smith, 1989; Rollinssmith and Blair, 1990) (Figure 1.3).

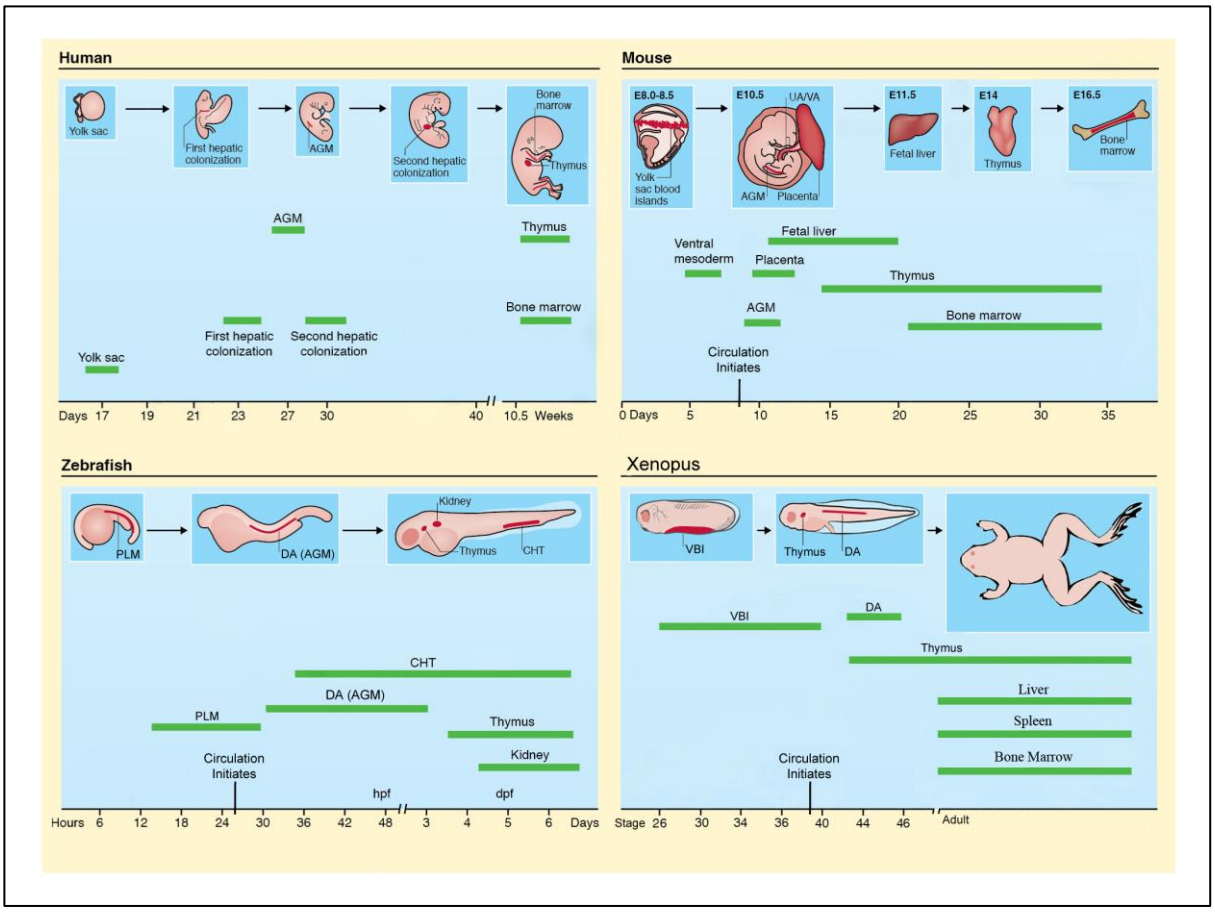



Figure 1-3: Vertebrate sites of haematopoiesis

Scheme shows sites of haematopoietic activity in the developing embryos leading to the establishment of the adult haematopoietic niche. Green bars show the approximate time and duration of haematopoietic activity at these sites. AGM: aorta gonad mesonephros, PLM: posterior lateral mesoderm, DA: dorsal aorta, CHT: caudal haematopoietic tissue, VBI: ventral blood island.

Image modified from (Jagannathan-Bogdan and Zon, 2013) with the addition of information from (Kau and Turpen, 1983; Maeno et al., 1985a; Turpen and Smith, 1989; Rollinssmith and Blair, 1990; Bechtold et al., 1992; Ciau-Uitz et al., 2000b; Walmsley et al., 2002a; Patient et al., 2003; Kissa et al., 2008; Kissa and Herbomel, 2010b; Lee et al., 2013).

### 1.3.1 The embryonic haemangioblast: a common origin of blood and endothelium

The close physical and temporal embryonic development of blood and endothelium has been recognised for almost a century (Sabin, 1917). Later cell culture assays saw blood and blood vessels growing from cultured mouse primitive streak cells, which suggested the presence of a cell with the potential to make both blood and endothelium, termed a 'haemangioblast' (Murray, 1932). Since then, gene expression analysis, *in vitro* differentiation assays and lineage tracing experiments have provided additional evidence for the existence of such a cell. Overlapping expression of different blood and endothelial genes has been shown in several developmental models, including mouse, zebrafish and *Xenopus* (Fina et al., 1990; Millauer et al., 1993; Kallianpur et al., 1994; Fong et al., 1995; Asahara et al., 1997; Kabrun et al., 1997; North et al., 1999; Brown et al., 2000; Walmsley et al., 2002a). Many of these genes have subsequently been shown to be essential for the development of both lineages (Godin and Cumano, 2002; Patterson et al., 2005; Patterson et al., 2007; Liu et al., 2008). These findings solidify the close embryonic relationship between blood and endothelium.

*In vitro* experiments have shown that mouse embryonic stem cells, under specific conditions, can form blast colony-forming cells (BL-CFCs), which are cells that have the potential to form both blood and endothelium (Choi et al., 1998; Palis et al., 1999). BL-CFC-like cells have also been isolated from mid-streak stage mouse embryos (embryonic day 7.5), giving additional strength to the existence of such cells during embryogenesis (Huber et al., 2004). *In vivo* lineage tracing experiments have proved more controversial. When single cells of the gastrulating zebrafish were tagged and followed using a photo activated fluorescein, a rare cell, present in ventral mesoderm, was identified as giving rise to both blood and endothelial cells (Vogeli et al., 2006). In contrast to this, *in vivo* lineage tracing experiments in mouse are

yet to identify such a cell, and cells in the yolk sac have been shown to give rise to either blood or endothelium but not both (Ueno and Weissman, 2006). One explanation for this discrepancy is that although a haemangioblast exists in mouse, no cell division takes place prior to lineage commitment. A lack of cell division of the haemangioblast would explain the close relationship between the tissues, the overlapping expression of genes and the inability to label a single cell in the embryo that gives rise to both blood and endothelium. However, as single cell labelling of a rare and transient population in the developing mouse embryo is fraught with technical challenges, it is fair to suggest that an absence of evidence is not evidence of absence, and such a cell may at some point be identified.

### 1.3.2 The adult haematopoietic stem cell and haemogenic endothelium

Labelling chick aortic endothelial cells using DiI acetylated low-density lipoprotein also labelled the haematopoietic clusters, suggesting that they were of endothelial origin (Jaffredo et al., 1998). Retroviral labelling of aortic endothelial cells also labelled these haematopoietic clusters (Jaffredo et al., 2000). This led to the theory that endothelial cells within the dorsal aorta transdifferentiate into HSCs from a cell with endothelial characteristics. Cells with this potential are called haemogenic endothelium and the transition from haemogenic endothelium to the HSC is termed the endothelial-to-haematopoietic transition (EHT). Subsequent to this initial hypothesis, time-lapse confocal imaging in zebrafish and mouse sections has allowed the budding of cells from the dorsal aorta to be visualised (Bertrand et al., 2010; Boisset and Robin, 2010; Boisset et al., 2010; Kissa and Herbomel, 2010a).

Transcription factor Runx1 is required for EHT. Runx1 is expressed in the ventral wall of the dorsal aorta, the haematopoietic clusters and the underlying mesenchyme (Cai et al., 2000; de Bruijn et al., 2002; North et al., 2002). When EHT is imaged in zebrafish embryos where

*runx1* has been inhibited, few EHT events are observed and those that do occur are incomplete and result in cell death immediately after budding (Kissa and Herbomel, 2010b). Runx1 has also been shown to be required for HSC emergence in mouse, chick and *Xenopus* embryos and is often used as a marker for HSCs in the dorsal aorta (Mukouyama et al., 2000; Chen et al., 2009).

## 1.4 *Xenopus laevis* as a model organism

### 1.4.1 The history of *Xenopus* as a model organism:

*Xenopus laevis* has long been used as a model organism for studying vertebrate embryonic development. The analysis of cell fate and embryonic patterning started with Wilhelm Roux, who destroyed one cell of the two-cell stage *Xenopus rana* embryo (Roux, 1888). The result was that only half of an embryo developed: either the right or the left side. This work from the late 1880s showed that, in the *Xenopus* embryo, cell fate decisions were made very early and that simple manipulations could be used to study these cell fate decisions. However, it wasn't until the 1930s that *Xenopus laevis* numbers increased in captive colonies for research. This was due to the observation that an adult female *Xenopus* would superovulate if injected with the urine from a pregnant woman (Bellerby, 1934; Robson, 1934; Shapiro and Zwarenstein, 1934). In the 1940s and 1950s this was the only available pregnancy test and *Xenopus* colonies were kept at many hospitals for this purpose (Newton, 2004). This use of frogs as pregnancy tests and the discovery that the hormone chorionic gonadotrophin was responsible for superovulating the frogs, gave researchers access to large numbers of

*Xenopus* eggs, which when fertilised with sperm from a male, would develop externally, in relative synchronicity, allowing for close observation and manipulation.

*Xenopus* embryos lent themselves well to lineage tracing and grafting experiments due to their large size (approximately 1 millimetre in diameter), their pigmentation and their *ex-utero* development. This allowed researchers to test when cell fate decisions were made in different cells during development and to analyse the role of signalling between tissues. *Xenopus* embryology has facilitated the discovery of germ layers (Nieuwkoop, 1985; Smith, 1987; Smith et al., 1990) and the exploration of developmental processes such as primordial germ cell formation (Blackler, 1958; Blackler, 1960; Blackler, 1962)..

### *1.4.2* Gene targeting in *Xenopus laevis*

Compared to other model organisms such as *Drosophila*, zebrafish or mouse, *Xenopus* has fallen behind in the development of genetic tools. This is due to *Xenopus laevis* being allotetraploid; having four copies of every gene (Graf and Kobel, 1991). The result of this is that the genomic sequencing of *Xenopus laevis* is still not complete at the time of writing and knocking out a gene requires the removal of all four copies of the gene targeted. Furthermore, as creating transgenic *Xenopus* is inefficient and *Xenopus* take at least a year to grow to sexual maturity, few transgenic lines exist (Chesneau et al., 2008). However, despite the lack of a finished genomic sequence, a large expressed sequence tag (EST) database exists. This allows for the identification of homologues from other species and for the production of RNA probes for use in *in situ* hybridisation analysis of gene expression.

*Xenopus* embryos can be manipulated in a number of ways. The embryo can easily be injected with DNA (Matthews and Colman, 1991), mRNA (Soreq, 1985), proteins (Dingwall

et al., 1982) and even polysaccharides (Paine et al., 1975). More recently, antisense morpholino oligonucleotides (morpholinos) have been injected into the *Xenopus* embryo to inhibit the expression of a specific gene of interest (Heasman et al., 2000). These are modified nucleic acids where the ribose ring is replaced with a morpholino ring and the phosphate backbone is replaced with one composed of phosphorodiamidate bonds. The result of these modifications is that short sequences of antisense morpholino (typically 25 bases) are able to bind to mRNA and are resistant to nucleases that would otherwise degrade them. This binding, when targeted to the 5' UTR of a gene, prevents ribosomal binding and so inhibits translation of that gene. Alternatively if a morpholino is targeted to a splice boundary, then this can prevent the splisosome from binding and can prevent proper splicing of the target (Heasman, 2002; Eisen and Smith, 2008) (figure 1.4).

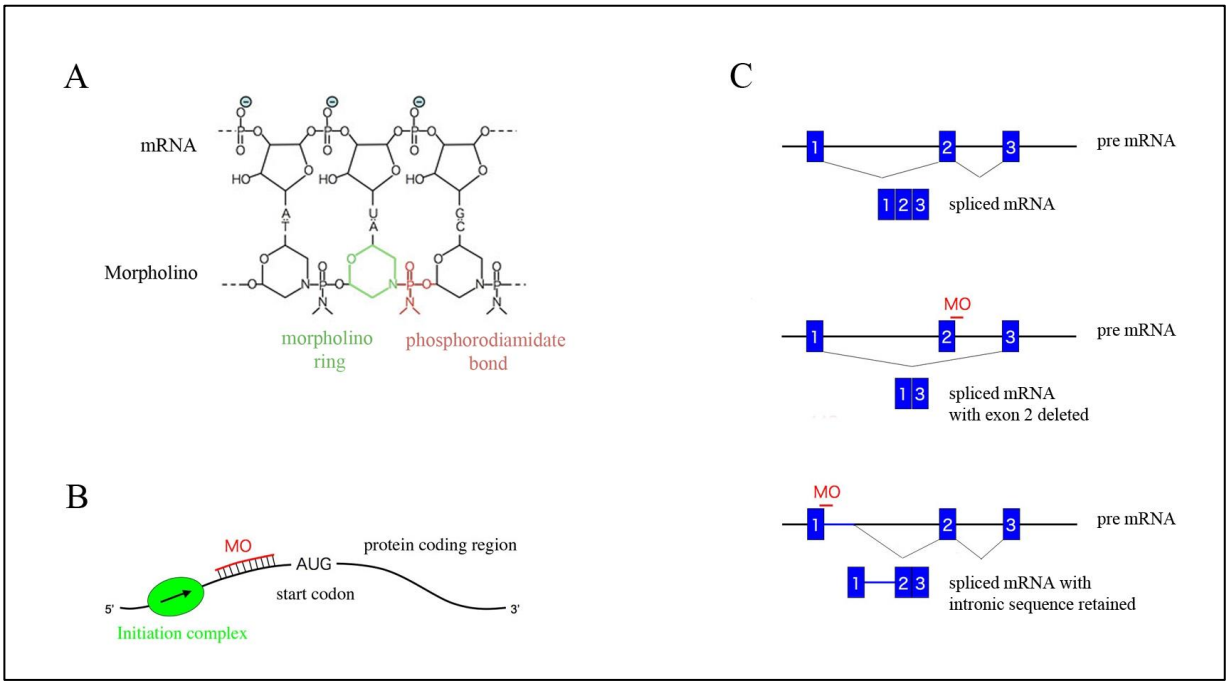



**Figure 1-4: Morpholino structure and function**

A) Diagram showing the structure of both mRNA and morpholino oligonucleotides. In green the morpholino ring replaces the ribose ring of mRNA and in red the phosphorodiamidate backbone replaces the phosphate backbone of mRNA. Image modified from (Yoshimura, 2012)

B) Translation-blocking morpholinos bind at, or close to, the ATG of the target mRNA. This binding physically inhibits the translation machinery and results in decreased levels of protein production of that target. Image modified from (Yoshimura, 2012)

C) Typical mRNA processing results in the exonic coding regions of the pre-mRNA combining to form the mature mRNA. Morpholino targeting of splice boundaries can physically inhibit binding of the splicosome and can result in the loss of exonic sequence or the inclusion of intronic sequences, which can produce truncated products when the frame is shifted. Image modified from (Yoshimura, 2012)

Tissue specificity of morpholinos, and other injected substances, can be achieved by injection into specific regions of the early embryo (Heasman, 2002). Well-defined regions or tissues of the developing embryo can be attributed to individual cells of a regularly cleaving 32-cell stage embryo (Dale and Slack, 1987b; Dale and Slack, 1987a; Kline and Moody, 1987; Moody, 1987b; Moody, 1987a). In addition to morpholinos, small molecule inhibitors are used to inhibit specific pathways during development. The *ex utero* development of *Xenopus* embryos allows for the addition of these small molecule inhibitors and although lacking spatial specificity, temporal specificity can be achieved, inhibiting pathways during specific developmental processes (Wheeler and Liu, 2012).

### 1.4.3 *Xenopus* as a model for blood development

*Xenopus* embryos are particularly suited for the study of blood development. As oxygen is able to diffuse through the epidermis of the developing embryo they do not require a functioning cardiovascular system until later in development relative to other model systems such as mouse. This allows the in depth analysis of phenotypes resulting in a loss of blood or vasculature. In addition, unlike other developmental models, the source of primitive and definitive blood is spatially distinct through embryonic development (Ciau-Uitz et al., 2000b), enabling the developmental requirements for primitive and definitive blood formation to be probed independently. This was first shown through transplantation experiments between diploid and triploid embryos (Turpen et al., 1981a) and later by lineage tracing (Ciau-Uitz et al., 2000a). These experiments also showed that the ventral blood island (VBI), which is the equivalent of the yolk sac in humans and mice, is comprised of two distinct populations, the anterior and posterior VBI. Briefly, *LacZ* mRNA injection into the C1 and D1 dorsal blastomere cells of a regularly dividing 32-cell *Xenopus* embryo labelled

the anterior VBI and *lacZ* mRNA injection into the ventral blastomere cell D4 labelled the posterior VBI (figure 1.5).

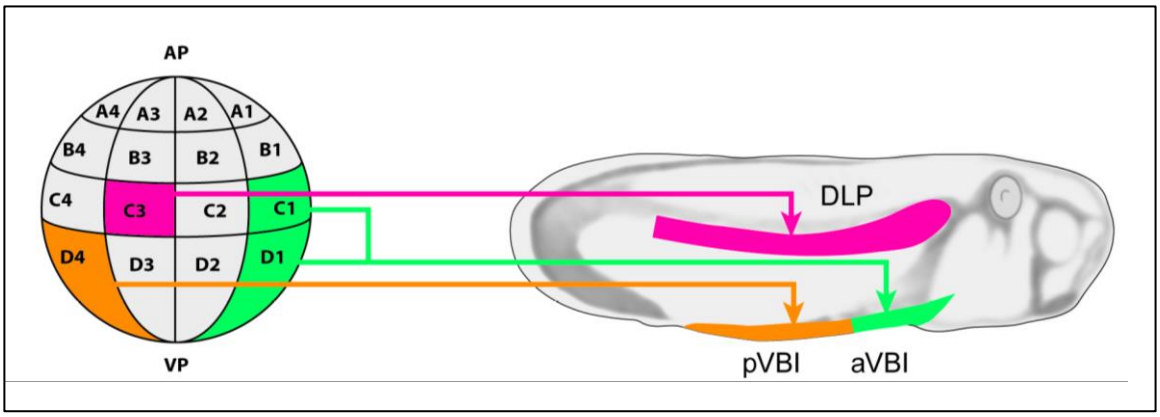



**Figure 1-5: Spatially separate origins of primitive and definitive blood**

Image shows which cells of the 32-cell Xenopus embryo contribute to haematopoietic tissues during embryonic development. C1 and D1 labelled in green give rise to the anterior ventral blood island. D4 labelled in orange gives rise to the posterior ventral blood island and C3 labelled in pink will form the dorsal lateral plate, and later the dorsal aorta, the underlying mesenchyme and the haematopoietic clusters. Image taken from (Ciau-Uitz et al., 2010).

Labelling of the C3 blastomere labels the dorsal aorta, the underlying mesenchyme and the haematopoietic clusters. Prior to this, during development the precursors of the dorsal aorta and HSCs are seen in the dorsal lateral plate mesoderm (figure 1.5). Transplantation experiments were used to show that cells from the dorsal lateral plate mesoderm (DLP) contributed to the adult haematopoietic system in *Xenopus* (Turpen et al., 1981b; Kau and Turpen, 1983; Turpen et al., 1997b). All dorsal aorta endothelial cells have been shown to derive from lateral plate mesoderm in mouse and this lateral plate mesoderm is also responsible for generating all HSCs found in the adult bone marrow (Wasteson et al., 2008; Zovein et al., 2010).

The transcription factor *fli1* is expressed in the DLP and is required for both blood and endothelial development (Ciau-Uitz et al., 2013). Careful analysis of sectioned embryos stained for *fli1* shows these cells migrate to the midline where they form the dorsal aorta below the hypocord (Cleaver and Krieg, 1998; Ciau-Uitz et al., 2000b; Walmsley et al., 2002b). Prior to this, *fli1* sits at the top of a gene regulatory network required for programming cells in the DLP (Liu et al., 2008). Temporal and epistatic analysis via morpholino knockdown has allowed for a detailed gene regulatory network to be proposed for programming cells in the *Xenopus* DLP (Ciau-Uitz et al., 2013). *Fli1* is required to initiate expression of *gata2* in the DLP. Gata2 is a haematopoietic transcription factor, which when knocked-out in mice results in death due to anaemia at embryonic day 10.5/11.5 (Tsai and Orkin, 1997). In combination with *fli1, gata2* is required for the expression of the Vegf receptor *flk1* and the Ets transcription factor *etv2*. *Flk1* expression in the DLP allows these cells to sense and respond to the paracrine Vegfa secreted by the adjacent somites. This paracrine signal is converted into a 'private' Vegfa autocrine loop, by initiating *etv6* expression, which then stimulates Vegfa production in the DLP. Etv2 has been identified in mouse as having a key role in yolk sac haematopoiesis and endothelial development (Lee et

al., 2008; Kataoka et al., 2011) and, although not required for erythroid development in zebrafish, its overexpression rescues the zebrafish *cloche* mutant, which has no blood or endothelium, suggesting that the role of etv2 in zebrafish is performed by another closely related gene (Xiong et al., 2008). After stage 24, *gata2* no longer requires *fli1* for maintenance and this job moves to *etv2.* At this point the Vegfa-dependent pathway synergises with the Vegfa-independent, *fli1, gata2, etv2* pathway to initiate expression of *scl* and *hex. Lmo2* expression also starts at this point and requires *gata2* and *etv2* in a Vegfa-independent manner. The mouse knockouts for Scl and Lmo2 both die at embryonic day 9.5/10.5 with haematopoietic precursors having never been specified (Shivdasani et al., 1995; Porcher et al., 1996; Yamada et al., 1998b). Crucially, interference with the expression of any of the genes in the DLP gene regulatory network results in the misprogramming of the adult haemangioblast in the DLP and as a result HSCs are not formed from the dorsal aorta (Figure 1-6, Figure 1-7). *Fli1* is clearly a key regulator in haematopoietic programming in the DLP as it sits at the top of both the Vegfa-dependent and -independent pathways, however it is not clear what regulates *fli1* expression in these cells. Finding what signals are required to initiate this gene regulatory network (Figure 1-7) in the DLP may inform our protocols for HSC generation *in vitro* and may provide a novel drug target(s) for the treatment of leukaemia.

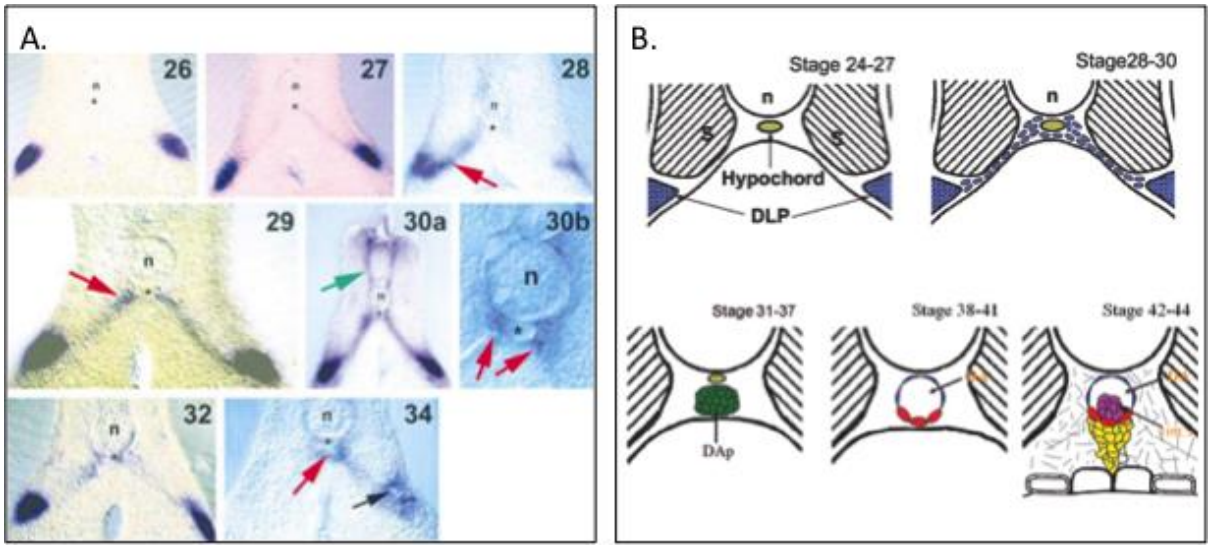



**Figure 1-6: The migration of *fli1*-expressing cells from the DLP to the midline**

A) *In situ* hybridisation staining of *fli1*-expressing cells migrating from the DLP to the midline. B) Schematic representation of cell migration from the DLP to the midline and the formation of the DA. At stages 26 and 27, *fli1*-expressing cells are clearly seen in the DLP. At stage 28 cells begin to migrate from the DLP to the midline (A. red arrow). At stage 30, *fli1*-expressing cells can be seen migrating from the dorsal fin to the midline (A. green arrow) in response to Vegf secretion from the hypochord. No migrating cells are seen at stage 32, suggesting that migration is complete at this stage. At stage 34, the migrated cells at the midline have formed a cord that has started to luminise and form the dorsal aorta (A. red arrow). The *fli1*-expressing cells that do not migrate form the posterior cardinal vein (A. black arrow, stage 34). Stages are shown on the top right corner of each image. Notochord (n) and hypochord (A. black asterisk, *) are indicated on each image. Images taken from (Ciau-Uitz et al., 2000b and from personal communication)

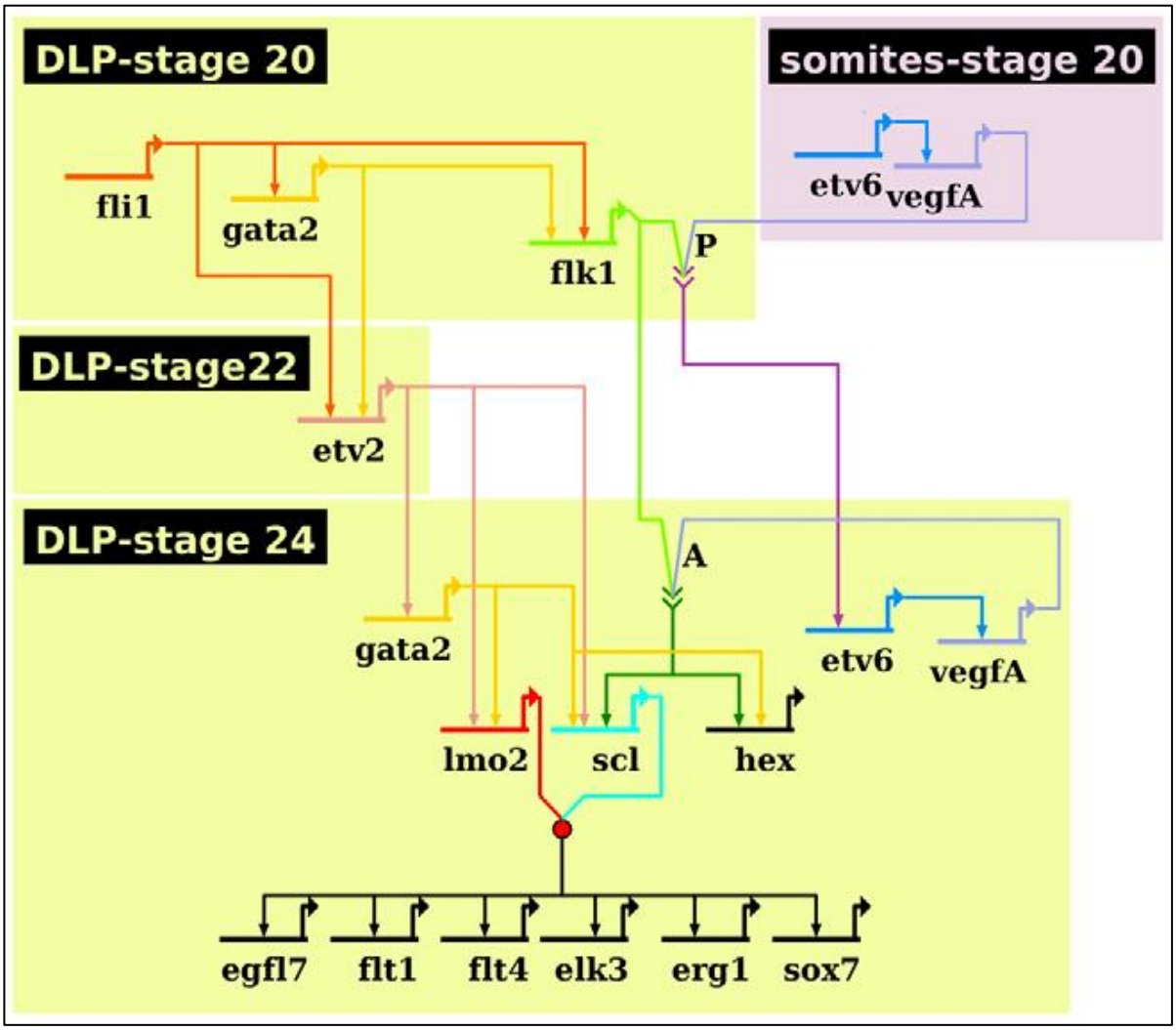



**Figure 1-7: The dorsal lateral plate gene regulatory network**

Genetic network diagram summarising the known requirements for programming in the dorsal lateral plate. The timing and tissue of expression is shown by the position of the gene name with gene inputs shown as arrows. Note that arrows do not necessarily signify direct interactions but indicate requirements for expression. Where two gene products physically interact for the expression of a target their arrows converge on chevrons or a circle. Where a gene is shown twice this indicates a change in its regulation (*gata2* is initiated by *fli1* but later requires *etv2* for maintenance). Image taken from (Ciau-Uitz et al., 2013)

## 1.5 BMP signalling

### 1.5.1The TGF-β superfamily and an overview of BMP signalling

The TGF-β superfamily consists of 4 main signalling pathways; TGF-β, BMP, activin and nodal. The signalling cascade from surface to nucleus is comparable between these 4 pathways, with different proteins having the same or similar roles in each. Secreted, diffusible ligands bind to serine/threonine transmembrane receptors. These receptors transfer the extracellular stimuli into an intracellular signalling cascade by phosphorylating receptor Smads (R-Smads). These R-Smads then bind the common Smad, Smad4, and together bind DNA with cofactors to modulate gene expression of targets. These pathways are regulated at multiple stages in the signalling cascade, by diffusible inhibitors that bind and sequester ligands and inhibitory Smads (e.g. Smad6), which interfere with DNA binding or protein-protein interactions. The BMP signalling pathway is the focus of this thesis with an emphasis on its role in haematopoietic programming during development (figure 1.8).

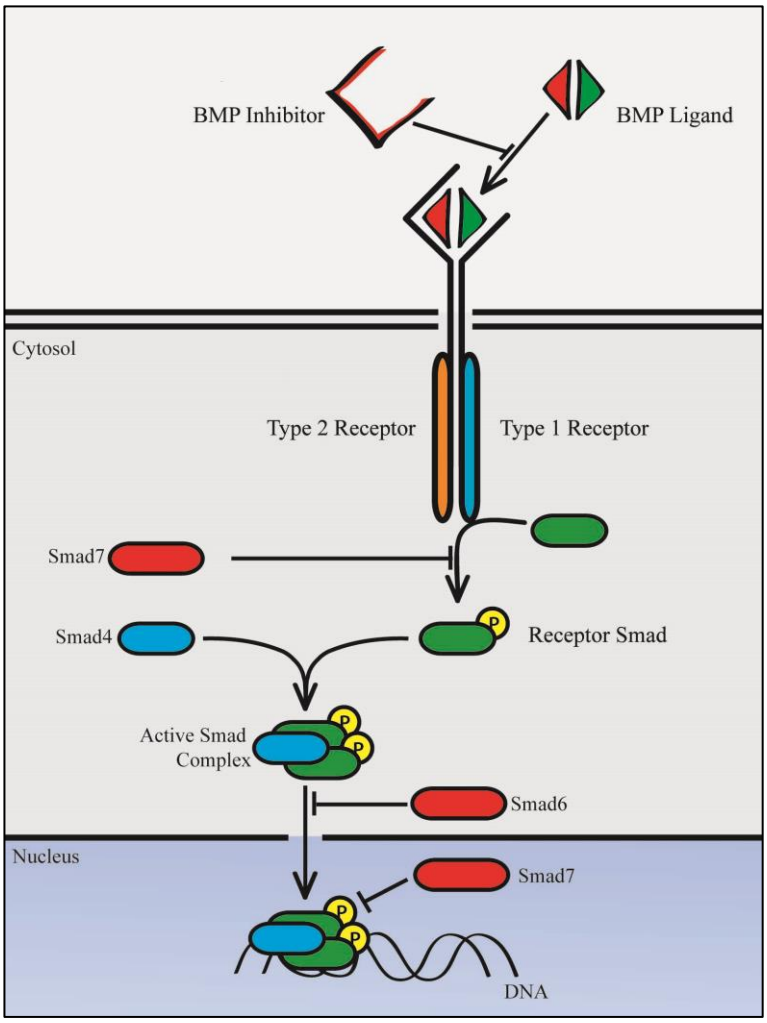



**Figure 1-8: The BMP signalling pathway.**

Schematic representation of the BMP signalling cascade, reviewed in (Reddi and Reddi, 2009). BMP ligands bind each other to form a dimer that is then bound by type I and type II receptors in the plasma membrane. This binding stabilises a complex consisting of two BMP ligands, two type I receptors and two type II receptors. This allows the constitutively active type II receptor to phosphorylate the type I receptors, thereby activating its serine/threonine kinase domain. The external stimulus is then internalised via the phosphorylation of the receptor-activated smads (R-Smad), which then bind to the co-smad, Smad4, and bind DNA. In doing so it recruits other cofactors and inhibits or promotes gene expression of target genes. The inhibitory smads, Smad6 and Smad7, inhibit signalling at multiple points, by interfering with binding events and labeling receptors for degradation.

### 1.5.2 The role of BMP signalling in haematopoiesis

Maternally-expressed *bmp4* is required to pattern the developing ventral mesoderm (Dale et al., 1992; Jones et al., 1992; Ueno et al., 1992). As all haematopoietic tissue comes from mesoderm, and as BMP signalling is required for mesodermal patterning, it logically follows that BMP signalling is required for blood development. There are multiple reports of increased blood marker expression where BMP signalling has been induced (Dale et al., 1992; Maeno et al., 1994; Johansson and Wiles, 1995; Maeno et al., 1996; Zhang and Evans, 1996) and the opposite is true when BMP signalling is inhibited (Graff et al., 1994; Maeno et al., 1994; Walmsley et al., 1994; Maeno et al., 1996; Zhang and Evans, 1996).

Mouse, monkey and human *in vitro* stem cell assays have all shown that BMP is required for the induction of mesoderm, including the expression of blood-specific genes (Johansson and Wiles, 1995; Bhatia et al., 1999; Adelman et al., 2002; Chadwick et al., 2003; Park et al., 2004; Pearson et al., 2008). In zebrafish, several BMP mutants have been identified and all have a dorsalised phenotype, resulting from a requirement for BMP signalling in embryonic patterning. These include the alk8 mutant 'lost-a-fin', bmp2b mutant 'swirl', bmp7 mutant 'snailhouse', tolloid mutant 'mini fin' and the smad5 mutant 'somitabun'(Kishimoto et al., 1997; Connors et al., 1999; Hild et al., 1999; Dick et al., 2000; Bauer et al., 2001). Separate from the role of BMP signalling in mesoderm induction and patterning, BMP signalling is required during gastrulation for cell movements (Heisenberg and Solnica-Krezel, 2008). These requirements combine and result in BMP patterning the dorsal-ventral axis in a temporally progressive manner along the anterior-posterior axis during gastrulation. Low BMP signalling at the dorsal lateral side of the embryo promotes convergence and extension movements whilst high BMP signalling ventrally promotes cell migration. It is thought that

this process is partially mediated by BMP signalling modulating cadherin-mediated cell adhesion (von der Hardt et al., 2007).

As BMP is required for embryonic patterning prior to HSC programing, it is difficult to draw meaningful conclusions from experiments where BMP signalling is blocked or induced prior to these inductive events. Increased expression of blood markers when BMP signalling is induced (Graff et al., 1994; Maeno et al., 1994; Walmsley et al., 1994; Maeno et al., 1996; Zhang and Evans, 1996) is likely due to an increase in all ventral posterior mesodermal derivatives and is not necessarily indicative of a specific effect on the HSC itself.

Some attempts have been made to investigate the later requirements for BMP signalling in HSC programming (Walmsley et al., 2002a). When the role of BMP signalling was investigated with specific interest in the *Xenopus* ventral blood island, BMP signalling was shown to be required for expression of *scl* and *gata2*, whilst expression of the endothelial gene *fli1* was unaffected. In contrast, when the dorsal lateral plate population was analysed, *fli1* expression was BMP-dependent. An important caveat to this statement is that by the stage of development when the DLP population begins to form, embryos injected with mRNA for the dominant negative *bmp* receptor had gross morphology defects too severe to allow for the proper analysis of the DLP.

Temporal inhibition of BMP signalling was first attempted by fusing the inhibitory Smad6 to the estrogen receptor ligand-binding domain (Schmerer and Evans, 2003). This allowed for the induction of BMP inhibition by negative Smad6 activity via estradiol treatment of injected embryos. Although this paper reports a role for BMP signalling post gastrulation, it focuses on the contribution of BMP signalling to the primitive ventral blood island and the results reported with reference to DLP stages are somewhat confusing and require further analysis.

Our own group has investigated the role of BMP signalling in the initiation and maintenance of *runx1* expression in the zebrafish dorsal aorta (Wilkinson et al., 2009). A transgenic zebrafish line with a heat-shock promoter driving the expression of a dominant-negative truncated BMP receptor (tBR) (Pyati et al., 2005) allowed temporal inhibition of BMP signalling. In these experiments, when tBR expression was induced at 22 hr post fertilisation *runx1* expression was lost from the dorsal aorta, suggesting that BMP signalling is required for *runx1* expression in the dorsal aorta. Another group has shown that when tBR is expressed in the *lmo2* lineage in zebrafish, the expression of blood markers (*scl*, *gata1*, *flk1*, *fli1*, *mpo*) is increased (Gupta et al., 2006). This finding suggests that BMP signalling restricts haematopoietic development in *lmo2*-expressing cells. Both of these experiments have used the dominant-negative, truncated BMP receptor to inhibit BMP signalling. Recent experiments in our lab brought the specificity of tBR into question, and will be discussed later.

### 1.5.3 The BMP signalling pathway

As early as 1945 Pierre Lacroix suggested the hypothetical existence of a factor in bone, which he termed osteogenin, that had the capacity to initiate bone growth and was responsible for the bone's ability to repair itself after injury (Lacroix, 1945). Experimental evidence supporting this claim was not published until Marshall Urist discovered that the injection of lyophilised demineralised bone into the muscle of rabbits was able to induce ectopic bone growth (Urist, 1965). Six years later, in another publication, he coined the term 'bone morphogenic protein' (Urist and Strates, 1971). This discovery led to major breakthroughs in the understanding of bone formation, however the observation that BMP homologues are found in the genomes of invertebrates, which have no bones, was a clear

indication that these signalling molecules have more divergent biological activities than merely bone formation (Lelong et al., 2001). The BMPs identified in these early experiments were BMP1, 2, 3 and 4. BMP1 was later identified as a metalloproteinase, where BMP2, 3 and 4 were identified as secreted signalling proteins and part of the TGF-β superfamily of signalling molecules. Other BMP ligands have subsequently been identified through their structural homology (McDonald and Hendrickson, 1993; Lelong et al., 2001).

BMP ligands are translated as inactive precursors with an N-terminal proregion and a C-terminal receptor-binding region, termed pre-BMPs (Figure 1-9) (Cui et al., 1998). These precursors form homo- or heterodimers which are subsequently cleaved by the serine protease furin at the conserved RXXR or RX[KR]R region to release the biologically active C-terminal dimer (McDonald and Hendrickson, 1993; Murray-Rust et al., 1993). The BMP ligand proregion shows little conservation, although the C-terminal domains are highly conserved. Included in this C-terminal domain are seven cysteine residues, one of which is required for dimerization while the other 6 have been shown to make three disulphide bonds and form a cysteine knot structure (McDonald and Hendrickson, 1993).

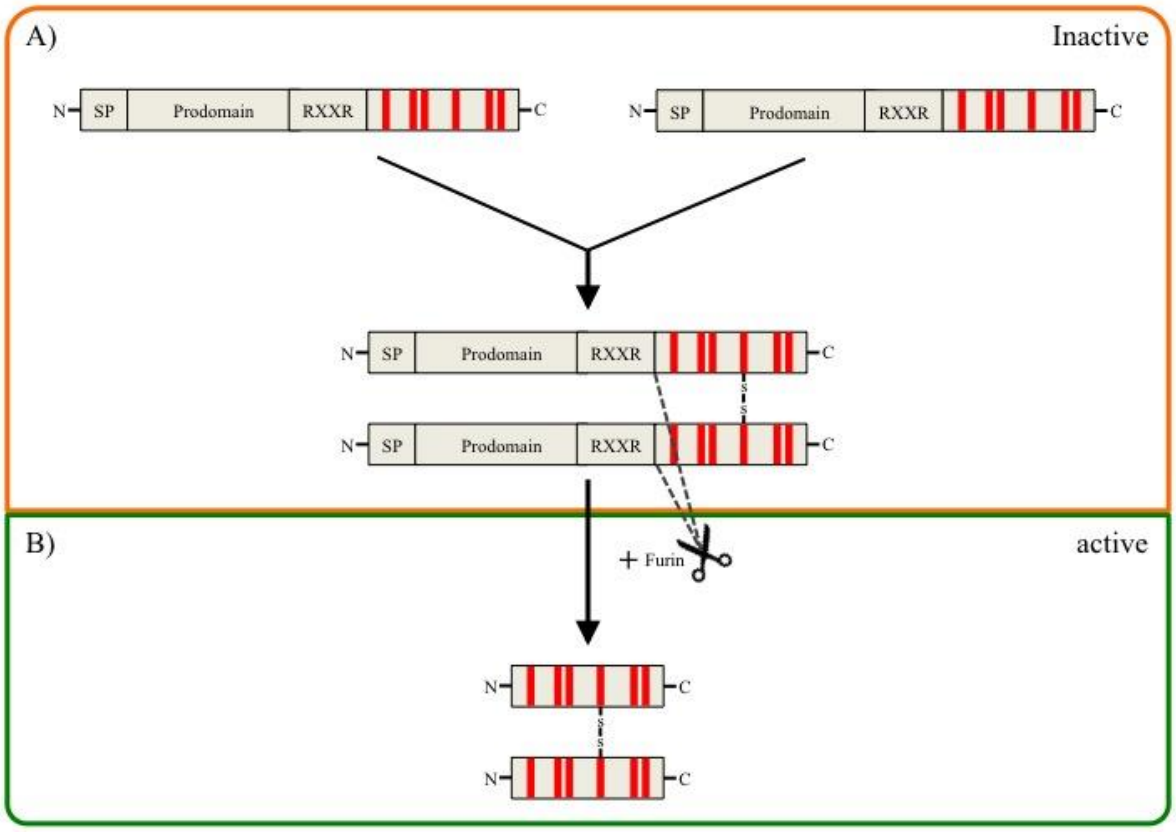



**Figure 1-9: BMP ligand dimerisation and cleavage**

BMP ligands form dimers via a disulphide bridge in the C-terminus of the protein. After dimerization the proteins are cleaved by the serine protease furin at the conserved RXXR or RX[KR]R region to release the biologically active C-terminal dimer.

### 1.5.4 BMP receptors and ligand binding

During signalling, BMP ligands are bound on the cell surface by the extracellular domain of serine/threonine kinase receptors. Two subgroups of BMP-specific receptors are responsible for BMP signalling; type I (TSR-1/Alk1, ActR-I/Alk2, BMPRIA/Alk3, BMPRIB/Alk6) and type II (BMPRII, ActRIIA and ActRIIB) receptors and both are required for signalling (Heldin et al., 1997). These receptors have similar structures: both have a short extracellular domain that facilitates ligand binding, a single pass transmembrane domain and an intracellular domain containing the serine/threonine kinase activity (Heldin et al., 1997). The kinase activity is constitutively active in the type II receptors and upon complex formation the type II receptor phosphorylates a glycine-serine rich domain on the intracellular part of the type I receptor. This phosphorylation results in the activation of the type I serine/threonine kinase domain, allowing it to phosphorylate downstream signalling molecules (R-Smads, see below) and transfer the external stimuli into an internal signalling cascade (Heldin et al., 1997).

BMP ligands are bound as dimers by two type I receptors and two type II receptors to form the active signalling complex (Heldin et al., 1997). The dimeric BMP ligand has two epitopes recognised by the receptors. The wrist epitope binds the type I receptor whilst the adjacent knuckle epitope binds the type II receptors, thus bringing both receptor types into close proximity and allowing transphosphorylation to occur (Kirsch et al., 2000). The BMP ligands exhibit varying affinities for specific type I and type II receptors. The combination of specific receptors expressed on the cell surface and the ligands present in the extracellular environment may in part contribute to the specific biological readout in the target cell.

The cytoplasmic components of BMP signalling are the Smad proteins. Smad proteins can be subdivided into three groups through their structure and function; R-Smads, co-Smad and I-

Smads (Massague et al., 2005). The receptor-regulated Smads (R-Smads) are the intracellular proteins responsible for transmitting the signalling events at the membrane to the nucleus. Their C-terminus contains a Ser-X-Ser motif, which is the target of the type I BMP receptor (Massague et al., 2005). The phosphorylation of this region results in a conformational change allowing the R-Smad to bind the co-Smad4 in a hetero-trimeric complex of two R-Smads and one co-Smad (Wu et al., 2001a; Wu et al., 2001b; Chacko et al., 2004). This is the complex that will bind DNA and effect expression of target genes.

The inhibitory Smads have evolved from a common ancestor with the receptor Smads, however, they act negatively to regulate the BMP signalling cascade. Many of their inhibitory activities are through competing for protein-protein interactions. Smad6 and Smad7 bind to the type I BMP receptor and block R-Smad phosphorylation and signal transduction (Hayashi et al., 1997). Smad7 is also able to bind to the Smad-binding elements in promoters and block the binding of the active Smad complex (Zhang et al., 2007). Smad6 has also been shown to disrupt the formation of the active Smad complex by binding the phosphorylated R-Smads and preventing them from binding Smad4 (Hata et al., 1998). In addition these inhibitory Smads have been shown to recruit the E3 ubiquitin ligases, Smurf1 and Smurf2, to the receptor to label the receptor for degradation (Kavsak et al., 2000; Ebisawa et al., 2001; Murakami et al., 2003).

The nuclear accumulation of Smad proteins was the initial finding which identified Smad proteins as part of the BMP signalling cascade (Hoodless et al., 1996; Liu et al., 1996). Whereas most proteins require the assistance of nuclear transport factors and a nuclear translocation signal to move from the cytoplasm to the nucleus, the phosphorylated Smads can directly interact with nucleoporins via a hydrophobic corridor that becomes accessible upon phosphorylation. This allows the phosphorylation state of the Smad protein to determine nuclear translocation rather than the constitutive nuclear localisation signal used by

other proteins. Although many diagrams of BMP signalling appear to show the R-Smad/Co-Smad complex forming in the cytoplasm, including the one presented here, this has not been empirically tested and it is possible that this complex actually forms in the nucleus immediately prior to DNA binding. The nuclear transfer of phosphorylated Smad1 can occur in the absence of Smad4, as shown in Smad4-deficient cells (Liu et al., 1997).

When the R-Smad/co-Smad complex becomes localised in the nucleus it is then able to bind DNA. DNA binding by the Smad complex is facilitated by a β-hairpin in the MH1 globular domain (Shi et al., 1998). *In vitro* analysis first identified 5'-CGCTAGAC-3' as the target of Smad binding (Zawel et al., 1998) although subsequent analysis has shown that 5'-AGAC-3' or its complement 5'-GTCT-3' is sufficient for binding (Massague et al., 2005). The binding affinity of the Smad complex to this element appears low when compared to other DNA binding proteins (Shi et al., 1998), and this may reflect the requirement of additional cofactors and explain why, at the time of writing, no crystal structure showing Smad-DNA binding has been described.

On binding DNA, the active Smad complex is able to regulate gene expression of target genes, positively or negatively. When activating target genes, the Smad complex recruits transcriptional activators. Acetyltransferases such as P300 and CBP have been found to bind in association with the Smad complex at Smad-binding elements (Massague et al., 2005). P300 and CBP activate gene expression by opening the chromatin structure at the promoter by recruiting histone modifiers and by recruiting the basal transcriptional machinery including RNA polymerase II (de Ruijter et al., 2003). When acting negatively the Smad complex recruits histone deacetylases, which increase the affinity of histones to DNA and as such restricts the access of DNA binding proteins to that region (Massague et al., 2005).

### 1.5.5 BMP signalling cofactors

Although overexpression assays have identified many potential Smad cofactors, very few of these have been studied *in vivo* at endogenous levels. One example that has been convincingly shown as a Smad cofactor is the zinc-finger protein, ZNF423 (Hata et al., 2000). This protein is required for the BMP activation of *vent2* expression during *Xenopus* ventral mesoderm formation. Another example is the protein Ecist, which acts with the Smad complex to activate expression of the homeobox gene *Tlx2* in the gastrulating mouse embryo (Xiao et al., 2003). These proteins act by increasing the affinity of the active Smad complex for DNA and by recruiting other factors to either inhibit or promote expression of the target gene.

Evi3 is a BMP cofactor that is potentially involved in programming the HSC. *Evi3* was first cloned as a frequent site of retroviral integration, causing B-cell lymphoma in the inbred mouse line, AKXD27 (Gilbert et al., 1993; Warming et al., 2004). This mouse line has a propensity to develop B-cell lymphomas and oncogenesis is accelerated following MuLV viral infection. The MuLV virus integrates its DNA into that of the mouse host, interfering with gene expression and leaving a DNA 'flag', allowing the identification of integration events. When using this method to look for secondary hits during oncogenesis, approximately 43 % of mice that develop B-cell lymphoma have integration in *Evi3*, causing overexpression of the gene. Similar results were found in a mouse line expressing the oncogenic fusion *E2A:HLF*. This mouse line does not normally develop leukaemia but when infected with MuLV it does so at an accelerated rate when compared to wild-type MuLV infected mice. This screen identified *Gfi1*, *Ikaros* and *Evi3* as integration sites that caused oncogenesis in these mice (Yamasaki et al., 2010); with Gfi1 and Ikaros having already been implicated in HSC programming (Willett et al., 2001; Wei et al., 2008). Concurrently *Evi3* was identified

as an mRNA transcript that was enriched in $CD34^+$ human haematopoietic progenitor cells compared to peripheral blood leukocytes (Bond et al., 2004) and is down regulated during maturation and differentiation (Bond et al., 2008). Evi3 also appears to have an important role in bone development. It has been shown to be required during early mesenchymal lineage commitment but is later inhibitory to osteoblast maturation (Hesse et al., 2010a). Later analysis of this phenotype linked Evi3 to inhibition of Runx2, a transcription factor that is vital for bone development (Ducy et al., 1997; Geoffroy et al., 2002; Wu et al., 2009; Hesse et al., 2010a).

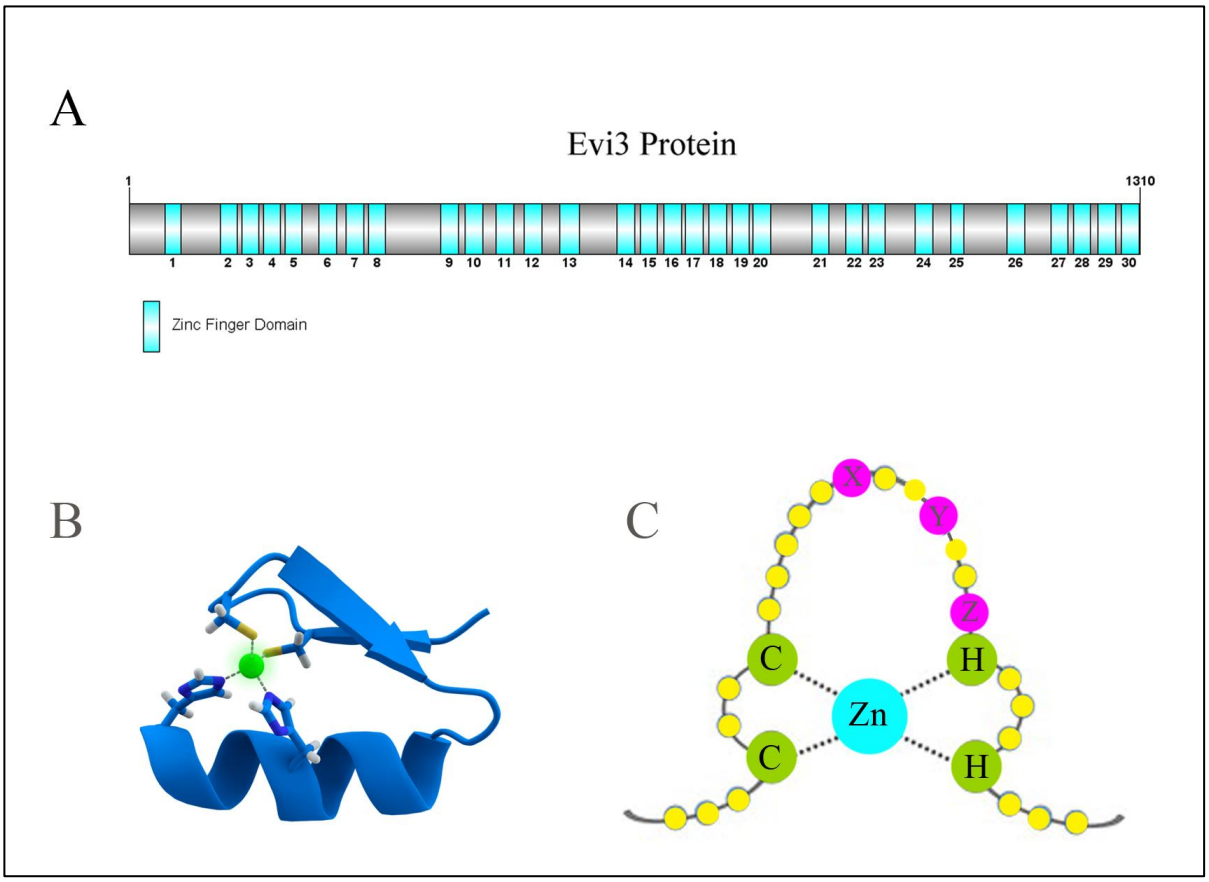



**Figure 1-10: *Xenopus* Evi3 protein structure**

A) Protein domains of Evi3, showing 30 C2H2 krüppel-like zinc finger domains. B) Three dimensional digital representation of a C2H2 zinc finger domain, showing the zinc ion in green and the cysteine and histidine residues that bind it. The alpha helix and two beta sheets, characteristic of C2H2 zinc fingers, are clearly visible. Image B was taken from (Splettstoesser, 2007). C) Schematic representation of a C2H2 zinc finger showing amino acids as coloured circles. Green circles show cysteine (C) and histidine (H) amino acids involved in zinc binding. Pink circles labeled X, Y and Z denote the location of amino acid residues that determine DNA binding specificity. Image C was modified from (Matharu et al., 2010).

Evi3 contains 30 C2H2 zinc fingers which are characterized by the amino acid sequence $X_2$-Cys-$X_{2,4}$-Cys-$X_{12}$-His-$X_{3,4,5}$-His (Pabo et al., 2001). In other proteins these zinc fingers are involved in protein:RNA, protein:DNA and protein:protein interactions, making proteins that contain them incredibly versatile. The α-helix of each domain can make sequence-specific contacts to DNA bases, and in Evi3 zinc fingers 2 to 8 are required for DNA binding. However, zinc fingers 14 to 19 are necessary for Evi3 to bind the Smad1/4 active complex (Hata et al., 2000).

Evi3 has also been shown to bind the histone deacetylase complex (Bond et al., 2004) and this binding is required for Evi3 to inhibit Runx2 during bone development (Hesse et al., 2010b). Evi3 has also been co-immunoprecipitated with the histone acetyltransferase P300, allowing it to activate neural genes in mouse embryonic stem (ES) cells (Kamiya et al., 2011a), thus Evi3 is able to recruit either positive or negative histone modifiers to gene targets.

### 1.5.6 Signalling regulation

As BMP ligands are secreted diffusible signalling proteins, the presence or absence of their receptors on a cell is one key method to ensure targeted signalling. Another method controlling signal transduction is through the binding of secreted ligands by extracellular inhibitors (Taipale and Keski-Oja, 1997). The list of ligand-binding extracellular proteins is ever expanding and at present includes diffusible proteins and membrane/matrix associated proteins. These proteins are summarised in table 1. In general the secreted diffusible inhibitors act by binding and sequestering BMP ligands and preventing them from binding to their receptors. Membrane- and matrix-associated proteins commonly act by limiting ligand

diffusion, however, some have been found to directly inhibit the formation of ligand receptor complexes or inhibit ligand processing and secretion.

| **Diffusible proteins** (Legend Reference) | **Properties/mechanisms** |
|---|---|
| Noggin [1/2/3/20] | Binds BMP2/4/5/6/7/13/14 |
| Follistatin [3] | Forms BMP trimer with receptors |
| Chordin/Sog [1/2/3] | Blocks BMP binding to receptors |
| Chordin-like/Ventroptin/Neuralin [1/2/3] | Binds BMPs |
| Chordin-like 2 [1/2/3] | Binds BMPs |
| CTGF [1/2] | Blocks BMP binding to receptors |
| Dan [2/3] | Binds BMPs |
| Cerberus/Caronte [3/5] | Binds BMP2/4/7 |
| PRDC [2] | Binds to BMP2/4 |
| Dand5/Coco/Dante [2] | Likely to bind BMPs |
| Gremlin [2] | Strong binding to BMP2/4/7 |
| Sclerostin domain containing 1 [3/4] | Binds BMPs |

| **Short range diffusible proteins** | |
|---|---|
| BMPER [17/18] | Binds BMP2/4/7, and BMP type I receptor |
| Tsukushi [12] | Binds BMP4/7 cooperatively with Chordin |

| **Membrane/matrix-associated proteins** | **Properties/mechanisms** |
|---|---|
| Glypicans [6/7/8/9/10] | Binds BMP2/4 and modulates diffusion |
| Biglycan [11] | Binds BMP4 cooperatively with Chordin |
| Crim1/Crm1 [13/14] | Binds BMP4/7 preBMPs, blocks there processing |
| Procollagen-IIA [15/16] | Binds BMP2 and potentially other BMP ligands |
| BAMBI [19] | Acts as nonfunctional type I pseudoreceptor |

**Table 1-1: Naturally occurring BMP ligand binding inhibitors**

This table is modified from (Umulis et al., 2009) with the addition of material from the following references; [1](Garcia Abreu et al., 2002), [2](Gazzerro and Canalis, 2006) [3](Balemans and Van Hul, 2002) [4](Yanagita, 2005) [5](Yu et al., 2008b) [6](Paine-Saunders et al., 2000) [7](Fujise et al., 2001) [8](Kirkpatrick et al., 2006) [9](Gumienny et al., 2007) [10](Fisher et al., 2006) [11](Moreno et al., 2005) [12](Ohta et al., 2004) [13](Fung et al., 2007) [14](Wilkinson et al., 2003) [15](Sieron et al., 2002) [16](Zhu et al., 1999) [17](Moser et al., 2003) [18](Serpe et al., 2008) [19](Lin et al., 2005) [20] (Zimmerman et al., 1996)

One example of a secreted diffusible ligand-binding protein is Noggin. Noggin was first identified in *Xenopus* where cDNA injection induced excessive head development (Smith and Harland, 1992); hence the name Noggin, meaning head. Noggin is thought to have evolved from the same common ancestral protein as BMP7 and as such has a similar structure and acts as a dimer (Groppe et al., 2002). Noggin binds to and inhibits BMP2, BMP4, BMP5, BMP6, BMP7 BMP13 and BMP14 with varying affinities (Zimmerman et al., 1996; Groppe et al., 2002; Seemann et al., 2009; Song et al., 2010). As mentioned previously (See section 1.5.3), BMP ligands bind to their receptors using a hydrophobic pocket. Noggin inhibition of these ligands occurs via a proline residue in Noggin. The hydrophobic ring of this proline is inserted into the hydrophobic pocket in the BMP ligand, preventing the ligand from binding to its receptor (Groppe et al., 2002). During embryogenesis Noggin is expressed in, and diffuses out from, the Spemann organiser. This contributes towards creating a dorsal-ventral gradient of BMP activity in the early embryo. This gradient is essential for patterning the embryo and for subsequent germ layer formation (Zimmerman et al., 1996). Mouse models have shown that Noggin is later required for the growth and patterning of the neural tube, somatic differentiation and for skeletal growth (McMahon et al., 1998; Lories et al., 2006; Tylzanowski et al., 2006). This once again highlights the pleotropic requirement for faithful BMP signalling during development and explains the many layers of regulation.

### 1.5.7 Crosstalk between BMP signalling and other pathways

As our understanding of cellular signalling develops, it has become increasingly evident that the pathways we once thought were distinct entities are in fact an interwoven network of signals and responses. Acting in a spatial- and temporal-specific context, these pathways act with synergistic or antagonistic effects to enable a plethora of biological outcomes. BMP

signalling has been linked to the notch pathway (Blokzijl et al., 2003), PI3K/Akt (Ghosh-Choudhury et al., 2002), MAPK (Moustakas and Heldin, 2005; Yang et al., 2010), Wnt (Hoppler and Moon, 1998; Itasaki and Hoppler, 2010), interferon-γ (Ulloa et al., 1999), NF-kB and of course TGF-β signalling. These interactions are almost overwhelmingly complex and have been well reviewed previously (Guo and Wang, 2009).

An interesting interaction is found between BMP signalling and the micro RNA pathway. BMP4 is required for the differentiation of vascular smooth muscle cells. This has been shown to be through BMP4 upregulation of the micro RNA miR-21, and subsequent inhibition of Pdcd4 (programmed cell death protein-4) by miR-21 (Davis et al., 2008). Surprisingly, Smad4 knockdown in cell culture had no effect on miR-21 levels despite inhibition of the R-Smads resulting in decreased levels of mature miR-21. When R-Smad inhibition was studied in more detail, although the levels of mature miR-21 were decreased, the levels of pre-miRNA-21, from which miR-21 is processed, were unchanged. This suggested that the R-Smads were required for the processing of this miRNA from its pre-miRNA. Co-immunoprecipitation of the R-Smads and the pre-miRNA processing complex (Drosha and p68) indicated that this was a direct and novel role for the R-Smads in regulating gene expression through miRNA processing (Warner et al., 2004). Subsequent analysis of miRNA levels in muscle cell culture has shown that 5 % of the miRNAs tested (20 of 377) are regulated by R-Smad levels (Hata and Davis, 2010).

As the role of these signalling pathways has been tested in different systems and using different approaches, it is perhaps unsurprising to find different results arising due to varying contexts. The crosstalk mentioned here, and the varying expressions of co-factors, both contribute to highly specific, contextually-dependent outcomes. As such, we cannot infer, for example, that as BMP is required for the induction of primitive blood in *Xenopus* embryonic development (Walmsley et al., 2002a) it necessarily plays a role in the later programming of

the adult blood lineage. This conclusion can only be drawn through specific experiments targeted at addressing this question and not via inference.

### 1.5.8 BMP mouse knockout lines

Mutant and transgenic mouse lines exist and have been used to study the role of BMP signalling during early embryogenesis (Table 1-2). Most of these lines die early in embryogenesis, presumably due to early patterning defects. To date, no mouse line has been created which allows the temporal inhibition of BMP signalling that would be required to probe the requirement for BMP signalling in HSC programming.

| Mouse line | Phenotype | Reference |
|---|---|---|
| **BMP2-/-** | Most embryos die at 7.5 dpc from a failure to close proamniotic canal. Approximately 30 % survive to 10.5 dpc and either do not develop a hear or the heart develops in the coelomic cavity | (Zhang and Bradley, 1996; Ma et al., 2005; Ma and Martin, 2005) |
| **BMP4-/-** | Most embryos die at 6.5 dpc with little or no mesoderm. Some survive pas this stage but late die due to defective vasculature and dramatically reduced red blood cell numbers. | (Winnier et al., 1995) |
| **Nkx2.5:cre/BMP4** | Deleting BMP4 in heart mesoderm results in abnormal branchial arch arteries and defective outflow tract myocardium | (Liu et al., 2004) |
| **BMP5 (shortear mutation)** | BMP5 null 'shortear' mutant mice have several small bones missing, one pair of ribs missing and an abnormal xiphoid process at the end of the sternum. | (Lynch, 1921; Kingsley et al., 1992; Kingsley, 1994) |
| **BMP7 -/-** | Homozygous mice die shortly after birth with defective kidneys. The kidneys were found to have little or no glomerula. Eye lens formation was also defective and skeletal defects affecting the rib cage, limbs and skull were also observed. | (Dudley et al., 1995; Luo et al., 1995) |
| **GDF5 -/-** | GDF -/- mice are viable but lack proximal interphalangeal joints, have fused wrist and ankle joints. | (Storm et al., 1994; Daans et al., 2011) |
| **BMPRI -/-** | Embryos die at 7.5/8.5 dpc with no mesoderm. | (Mishina et al., 1995) |
| **Smad4 -/-** | Embryonic death 6/7 dpc due to impaired extra embryonic membrane formation and decreased epiblast formation. | (Sirard et al., 1998; Yang et al., 1998; Yang et al., 2002) |
| **Smad1-/-** | Embryonic death 6/7 dpc with defective allantois formation and a lack of primordial germ cells. | (Tremblay et al., 2001; Huang et al., 2002; Aubin et al., 2004) |
| **Smad5 -/-** | Variable phenotype with embryonic death at 9.5/11/5 dpc. Common features include abnormal amnion morphology, vestigial gut, and heart development in the coelomic cavity | (Chang et al., 1999) |
| **Bambi -/-** | No detectable phenotype either embryonically or postnataly | (Chen et al., 2007) |
| **Noggin -/-** | Embryos die perinatally (developmental stage not reported). Embryos neural plate fails to close, and limbs are club shaped. | (McMahon et al., 1998) |

**Table 1-2 Mouse BMP knockout lines and their phenotype**

### 1.6 Objectives

Whilst several studies have indicated a role for BMP signalling in mesoderm induction, and there have been reports suggesting that BMP is specifically required for haematopoietic programming in the primitive VBI in *Xenopus*, the role of BMP signalling post-gastrulation has been poorly studied. It is not possible to study haematopoietic programming in the precursors of the dorsal aorta in any organism other than *Xenopus*, where detailed knowledge of these cells and their spatial separation from the primitive blood lineage allows precise questions to be addressed.

The questions addressed in this thesis are:

1) Is BMP signalling required post-gastrulation for HSC programming?
2) If so, when is BMP signalling required?
3) What is the role of BMP signalling in programming the HSC?
4) What gives BMP signalling its specificity in these cells?

# Chapter 2

# Materials and Methods

# Chapter 2 : Materials and Methods

## 2.1 Solutions

All solutions were prepared with autoclaved Mili-Q water and were made immediately prior to use unless storage conditions are mentioned.

| Solution | Composition |
|---|---|
| AP buffer | 0.1 M Tris pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl, 0.1 % (v/v) Tween 20 |
| Bleaching solution | 5 % (w/v) formamide, 0.5 % SSC, 33 % $H_2O_2$ |
| Denhardt's solution (50 X) | 2 % (w/v) BSA (Fraction V), 2 % (v/v) ficoll 400, 2 % (w/v) polyvinylpyrrolidone. (Stored at – 20 °C) |
| De-jellying solution | 2% (w/v) cysteine in 1 x MBS (pH 8.0) |
| Electrophoresis agarose | 1 % (w/v) agarose and 0.005 % (v/v) GelRed nucleic acid stain, 0.5 x TAE |
| Hybridisation solution (sections) | 50 % formamide, 5 X SSC pH 4.5, 50 µg/ml torula yeast RNA, 1% SDS, and 50 µ g/ml heparin |
| Hybridisation solution (WISH) | 50 % (v/v) formamide, 5 X SSC, 1mg/ml tolura yeast RNA, 100 µg/ml heparin. (Stored at – 20 °C) |
| Injection solution (ficol) | 4 % (w/v) ficol 400, 1 x MBS |
| Lysogeny broth (LB) | 10 g/L bactotryptone, 5 g/L bactoyeast, 10 g/L NaCl in $H_2O$ |
| Lysogeny broth agar plates (LB agar) | Lysogeny broth containing 15 g/L bactoagar |
| Maleic acid buffer (MAB) | 100 mM maleic acid, 150 mM NaCl pH 7.5 (Stored at 4 °C) |
| MAB/Tw | MAB containing 0.2 % (v/v) Tween 20 |
| MAB/block | MAB containing 2 % (w/v) boehringer block (dissolved at 67 °C and stored at – 20 °C) |
| MEMFA | 0.1 M MOPS pH7.4, 2 mM EGTA, 1 mM $MgSO_4$ (stored at room temperature, folmaldehyde is added to 3.7 % (v/v) immediately prior to use) |
| Modified Barth's saline (MBS) | 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM HEPES, 0.3 mM $CaNO_3$:$4H_2O$, 0.41 mM $CaCl_2$:$6H_2O$, 0.82 mM $MgSO_4$:$7H_2O$ pH 7.6. (Stored at 4 °C, penicillin/streptomycin added to 10 µg/ml prior to use) |
| MS222 | 5 g/L MS222 (tricaine methanesulfonate) pH 7.0 |
| PBS/Tw | PBS containing 0.1 % (v/v) Tween 20 |
| PFA (4 %) | 4 % (w/v) paraformaldehyde in 1 X PBS |
| Pronase solution | 0.1 x MBS, 200 ng/ml streptomyces griseus protease |
| Slide *in situ* solution I | 50 % formamide, 5 X SSC pH 4.5, 1 % (v/v) SDS |
| Slide *in situ* solution II | 50 % (v/v) formamide, 2 X SSC pH 4.5 |
| SSC (20 X) | 3 M NaCl, 0.3 M sodium citrate |
| TAE (25 X) | 2 M tris-acetate, 50 mM EDTA |
| TEA | 0.1 M triethanolamine pH 7.9 |
| Testes media | 70% (v/v) Liebowitz growth medium (Life Technologies 11415), 30% (v/v) $H_20$ and 10 µg/ml penicillin/streptomycin |

## 2.2 Preparation and manipulation of nucleic acids

### 2.2.1 Transforming and culturing bacteria

For standard transformations, DH5α chemically competent cells (Life Technologies 18263-012) were transformed by incubating with DNA on ice for 30 min before being subjected to a 42 °C heat shock in a dry block heater (Techne DB-3D) for 30 seconds followed immediately by 2 min on ice. Five hundred µl SOC medium (Invitrogen 15544) was then added and the cultures allowed to grow for 1 hr in a 37 °C water bath (Grant JB1) before plating out onto LB agar plates (Merck KGaA 110283) containing 50 µg/ml ampicillin (Sigma A5354), 50 µg/ml kanamycin (Sigma K0254) or 50 µg/ml chloramphenicol (Sigma R4408) as required.

For simple transformations (e.g. maintaining plasmid stocks) NEB 5-alpha competent E.coli cells (NEB C2987) were used following the same protocol.

### 2.2.2 Plasmid preparations

Plasmids were isolated from bacterial cultures using the Quicklyse Miniprep Kit (Quiagen 27406) for small-scale preparations and using GenElute HP Plasmid Kit (Sigma NA0310) for large-scale preparations. Both kits were used in accordance with the manufacturer's protocol.

### 2.2.3 Determination of DNA and RNA concentrations

DNA and RNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer in accordance with the manufacturer's protocol. The ratio of absorbance

at 260 nm and 280 nm was used to assess the purity of the DNA and RNA samples. A ratio of ~ 1.8 was accepted as pure for DNA and a ratio of ~2.0 was accepted as pure for RNA.

### 2.2.4 Restriction enzyme digestion

DNA was digested using New England BioLabs restriction enzymes in accordance with manufacturer's guidelines using the provided buffers and BSA where required. Complete digestion was confirmed by agarose gel electrophoresis (see section 2.2.5) by comparison with 100 ng undigested DNA and a 1 Kb DNA Ladder (NEB N3232). Where a single band was observed on the gel, the digested DNA was cleaned for downstream reactions using the QIAquick PCR Purification Kit (Qiagen 28104). Where multiple bands were observed on the gel, the desired band was cut from the gel using a scalpel (Appleton Woods GC685), and purified using the QiaexII Gel Extraction Kit (Qiagen 20021).

### 2.2.5 Analysis of DNA and RNA by agarose gel electrophoresis

Analysis of both DNA and RNA was carried out by agarose gel electrophoresis (Sambrook, 2001). Typically gels containing 1% (w/v) agarose (Invitrogen 16500) and 0.005 % (v/v) GelRed nucleic acid stain (Biotium 41003) in 0.5 x TAE were prepared and run in 0.5 x TAE buffer using a mini gel electrophoresis trough (i-Mupid i-CE) for 30 min at 100V. For mRNA or RNA antisense probes the electrophoresis equipment was cleaned prior to use using RNaseZap (Life Technologies AM9780) to remove RNase contamination. Gels were imaged using a Biorad Gel Doc XR+ system and images were annotated using Biorad Image Lab 4.0 software.

### 2.2.6 Concentration of DNA and RNA by ethanol precipitation

Where DNA or RNA required concentration, the DNA or RNA was precipitated using ethanol and then suspended in a smaller volume. Sodium acetate (pH 5.2) was added to the solutions to a concentration of 0.3 M followed by the addition of 2.5 volumes of ethanol. The solution was then mixed and placed on dry ice for at least 30 min. Samples were centrifuged at 4 °C at 12,000 g for 30 min to collect the precipitated DNA or RNA. The supernatant was removed and the pellet washed with 70 % ethanol. To assist the drying of the precipitate the sample was briefly centrifuged 3 times, removing as much supernatant as possible each time. The sample was allowed to air dry before suspension in autoclaved Milli-Q water.

### 2.2.7 Identification of bacterial clones containing DNA of interest

To identify bacterial colonies that contained the DNA fragment of interest, colony PCR was performed. Individual colonies were picked from the agar plates using a pipette tip and were transferred to a 1.5 ml tube containing 30 µl dH2O. Eight µl of this was used as a template in a 20 µl PCR reaction using appropriate primers, JumpStart PCR master mix (Sigma P0982) and a thermal cycler (Bio-rad Dyad). This was then analysed by agarose gel electrophoresis, looking for bands correlating to the expected size of amplicon. The plasmid DNA of positive clones was purified from a 200 ml LB culture using the GenElute plasmid midiprep kit (Sigma NA0310) in accordance with the manufacturer's protocol. Purified DNA was then sent for DNA sequencing for confirmation.

### 2.2.8 DNA sequencing

All sequencing was carried out by the WIMM DNA Sequencing Service. This in-house service uses an ABI-3730 DNA analyser (Applied Biosystems 3730), calibrated to use Applied Biosystems BigDye Terminator v3.1 chemistry for sequencing and the G5 dye set from ABI for fragment analysis. Sequence reads were analysed using APE software and other online resources such as BLAST (NCBI) and ClustalW2 (EMBL-EBI).

### 2.2.9 Amplification of DNA by polymerase chain reaction (PCR)

Primers were designed using ApE software and other online resources such as Primer 3 and BLAST (NCBI). For the generation of short amplicons or for PCR as a test rather than used for the creation of a new fragment, JumpStart PCR master mix (Sigma P0982) was used in accordance with the manufacturer's protocol and with appropriate template and primers. Where a high-fidelity polymerase was required, Platinum Pfx DNA polymerase (Invitrogen 11708-013) was used in accordance with the manufacturer's protocol. All PCR was performed using a thermal cycler (Bio-rad Dyad). Generally, template DNA was mixed with 0.2 mM dNTPs, 10 pM forward primer, 10 pM reverse primer, 2 units of DNA polymerase, to a total volume of 50 µl in PCR amplification buffer appropriate to the polymerase used. A typical cycle was as follows: 4 min at 94 °C followed by 35 cycles of 94 °C for 30 sec, 55 °C for 1 min, and 72 °C for 1 min/kb amplicon. A final 72 °C for 10 min was then performed prior to cooling to 4 °C.

### 2.2.10 RNA quantification by quantitative PCR (q-PCR)

RNA was isolated as in section 2.4.8 and cDNA synthesised as in section 2.3.4.

Q-PCR was performed using the ABI Prism 7700 Sequence Detector using the SYBR green assay. For each assay 6 μl cDNA was added to 4 μl SYBR Select Master Mix and 5 pmol of each primer was added. Primers are shown in table 3. All assays were done in both technical and biological triplicate. Quantification was normalised by comparison to ODC and levels measured as relative quantification. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec, 55 °C for 15 sec, 60 °C for 1 min. This was then followed by a melting curve to ensure cycling resulted in the amplification of a single product.

Prior to Q-PCR all primer pairs were assessed using a dilution series of cDNA. The log of the input amount was plotted against the threshold cycle value (Ct). A gradient equal to 1 shows that across the cDNA dilution series, the efficiency of amplification is constant, and as such these primers are suitable for the quantification of cDNA within that range.

**Table 3: Q-PCR primers**

| Gene name | Forward primer | Reverse primer |
|---|---|---|
| ***Fli1*** | 5’ aatgcaattatctgttggtcctg 3’ | 5’ aaagcagcaaagcattagcataatac 3’ |
| ***Gata2*** | 5’ gatagaggaaggtaatccatgtgc 3’ | 5’ cacacataggatagtgtggcagt 3’ |
| ***Flk1*** | 5’ acattcctgtagagcctgtggt 3’ | 5’ ggactggtagtcgctagtttgg 3’ |
| ***Odc*** | 5’ gtcaatgatggagtgtatggatc 3’ | 5’ tccattccgctctcctgagcac 3’ |

## 2.3 *In vitro* synthesis reactions

### 2.3.1 *In vitro* transcription of capped mRNA for embryo injections

Template plasmids were digested as previously described (see section 2.2.4) to produce a linear template strand. This was then cleaned (see section 2.2.2) and the concentration determined by nanodrop analysis (see section 2.2.3). Capped mRNA was made using the mMessage mMachine kit (Life Technologies AM1340, AM1348, AM1344) in accordance with the manufacturers protocol. This mRNA was then purified using the RNeasy micro kit (Qiagen 74004) before aliquoting and storing at -80 °C. The quality and concentration of the mRNA produced was analysed by both gel electrophoresis (looking for a single clean band) (see section 2.2.5) and by nanodrop (see section 2.2.3)

### 2.3.2 *In vitro* transcription of RNA probes for *in situ* hybridisation

Template plasmids were linearised by restriction enzyme digestion (see section 2.2.4) and cleaned by ethanol precipitation (see section 2.2.6). Probes were synthesised using an RNA polymerase appropriate to the promoter in the template DNA. The following were mixed in a tube and RNase-free $dH_2O$ added to a final volume of 50 µl: 10 µl 5X transcription buffer, 10 µl 0.1 M DTT, 20 µl 2.5 mM dNTPs, 2 µl 20 U/ µl Rnasin, 3 µl polymerase (T7 (Roche 10881767001), T3 (Roche 11031163001) or Sp6 (Roche 11487671001)), 5 µl 10X DIG RNA labelling mix. This reaction was incubated at 37 °C for at least 2 hr. Following this the DNA was degraded by the addition of 1 µl DNase1 and the reaction incubated for a further 30 min at 37 °C. The reaction was then stopped by the addition of EDTA to a final concentration of

10 mM. The DIG labelled probe was then purified by ethanol precipitation (see section 2.2.6) and resuspended in 200 µl hybridisation solution. Two µl of probe was analysed by gel electrophoresis (see section 2.2.5) to ensure that the probe had been synthesised and to check for degradation of the probe; which would appear as a smear on the gel.

### 2.3.3 Probe templates

| Gene name | Gene symbol | Linearization enzyme | RNA polymerase | Reference |
|---|---|---|---|---|
| **GATA binding protein 2** | *gata2* | Xbo1 | SP6 | (Walmsley et al., 1994) |
| **Friend leukemia virus integration 1** | *fli1* | Sma1 | T3 | (Meyer et al., 1993) |
| **hematopoietically expressed homeobox** | *hex* | BamH1 | T7 | (Jones et al., 1999) |
| **runt-related transcription factor 1** | *runx1* | Sal1 | T7 | (Tracey et al., 1998) |
| **T-cell acute lymphocytic leukemia 1** | *tal1 (scl)* | Xho1 | SP6 | (Mead et al., 1998) |
| **kinase insert domain receptor** | *kdr (flk1)* | Not1 / Sac1 / Sma1 | T7 | (Cleaver et al., 1997) |
| **LIM domain only 2** | *lmo2* | EcoR1 | T7 | IMAGE EST 4174203 |
| **growth factor independent 1 transcription repressor** | *gfi1* | EcoR1 | T7 | IMAGE EST 8547327 |
| **ets variant 2** | *etv2* | Not1 | T7 | IMAGE EST BC099054 |
| **homeobox C10** | *hoxc10* | BsrG1 | T7 | (Christen et al., 2003) |
| **forkhead box F1** | *foxf1* | EcoR1 | T7 | (Deimling and Drysdale, 2011) |
| **heart and neural crest derivatives expressed 1** | *hand1* | BamH1 | T7 | (Sparrow et al., 1998) |

### 2.3.4 cDNA synthesis

RNA was isolated (see section 2.4.8) and was reverse transcribed to cDNA using the Superscript III RT-PCR system (Invitrogen 18080051) in accordance with the manufacturers protocol using random primers and 1 μg total RNA as starting template. The resulting cDNA was diluted to 300 μl in autoclaved $dH_2O$.

## 2.4 Frog husbandry and the manipulation of frog embryos

### 2.4.1 Frog husbandry

Mature *Xenopus laevis* were purchased from Nasco (LM00531, LM00715M) and were maintained on a 12 hr light cycle, in a flow through system using re-circulating water (Marine Biotech X-Rack system system). The water was kept at 19 °C, pH 7.0, and frogs were fed twice a week on liver or heart (local butcher) and once on pellets (Blades Biological Ltd DTS030). Females were fed four times a week for one month following induced superovulation.

Frogs were kept in quarantine, tested for *Batrachochytrium dendrobatidis* and treated for parasitic worms by four-day treatment in 3.39 M Levamisole hydrochloride (Sigma-Aldrich 196142) before being introduced to the main system.

### 2.4.2 Inducing superovulation and the preparation of embryos

In order to obtain embryos, 600 U of human chorionic gonadotrophin (Intervet) was injected into the dorsal lymph sac of adult female *Xenopus laevis*. These females were then kept in a 17 °C incubator in system water overnight. Eggs were squeezed from the females the following day and were fertilised using macerated testes in 0.1 x MBS solution. The testes were harvested from a Schedule 1 male and were kept in 70 % (v/v) Liebowitz growth medium (Life Technologies 11415), 30% (v/v) $H_20$ and 10 µg/ml penicillin/streptomycin. Eggs were flooded with 0.1 x MBS after 20 min and after 1 hr embryos were de-jellied in 2 % (w/v) cysteine in 1 x MBS (pH 8.0) with gentle agitation. Embryos were then washed three times in 1 x MBS and kept at 19 °C until required. Embryos were kept in 1 x MBS until 8 hr post-fertilisation at which point the embryos were washed into 0.1 x MBS for the remainder of their development.

### 2.4.3 Injection of mRNA or morpholinos into embryos

Needles were made using borosilicate glass capillaries (Harvard Apparatus 30-0017) and a micropipette puller (Shutter Instrument Co. P-97) and were mounted onto a micro-injector (Medical Systems Corporation PLI-100). Embryos were aligned on a 500 µm mesh (Spectrum Labs) which was glued to the bottom of a 900 mm petri dish (Thermo scientific SC261). Embryos were washed into 4 % (w/v) ficol 400 (Sigma F9378) 1 x MBS solution prior to injection and were kept in 4 % (w/v) ficol (Sigma F9378), 1 x MBS until 6 hr post-fertilisation at which point the embryos were washed into 0.1 x MBS for the remainder of their development. The injection volume was calibrated by counting the number of injections obtained whilst expelling 1 µl of water. The injection time was then altered to compensate for

any discrepancy from the desired count. Typically 5 nl was injected into both cells of the two cell stage embryo.

### 2.4.4 Treatment of embryos with small molecules

DMH1 was dissolved in DMSO to make a stock concentration of 10 mM. This was alliquoted and stored at – 20 °C. Prior to use, alliquotes of DMH1 were warmed to 35 °C for 10 min to ensure the DMH1 was fully dissolved. Embryos were treated in batches of 50 to 100 embryos per petri dish. Embryos were washed into 0.1 X MBS containing 100 μM DMH1, using the same volume of DMSO in control treatments. Dishes were kept at 19 °C as lower temperatures resulted in precipitation of the DMH1 and consistency was required between experiments. Before collecting, embryos were briefly washed twice in 0.1 X MBS to remove DHM1 from the solution to prevent any possible interference with downstream reactions.

Dexamethasone was dissolved in ethanol to a stock concentration of 10 mM and was used at a concentration of 30 μM. As with DMH1, embryos were treated in batches of 50 to 100 embryos per petri dish and control embryos were similarly treated with ethanol.

### 2.4.5 Heat-shock induction of BMP inhibitor *noggin* in transgenic frog embryos

Eggs collected from heat-shock inducible *noggin* transgenic frogs were fertilised using wild-type testes harvested from a Schedule 1 male. As the heat-shock inducible *noggin* transgenic frogs are heterozygous this results in approximately 50 % transgenic, 50 % wild-type

embryos. Wild-type embryos underwent the same heat-shock treatment as a control. The *hsp70* promoter driving *noggin* expression was activated by transferring the embryos into 35 °C 0.1 x MBS in a 50 ml falcon tube (Sigma-Aldrich CLS430921) and placed in a 35 °C incubator (Techne FHB1DE) for 30 min. After this the embryos were washed into 19 °C 0.1 x MBS and returned to a 90 mm petri dish (Appleton Woods SC261) to continue developing prior to collection. Transgenic embryos were separated from their wild-type siblings based on GFP expression in the developing eye, driven by a *gamma-crystallin* promoter.

### 2.4.6 Dissections

Embryos were dissected in 90 mm petri dishes (Appleton Woods SC261) containing 1 X MBS and coated in 2 % (w/v) agarose (Invitrogen 16500) 1 x MBS. Tissue was dissected using micro-scalpels (Fine Science Tools 26007-04 & 26018-17). Tissue samples were processed or fixed in small batches of 5 to 10 embryos to minimise sample degradation.

### 2.4.7 Manual and enzymatic removal of vitelline membrane from frog embryos

The vitelline membrane of embryos older than stage 20 was removed prior to fixing to avoid curvature of the embryo post-fixation. This was done manually for small numbers of embryos (<200) using Dumont tweezers (WPI 500342). For larger numbers, embryos were washed into 0.1 x MBS containing 200 ng/ml protease from *Streptomyces griseus* (Sigma P5147) for approximately three min with intermittent swirling. Embryos were then carefully washed several times with 0.1 x MBS and either fixed or allowed to continue growing.

### 2.4.8 RNA extraction from whole embryos or tissues

RNA was isolated using the RNeasy Micro Kit (Qiagen 74004) following the "fibrous tissue" protocol, which incorporates a proteinase K step to degrade protein and prevent clogging of the column during purification. The amount of starting material used varied depending on whether whole embryos or dissected tissues were used. In general no more than 15 whole embryos or an equivalent amount of dissected tissue were used.

## 2.5 Analysis of embryos

### 2.5.1 Whole-mount *in situ* hybridisation (WISH)

#### 2.5.1.1 Fixation and storage of samples

Embryos had their vitelline membranes removed as previously described (see section 2.4.7) and were fixed in 1.5 ml tubes in 1 ml MEMFA, with a maximum of 20 per tube. Samples were fixed overnight at room temperature before being washed three times for 30 min in 100 % methanol. Embryos were then stored at – 20 °C at least overnight and could be stored this way for several months prior to staining.

#### 2.5.1.2 Hybridisation of DIG labelled probe with mRNA

All washes were done at room temperature in a volume of 1 ml when performed in tubes by hand, 40 ml when in a 24 well plate and 80 ml when in a 48 well plate using an *in situ*

machine (Intavis). All washes were for 10 min unless stated otherwise. All steps performed at 67 °C were done by hand in 1.5 ml tubes kept on their side in a dry block heater (Techne DB-3D).

Embryos were rehydrated in a methanol series: (75 % (v/v) methanol, PBS/Tw; 50 % (v/v) methanol, PBS/Tw; 25 % (v/v) methanol, PBS/Tw) followed by three washes in PBS/Tw, 5 min in each. Embryos were then bleached in bleaching solution on a light box until all pigment was removed. Samples were immediately washed three times in PBS/Tw to remove all bleaching solution. Embryos older than stage 34 were then permeabilised in 8 μg/ml proteinase K, PBS/Tw for 1 min before being washed three times in 2 mg/ml glycine PBS/Tw to stop the proteinase K reaction and then three times in PBS/Tw to completely remove the glycine.

Embryos were then acetylated by washing three times in 0.1 M TEA with 2.5 μl/ml acetic anhydride added to the second wash and 5 μl/ml added to the third. This was followed by three washes in PBS/Tw. Embryos were equilibrated in 67 °C hybridisation buffer for 30 min before washing into 67 °C hybridisation buffer for 6 hr. This hybridisation buffer was then replaced with hybridisation buffer containing DIG-labelled probe (see section 2.3.2) and incubated overnight at 67 °C.

#### 2.5.1.3 Antibody binding of DIG-labelled probe

Hybridisation buffer containing probe was removed and stored at - 20°C for use in future experiments. Excess probe was removed by washing twice in 50 % formamide (v/v) (VWR 24311.320) 5 x SSC at 67 °C followed by 25 % (v/v) formamide, 2 x SSC at 65 °C then 12.5 % formamide (v/v) 2 x SSC then 2 x SSC at 63 °C, 0.1 % (v/v) Tween 20 and finally 0.2 x

SSC, 0.1 % (v/v) Tween 20 for 30 min at 61 °C. Embryos were then washed three times in PBS/Tw followed by three times in MAB/Tw.

Samples were then blocked by incubating for 4 hr in MAB/block before washing into MAB/Tw containing 1/2000 (v/v) alkaline phosphatase conjugated anti-digoxigenin Fab antibody fragments (Roche 11082736103) for 12 hr at 4 °C. Excess antibody was removed by washing five times in MAB/Tw for 90 min, before washing over night in MAB/Tw at 4 °C.

#### 2.5.1.4 Staining using BM purple

Samples were washed three times in AP buffer before the addition of 50 % (v/v) AP buffer 50 % (v/v) BM purple (Roche 11442074001). At this point the reaction was light sensitive so the samples were stored wrapped in tinfoil. Staining time varied depending on the probe used and the embryonic stage. Where staining was left overnight the samples could be placed at 14 °C or 4 °C to slow or stop the staining reaction to prevent over-staining. When staining was complete embryos were fixed by washing into MEMFA for 1 hr. Excess stain was removed by repeated washing in methanol before the staining was photographed in ethanol (see section 2.5.5).

### 2.5.2 Clearing WISH embryos

To visualise genes expressed at the midline, embryos were cleared by adding a 2:1 solution of benzyl benzoate/benzyl alcohol (Sigma). As much methanol as possible was removed from embryos using a 200 µl pipette before equilibrating embryos in 500 µl benzyl benzoate/benzyl alcohol before replacing with fresh benzyl benzoate/benzyl alcohol after 15

min. At this point it is possible to photograph embryos with the midline clearly visible. After photography embryos were washed three times for 10 min in methanol and stored at – 20 °C.

### 2.5.3 *In situ* hybridisation on embryo sections

#### 2.5.3.1 Fixation

Embryos were washed three times for 20 min in 4 % PFA before storing individual embryos in 1.5 ml tubes containing 1 ml 4 % PFA. Samples were stored at 4 °C for at least 1 week prior to sectioning.

#### 2.5.3.2 Wax Embedding and Sectioning

All washes were for 10 min using 1 ml unless stated otherwise. Embryos were washed three times in PBS/Tw and dehydrated by washing in an ethanol series: (25 % (v/v) Ethanol, PBS/Tw; 50 % (v/v) ethanol, PBS/Tw; 75 % (v/v) Ethanol, PBS/Tw). Samples were then washed three times in 100 % ethanol followed by two washes in xylene. Embryos were then washed into 50 % (v/v) xylene, 50 % (v/v) paraffin for 45 min at 70 °C followed by three washes in 100 % paraffin for 30 min at 70 °C. Embryos were then placed in plastic containers, orientated and set in paraffin using a tissue embedding machine (Leica EG1150). Embryos were left to fully set overnight.

A microtome (Leica, RM2165) was used to cut the embryo into 10 μm sections, which were transferred to a TESPA-coated glass slide with a thin layer of $H_2O$ on the slide. This $H_2O$ was then allowed to evaporate by placing the slide on a heated flattening table (Leica HI1220) at

37 °C and leaving it overnight to dry. Slides were then stored at room temperature prior to staining.

#### 2.5.3.3 De-waxing and *in situ* hybridisation

Slides were washed for 5 min in 50 ml glass coplin jars by transferring slides from one coplin jar to another, unless stated otherwise. Slides were washed three times in 100 % xylene to remove the wax from the sections. Samples were then washed twice in 100 % ethanol before being incubated in 6 % (v/v) hydrogen peroxide, methanol for three hr. Sections were then rehydrated to PBS/Tw using a methanol series (75 % (v/v) methanol, PBS/Tw; 50 % (v/v) methanol, PBS/Tw; 25 % (v/v) methanol, PBS/Tw) then washed three times in PBS/Tw.

Slides were incubated in 12.5 µg/ml Proteinase K, PBS/Tw for 15 min before inactivating the proteinase K by washing twice with 2 mg/ml glycine, PBS/Tw. Sections were then refixed at room temperature for 30 min in 4 % (v/v) PFA, 0.2 % (v/v) glutaraldehyde (Sigma G-5882), PBS. This step was performed in a sealable plastic box with the slides held horizontally, adding 1 ml of fixative on the surface of the slide. Slides were then washed twice in coplin jars with PBS/Tw and then were prehybridised in a sealable plastic box with the slides held horizontally, adding 300 µl hybridisation solution (50 % (v/v) formamide, 5 X SSC pH4.5, 50 µl/ml torula RNA, 1 % SDS, 50 µg/ml heparin) covering the slide with a cover slip (VWR 631-0135) and incubating at 70 °C for 2 hr. Hybridisation solution was then replaced with hybridisation solution containing probe and incubated at 70 °C overnight. After hybridisation, sections were washed twice for 30 min at 70 °C in solution I (50 % formamide, 5 X SSC pH 4.5, 1 % (v/v) SDS) and twice for 30 min at 70 °C in solution II (50 % (v/v) formamide, 2 X SSC pH 4.5). Slides were then washed three times in MAB and then were blocked for 2 hr in MAB/block. Slides were then incubated for 2 hr with MAB/block

containing 1/2000 (v/v) alkaline phosphatase conjugated anti-digoxigenin Fab antibody fragments (Roche 11082736103). Sections were then washed 4 times in MAB/Tw and then 3 times in AP buffer. Staining was developed by incubating in 75 % (v/v) BM purple, 25 % (v/v) AP buffer 10 % PVA.

At this point the reaction was light sensitive so the samples were stored wrapped in tinfoil. Staining time varied depending on the probe used and the embryonic stage. When staining was complete embryos were fixed by washing into MEMFA for 1 hr. Excess stain was removed by repeated washing in PBS/Tw before the samples were mounted in 80 % glycerol and the staining photographed (see section 2.5.5).

### 2.5.4 Western blotting

Samples for western blotting were snap frozen in liquid nitrogen and stored at – 80 °C to minimise protein degradation. Samples were homogenised in oocyte lysis buffer (OLB) (10 μl/embryo or 50 μl/ 10 dissected DLPs) on ice by pipetting up and down. Samples were then spun for 20 min at 4 °C. The supernatant was then removed and mixed with equal volume of 2 x RSB (reticulocyte standard buffer), boiled at 98 °C for 5 min then 10 μl was loaded into the wells of a 10 % Bis-Tris gel. One well was loaded with protein ladder (full range rainbow ladder, Amersham RPN 800E) to allow protein band estimation. Where there were insufficient samples to fill all the wells of the gel, 50 % (v/v) RSB, 50 % (v/v) OLB was loaded to prevent uneven separation of the protein sample on the gel.

The gel was first run at 50 V for 30 min to stack the samples into the gel and was then run at 200 V for 2 hr.

The protein was then transferred from the gel to nitrocellulose membrane using a Trans-Blot Turbo Transfer system (Biorad). The turbo protocol was in accordance with the manufacturers instructions.

The membrane was then transferred to a 50 ml tube containing TBS/Tw and washed twice briefly in TBS/Tw before blocking the membrane with 5 % (w/v) marvel, TBS/Tw overnight at room temperature. After blocking, the primary antibody was applied in 7 ml of 0.5 % (w/v) marvel, TBS/Tw at 4 °C overnight. The antibody containing solution was then removed and stored at 4 °C and the blot washed three times in TBS/Tw to remove excess antibody from the blot. The secondary antibody was then applied in 7 ml of 0.5 % (w/v) marvel, TBS/Tw and left to incubate for 1 hr. Excess secondary was removed by washing 4 times in TBS/Tw. Detection of the secondary antibody was visualised using western blot luminol reagent (Santa Cruz SC-2048) in accordance with the manufacturers protocol using Biomax light film for chemiluminescence (Kodak) and exposing the blot for different time intervals to ensure correct exposure.

### 2.5.5 Imaging frog embryos

Images of live or fixed and stained embryos were captured using a Nikon SMZ-U 1500 dissecting microscope with a Q Imaging Micropublisher 5.0 Uncooled CCD Colour Camera using Q-Capture Pro 7 software. Fluorescent embryos were imaged using a Nikon Eclipse E600 compound microscope equipped with a Nikon Plan Fluor 10 x/0.30 DIC L lens and photographed using an Olympus Camedia C-3030 zoom digital camera. Where additional lighting was required, embryos were illuminated using SCHOTT KL1500 light source.

### 2.6 Preparation of images for publication

Adobe Photoshop CS4 and Adobe Illustrator CS4 were used to prepare images for print. Schematic images of pre-mRNA were drawn to scale using the ‘exon-intron graphic maker’ found at wormweb.org/exonintron.

# Chapter 3
# BMP signalling is required post-gastrulation for HSC ontogeny

# Chapter 3 BMP signalling is required post-gastrulation for HSC ontogeny

## 3.1 Introduction

BMP signalling is required for mesodermal patterning and cellular movements during gastrulation (see section 1.2.1), therefore exploring the role of BMP signalling post-gastrulation has been difficult without temporal control of BMP signal inhibition. Disrupting BMP signalling results in embryonic death in mice, where BMP inhibition blocks all mesoderm production (see section 1.2.8). Blocking BMP signalling in *Xenopus* by injection of mRNA coding for a dominant negative truncated BMP type II receptor, resulted in gross morphological defects that prevented the analysis of adult HSC programming (Ciau-Uitz et al., 2000b). Studies by our group have since suggested that BMP signalling is required for the expression of the critical HSC transcription factor *runx1* in the zebrafish dorsal aorta (Wilkinson et al., 2009). However if the role of BMP signalling in HSC ontogeny is to be fully explored, it must be studied in *Xenopus* where the spatial separation of primitive and definitive blood allows each population to be examined independently. Furthermore, the gene regulatory network describing programming events during HSC ontogeny in *Xenopus* is unparalleled in any other *in vivo* model of haematopoiesis, allowing the careful analysis of inputs into this network. With the aim of exploring the role of BMP signalling in HSC programming, new methods of inhibiting BMP signalling in *Xenopus* with temporal specificity were required.

Dorsomorphin dihydrochloride was identified as a BMP inhibitor in a chemical screen for small molecules that disrupt dorsal-ventral patterning in zebrafish (Yu et al., 2008a). It has

been used in many research areas such as neuronal signalling (Hegarty et al., 2013; Liu et al., 2013), oncology (Maegdefrau and Bosserhoff, 2012; Yan et al., 2012) and erythropoiesis (Hao et al., 2008; Ferri-Lagneau et al., 2012). The phenotype resulting from dorsomorphin dihydrochloride treatment has commonly been attributed to inhibition of BMP signalling. However dorsomorphin dihydrochloride also inhibits the Vegf receptor, Flk1 (Hao et al., 2010). Furthermore, dorsomorphin dihydrochloride inhibits Flk1 at a lower concentration than required to block the BMP type I receptor (Flk1 $IC_{50}$ 25.1 nM, BMPR-I $IC_{50}$ 148.1 nM) (Hao et al., 2010). Therefore, dorsomorphin dihydrochloride is not a suitable tool to study the role of BMP signalling during *Xenopus* haematopoietic programming.

In late 2009, Hao et al. descried an *in vivo* structure-activity relationship study of dorsomorphin dihydrochloride (Hao et al., 2010) where they used the BMP-dependent dorsal-ventral patterning defect and the *flk1*-dependent intersomitic vessel defect in the zebrafish (Habeck et al., 2002; Covassin et al., 2006) to test analogues of dorsomorphin dihydrochloride for specific BMP signalling or *flk1* inhibition. One of these analogues, DMH1, was identified as a potent ($IC_{50}$ 107.9 nm) and specific BMP inhibitor which dorsalised embryos without disrupting angiogenic sprouting of the intersomitic vessels. DMH1 functions through inhibition of BMP type I receptors *alk1*, *alk3* and *alk6* and thus blocks BMP-mediated Smad1/5/8 phosphorylation (Figure 3-1) (Hao et al., 2010).

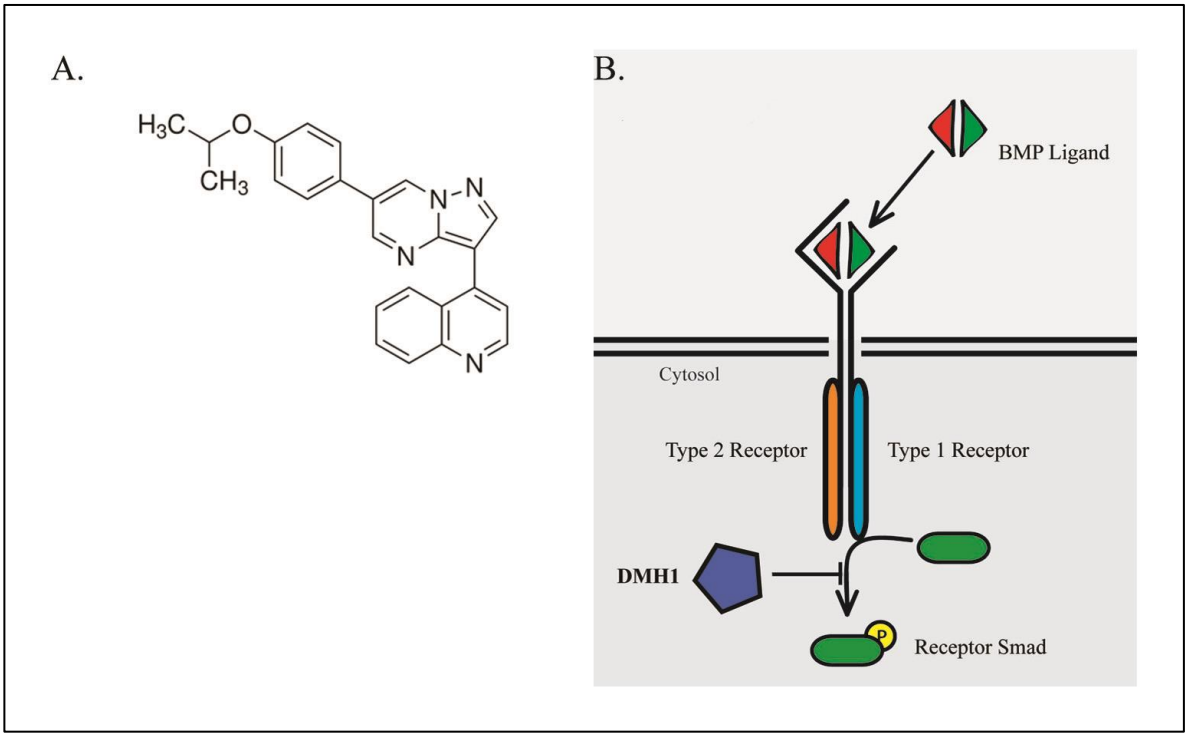



**Figure 3-1: DMH1 structure and scheme showing position in BMP signalling pathway**

A) Chemical structure of DMH1; 4-[6-(4-Isopropoxyphenyl)pyrazolo[1,5-a]pyrimidin-3-yl]quinoline. B) Scheme showing DMH1 (shown as blue pentagon) inhibiting BMP type I receptor (Alk1, Alk3 and Alk6) mediated R-Smad (Smad 1/5/8) phosphorylation.

The discovery of DMH1 as a potent and selective BMP signalling inhibitor in zebrafish suggested that DMH1 could be used to block BMP signalling during *Xenopus* embryonic development. DMH1 has no inhibitory effect on Tgf-β, or Flk1 when tested using purified human enzymes in an *in vitro* kinase assay (Hao et al., 2010), however additional, untested, off-target affects may be present when treating embryos with DMH1. For this reason, a second, independent, method of temporal BMP signalling inhibition was required to strengthen and confirm any phenotype resulting from DMH1 treatment.

An alternative method for inhibiting BMP signalling with temporal control is using a transgenic *Xenopus* line. The *Xenopus* tail is able to regenerate during embryogenesis and in 2003 Jonathan Slack's group at the University of Bath showed that BMP is required for this process (Beck et al., 2003). They examined the role of BMP signalling in this process by creating a *Xenopus* transgenic line in which the *Xenopus* heat shock promoter (HSP70) drives the expression of the naturally occurring diffusible BMP inhibitor, *noggin.* The transgenic construct also contains a marker that expresses GFP under the control of the *γ-crystallin* promoter (Figure 3-2). This allowed transgenic offspring to be identified via GFP fluorescence in the developing eye. We therefore obtained this transgenic line to use in parallel with DMH1 to investigate the post-gastrulation role of BMP signalling in HSC specification.

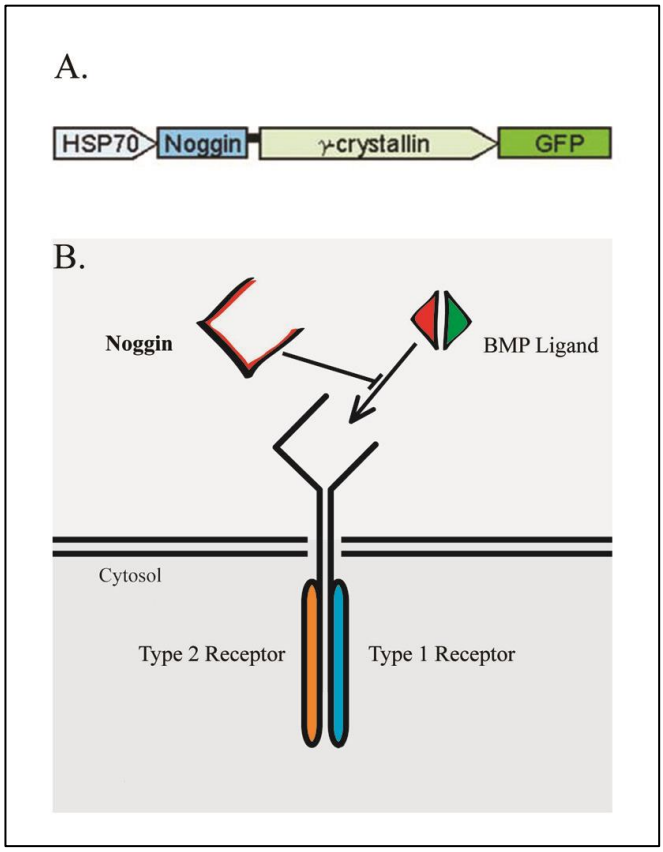



**Figure 3-2 Transgenic construct in heat-shock *noggin Xenopus* and scheme showing position in BMP signalling pathway**

A) Transgenic construct present in *Xenopus* heat-shock *noggin* transgenic line. The *Xenopus* heat-shock promoter HSP70 is used to drive expression of Noggin protein with temporal control. GFP under the control of the *γ-crystallin* promoter allows transgenic offspring to be identified via GFP fluorescence in the developing eye. B) Scheme showing Noggin protein inhibiting BMP signalling by binding and sequestering BMP ligands BMP2, BMP4, BMP5, BMP6, BMP7.1, BMP7.2 and Gdf6, preventing them from binding to the BMP receptor and therefore inhibiting BMP signalling.

The data presented in this chapter show, using the small molecule DMH1 and the heat-shock *noggin* transgenic *Xenopus* line, that BMP signalling can be inhibited during *Xenopus* embryonic development with temporal specificity. Using these methods to inhibit BMP signalling post-gastrulation, the patterning defects associated with pre-gastrulation BMP signalling inhibition can be avoided. Furthermore, shown here for the first time, using two independent methods of BMP signalling inhibition, is evidence that BMP signalling is required post-gastrulation for HSC programming in *Xenopus*.

### *3.2* BMP ligands and receptors are expressed in post-gastrulation haematopoietic tissues in *Xenopus*

BMP signalling requires the binding of BMP ligands to BMP receptors on the surface of the target cell. If BMP signalling is required post-gastrulation for haematopoietic stem cell programming, then it logically follows that BMP ligands must be expressed close to or by the target cell, and both type I and type II BMP receptors must be expressed in the haematopoietic stem cell precursor itself. BMP ligands and receptors are expressed in the *Xenopus* dorsal aorta prior to the expression of HSC markers (Aldo Ciau-Uitz, personal communication). To investigate whether the BMP signalling pathway is also likely to be active in the DLP when the precursors to the HSCs are being specified (stage 26), whole-mount *in situ* hybridisation was performed. The expression pattern of a panel of probes related to the BMP signalling pathway, including the BMP type I receptors *alk1*, *alk3*, and *alk6*, the BMP type I receptor *actRII*, and the BMP ligands *bmp2*, *bmp4*, *bmp7* and *gdf6*, were analysed in stage 26 embryos.

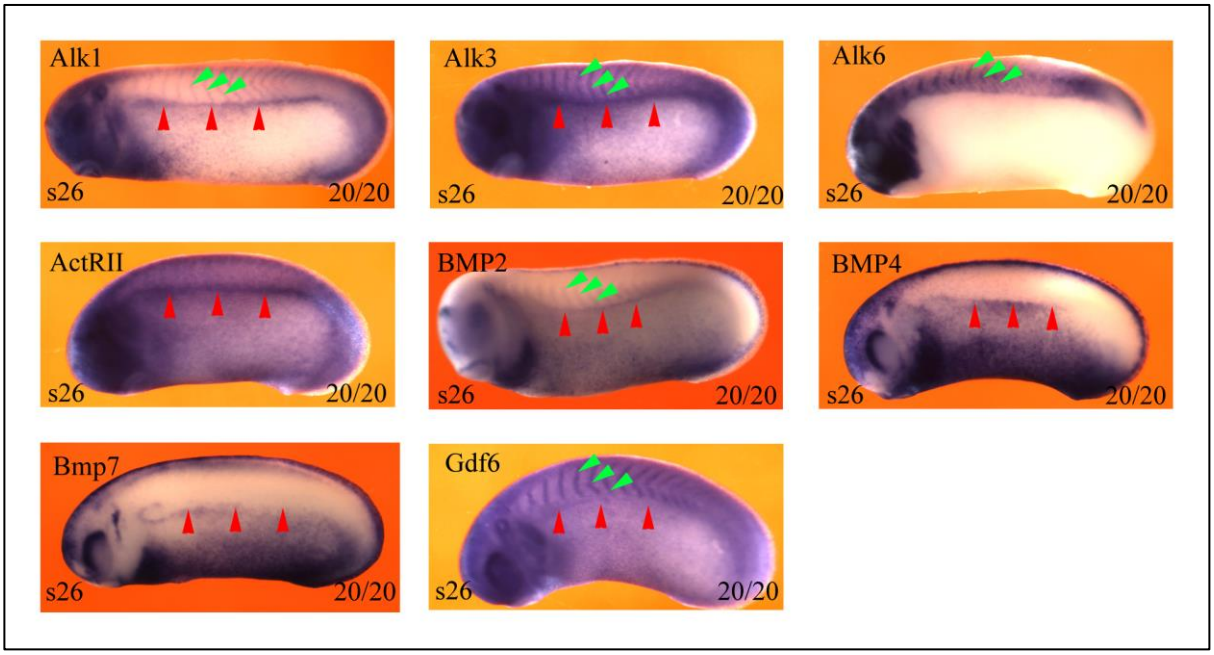



**Figure 3-3: BMP ligands and receptors**

Whole-mount *in situ* analysis of BMP receptors (*alk1*, *alk3*, *alk6*, *actRII*) and BMP ligands (*bmp2*, *bmp4*, *bmp7*, *gdf6*) at stage 26. Embryos were photographed from the lateral view, with anterior to the left and dorsal to the top. Text on individual images shows the probe used (top left), the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Red arrowheads indicate staining in the DLP. Green arrowheads indicate somitic expression. Images and numbers are from one experiment but are representative of at least three biological replicates.

Both type I and II BMP receptors and several BMP ligands are expressed in the DLP at stage 26, when HSC precursors in this population are being programmed. This suggests that the BMP signalling pathway is active at this stage. The type I BMP receptors *alk1*, *alk3* and the type II BMP receptor *actRII* are all clearly expressed in the DLP, making these cells sensitive to BMP ligands in the surrounding tissues. Furthermore, the BMP ligands *bmp2*, *bmp4*, *bmp7* and *gdf6* are also expressed in the DLP. *BMP2* and *gdf6* are also expressed in the somites. The expression domain of *alk6* is mostly in the head and in the somites, and does not extend ventrally to include the DLP. Vegfa, expressed by the somites is essential to haematopoietic programming in the adjacent DLP (Ciau-Uitz et al., 2013), therefore BMP signalling activity in the somites has potential to influence programming events in the DLP. In summary, WISH at stage 26 indicates that the genes required for BMP signalling are expressed in the DLP and have the potential to feed into the haematopoietic programming of these cells.

### 3.2.1 Induced Noggin protein is stable for at least 24 hr post-heat shock.

In order to test the temporal window required for haematopoietic programming during *Xenopus* embryonic development using the heat-shock inducible transgenic line, it is important to know the kinetics of Noggin transcription and stability. Embryos were heat-shocked at stage 14 (immediately post-gastrulation) for 30 min at 35 °C and protein levels were analysed by western blot (Figure 3-4). Noggin protein, produced by heat shock induction in this transgenic line, contains a C-terminal HA tag, which allowed the detection of induced Noggin protein by western, using an anti-HA antibody.

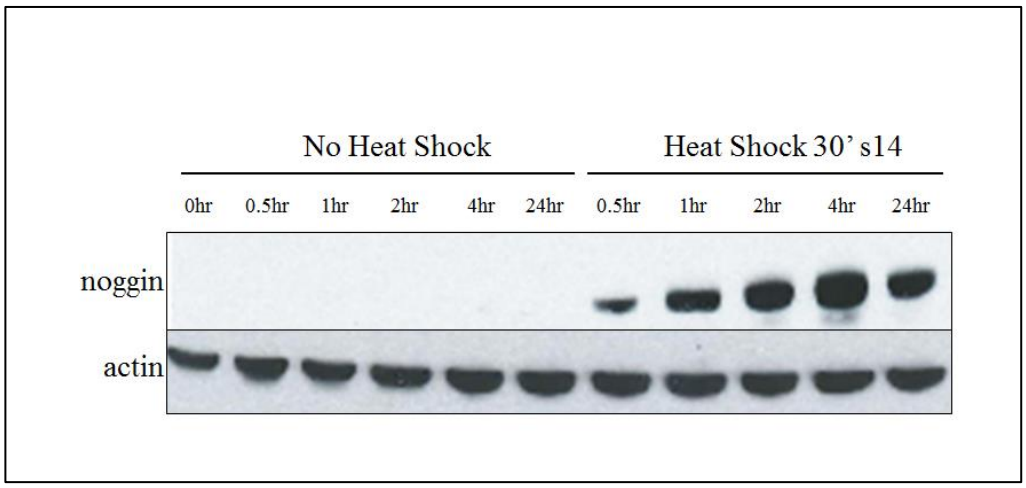



**Figure 3-4 Noggin protein stability post-heat shock**

Western blot probed for HA-tagged Noggin protein (top panel) and actin protein (bottom panel), which is used as a loading control. Samples were heat shocked at stage 14 for 30 min at 35 °C. Time points shown indicate the time following the beginning of heat shock at which samples were collected. Twenty embryos were collected for each time point.

From this we can conclude that no detectable Noggin protein is produced without heat shock induction. The membrane was overexposed after the above exposure was collected and no bands corresponding to Noggin protein were seen in samples without heat-shock. Noggin protein is produced within the 30 min of heat shock, with a band corresponding to Noggin protein visible when samples were collected immediately after heat-shock. The speed of Noggin protein production following heat-shock suggests that BMP signalling can be inhibited with good temporal control. Noggin protein levels decline between 4 to 24 hr however a significant level of Noggin protein is still visible at 24 hr post-heat shock. This finding informed the design of future experiments. Embryos kept past 24 hr post-heat shock had additional Noggin protein induced by heat shock every 24 hr.

### 3.2.2 Heat shock induction of *noggin* expression in transgenic *Xenopus* results in reduced levels of phosphorylated BMP R-Smads

To confirm that the Noggin protein produced via heat-shock was able to inhibit BMP signalling, the level of phosphorylated BMP R-Smads was also analysed by western blot. In this experiment, the dorsal lateral plate tissue was dissected from 10 stage 25 embryos in control and transgenic samples, following heat-shock at stage 14. No *Xenopus*-specific phosphorylated Smad antibodies have been generated. Two anti-phospho- smad1/5/8 antibodies from Cell Signalling (9516S and 9511S) gave multiple bands despite extensive troubleshooting, including varying the blocking substrate and altering antibody dilutions. These non-specific bands prevented the visualisation of the target band, therefore we turned to a polyclonal anti-phospho- smad1/5/8 antibody from Millipore (AB3848). Using this antibody, clear bands were detected (Figure 3-5) which correspond to *Xenopus* phosphorylated BMP R-Smads.

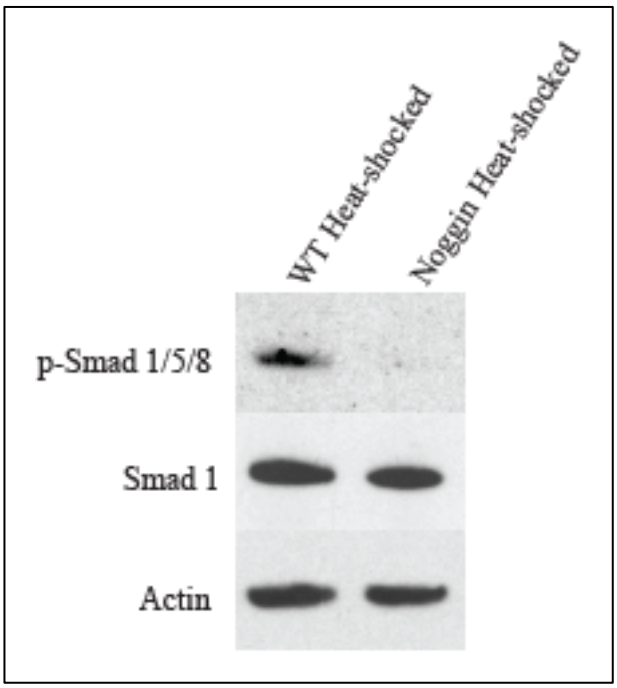



**Figure 3-5 Heat shock induction of Noggin protein inhibits BMP R-Smad phosphorylation in the developing *Xenopus* embryo**

Western blot analysing the effect of Noggin induction on BMP signalling. Samples were heat-shocked at stage 14 for 30 min at 35 °C and were collected at stage 25. DLPs were dissected from 10 embryos for each condition. The membrane was probed for phosphorylated R-Smads (Millipore AB3848, top panel), total Smad1 (Cell Signalling 6944P, middle panel) and actin (Sigma A3854, lower panel). The western shown is representative of three biological replicates.

As a band corresponding to phosphorylated BMP R-Smad protein is present in the control sample, we can conclude that BMP signalling is active in the DLP of stage 25 *Xenopus* embryos. When comparing the levels of phosphorylated BMP R-Smads with embryos that had Noggin protein induced from stage 14, we clearly see a loss of BMP R-Smad phosphorylation. Altogether the data show that heat-shock induction of Noggin protein is sufficient to block BMP R-Smad phosphorylation and therefore BMP signalling during *Xenopus* embryogenesis. Total Smad1 protein levels show that this is not coupled with a compensatory increase in Smad1 levels (Figure 3-5).

### 3.2.3 DMH1 treatment results in reduced levels of phosphorylated BMP R-Smads

Although DMH1 has been shown to inhibit BMP signalling, via decreased phosphorylation of BMP R-Smads, this was done *in vitro* in HEK293 cells (a human embryonic kidney cell line) (Hao et al., 2010). In zebrafish, DMH1 was shown to dorsalise embryos (indicating a block in BMP signalling), however phosphorylation levels of BMP R-Smads were not analysed (Hao et al., 2010). To investigate whether DMH1 was also able to inhibit BMP signalling in *Xenopus*, embryos were treated with 100 μM DMH1 at stage 14 (immediately post-gastrulation) and at stage 25 the DLP was dissected from these embryos and collected for analysis of BMP R-Smad phosphorylation by western blot (Figure 3-6).

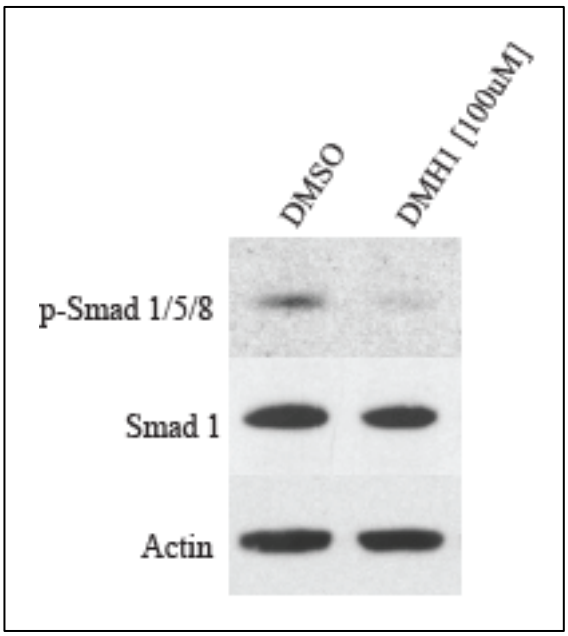



**Figure 3-6 DMH1 treatment inhibits BMP R-Smad phosphorylation in the developing *Xenopus* embryo**

Western blot showing the effects of DMH1 treatment on BMP signalling. Samples were treated with 100 μM DMH1 at stage 14 and were collected at stage 25. DLPs were dissected from 10 embryos for each condition. The membrane was probed for phosphorylated R-Smads (Millipore AB3848, top panel), total Smad1 (Cell Signalling 6944P, middle panel) and actin (Sigma A3854, lower panel). The western shown is representative of three biological replicates.

Treatment of embryos with 100 μM DMH1 resulted in a clear reduction of BMP R-Smad phosphorylation. This shows for the first time that DMH1 is able to block BMP R-Smad phosphorylation and therefore BMP signalling during *Xenopus* embryogenesis. In agreement with the previously described analysis of Smad1 protein levels in heat-shock Noggin induced embryos (see section 3.2.2), no compensatory increase in total Smad1 protein levels is observed in DMH1-treated embryos (Figure 3-6).

### 3.2.4 Inhibition of BMP signalling by DMH1 or Noggin induction prior to the completion of gastrulation results in expected gross morphological defects

Previously, mRNA coding for the truncated BMP receptor, a dominant negative form of the BMP type II receptor *bmprII*, was injected into *Xenopus* embryos to inhibit BMP signalling (Ciau-Uitz et al., 2000b). These embryos were dorsalised, with extra head tissue at the expense of tail. In order to test if DMH1 or heat shock-induced Noggin protein was able to phenocopy this previous experiment, BMP was blocked using these methods at stage 13 (immediately prior to the completion of gastrulation).

Consistently, when BMP signalling was inhibited prior to the completion of gastrulation, either by DMH1 treatment or in the *Xenopus* transgenic line, embryos were dorsalised (Figure 3-7). Although it is difficult to compare levels of BMP signalling inhibition, the results shown here indicate that DMH1 and *noggin* induction inhibits BMP signalling at least to the level required to induce dorsalisation of the embryo. Western analysis shows that BMP R-Smad phosphorylation is inhibited to an undetectable level (Figure 3-5, Figure 3-6).

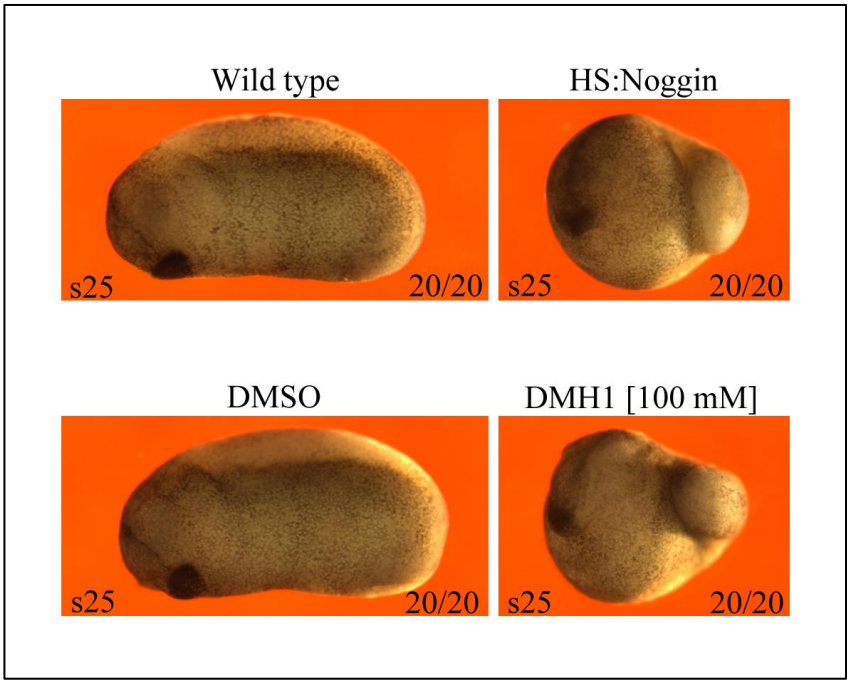



**Figure 3-7 Inhibition of BMP signalling by DMH1 or Noggin induction prior to the completion of gastrulation results in gross morphological defects**

Embryos were treated by heat-shock or small molecule inhibitor from stage 13 and collected at stage 24. Embryos were photographed from the lateral view, with anterior to the left and dorsal to the top. Text on individual images shows the stage imaged (bottom left) and the proportion of embryos represented by that image (bottom right). Images and numbers reflect one experiment that is representative of at least three biological replicates.

### 3.2.5 Induction of noggin post-gastrulation avoids gross morphological defects

To analyse the role of BMP signalling post-gastrulation, the gross morphological defects shown in Figure 3-7 had to be avoided. This was particularly relevant with respect to mesoderm. The adult HSC is derived from mesodermal tissues and so if post-gastrulation BMP inhibition resulted in abnormal mesodermal patterning, the role of BMP signalling in the HSC ontogeny could not be analysed. To test if post-gastrulation BMP-inhibited embryos had normal gross morphology and mesodermal patterning, embryos were treated at stage 14 (immediately post-gastrulation), collected at stage 25 and analysed by whole mount *in situ* hybridisation. The markers *hand1*, *foxf1* and *hoxc10* were analysed at stage 25 (Figure 3-8). These markers were selected based on their expression in the lateral plate mesoderm with distinct boundaries that could be used to assess correct axis patterning. *Hand1* and *foxf1* are normally expressed in the anterior two thirds of the embryo, with the upper boundary of expression bordering the dorsal lateral plate mesoderm (Deimling and Drysdale, 2011). *Hoxc10* is clearly expressed in the posterior half of the embryo, which is lost if BMP signalling is blocked during gastrulation (Deimling and Drysdale, 2011). Therefore these markers can be used to determine whether post-gastrulation inhibition of the BMP signalling pathway causes mesodermal patterning defects. If the patterning of these genes was perturbed in post-gastrulation BMP inhibition then that would preclude the study of HSC ontogeny with respect to BMP signalling.

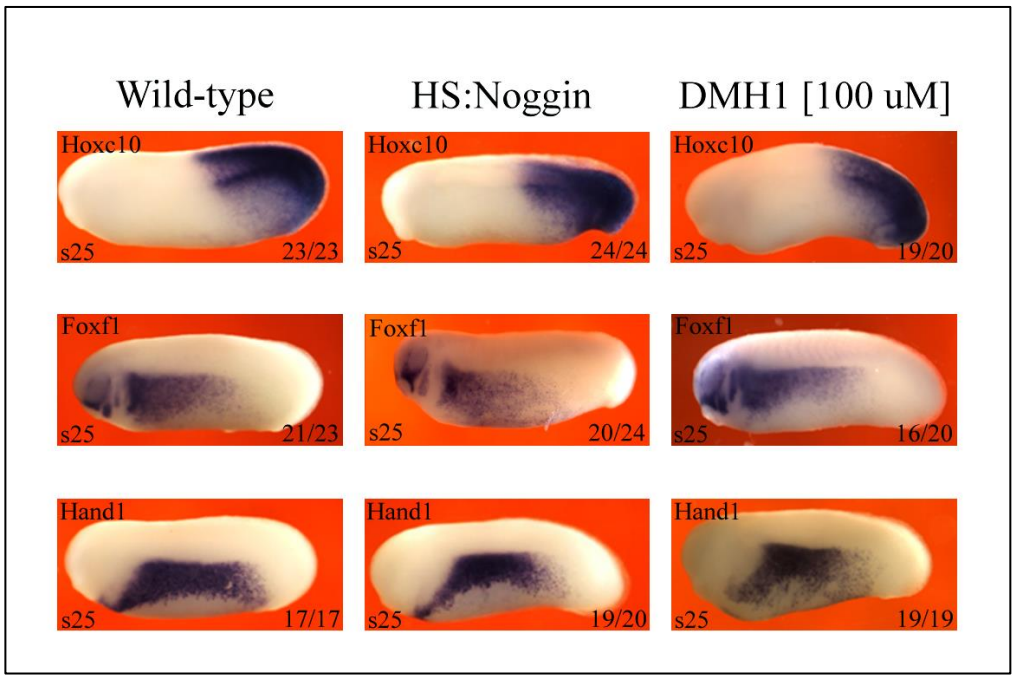



**Figure 3-8 Post-gastrulation BMP inhibition does not affect mesodermal patterning and dorsal ventral axis formation**

Whole-mount *in situ* analysis of mesodermal markers *hoxc10*, *foxf1* and *hand1* following BMP inhibition from stage 14 and collection at stage 25. Embryos were photographed from the lateral view, with anterior to the left and dorsal to the top. Text on individual images shows the stage imaged (bottom left), the probe used (top left) and the proportion of embryos represented by that image (bottom right). Images and numbers reflect one experiment that is representative of at least three biological replicates.

When BMP was inhibited at stage 14 following the closure of the blastocoel, no increased mortality, gross morphological defect or growth retardation was observed. A slight indentation can be seen in the developing tail from stage 24 onwards (Figure 3-7). No significant differences in the expression of *hand1*, *foxf1* and *hoxc10*, between control and BMP inhibited embryos, can be detected. This is particularly important with respect to the dorsal lateral plate tissue, which borders the expression of both *foxf1* and *hoxc10*. As BMP signalling inhibition does not affect the expression of these genes we can conclude that that the gross morphological defects associated with pre-gastrulation BMP signalling inhibition can be avoided by temporal inhibition from stage 14.

### 3.2.6 BMP is required post-gastrulation for HSC programming

In order to determine whether BMP signalling is required post-gastrulation for HSC programming, BMP signalling was inhibited by heat-shock induction of *noggin* expression in the *Xenopus* transgenic line or by treatment with 100 μM DMH1 from stage 14. Embryos were collected at stage 39 and stained for the haematopoietic markers, *runx1*, *spib* and *lmo2.* These haematopoietic markers are essential for HSC development. Runx1 mouse knockouts do not produce any definitive blood cells and die at embryonic day 12.5 (Chen et al., 2009). Furthermore *runx1* inhibition in zebrafish results in low numbers of HSCs budding from the dorsal aorta. The few cells that do bud burst immediately after budding (Kissa and Herbomel, 2010b). Lmo2 knockout mice have no haematopoietic precursors and die at embryonic day 9.5 (Yamada et al., 1998a). Lmo2 acts as a scaffold protein for a complex of haematopoietic transcription factors, including Scl, Gata1, Gata2 and Ldb1 (El Omari et al., 2011). Spib is another essential haematopoietic transcription factor, which when knocked down in *Xenopus* results in defective haematopoietic programming (Costa et al., 2008b).

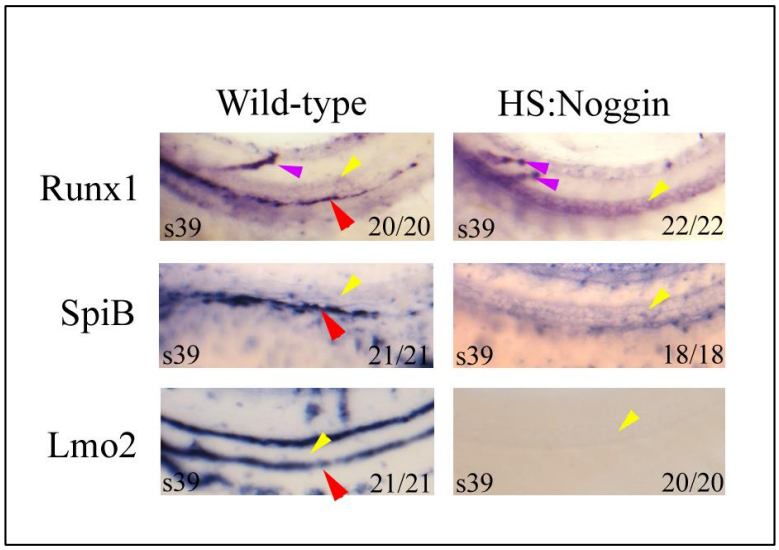



**Figure 3-9: Noggin inhibition of BMP signalling post-gastrulation blocks HSC programming**

Embryos were heat-shocked at stage 14 and cultured until stage 39, at which point they were collected for whole-mount *in situ* hybridisation analysis of the haematopoietic markers *runx1*, *spib* and *lmo2*. Embryos were photographed from the lateral view, with anterior to the left and dorsal to the top. Text on individual images shows the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Red arrowheads highlight the dorsal aorta, yellow arrowheads highlight the notochord and purple arrowheads highlight the developing lateral line. Images and numbers are from one experiment and are representative of at least three biological replicates.

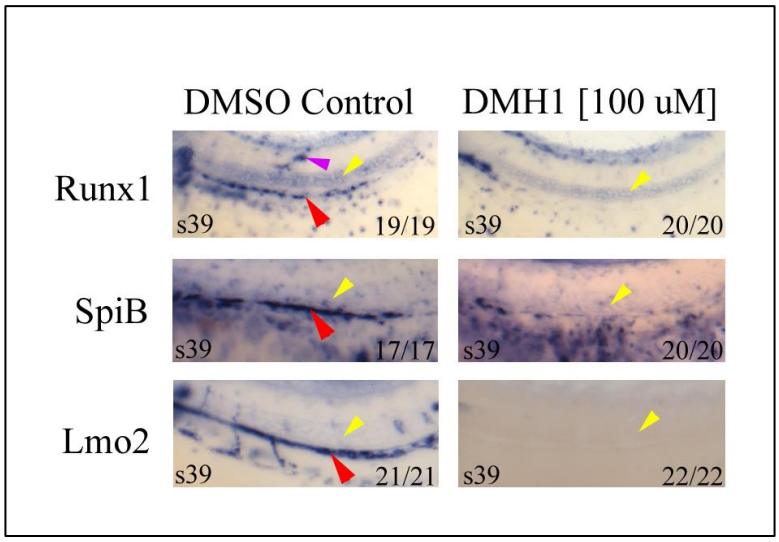



**Figure 3-10 DMH1 treatment post-gastrulation blocks HSC programming**

Embryos were heat-shocked at stage 14 and cultured until stage 39, at which point they were collected for whole-mount *in situ* hybridisation analysis of the haematopoietic markers *runx1*, *spib* and *lmo2*. Embryos were photographed from the lateral view, with anterior to the left and dorsal to the top. Text on individual images shows the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Red arrowheads highlight the dorsal aorta, yellow arrowheads highlight the notochord and purple arrowheads highlight the developing lateral line. Images and numbers are from one experiment and are representative of at least three biological replicates.

When BMP signalling was blocked from stage 14 by DMH1 or by heat-shock induced *noggin* expression in the transgenic line, HSC markers were lost from the stage 39 dorsal aorta. This shows, by two methods of BMP signalling inhibition looking at three HSC markers, that BMP signalling is required post-gastrulation for HSC development in *Xenopus*.

### 3.3 Discussion and conclusions

Whole-mount *in situ* hybridisation (WISH) staining of the developing *Xenopus* embryo shows that BMP ligands and type I and type II BMP receptors are all expressed in the DLP. More specifically, the BMP type I receptors *alk1* and *alk3*, the BMP type II receptor *actRII* and the BMP ligands *bmp2*, *bmp4*, *bmp7* and *gdf6* are all expressed in the DLP. This shows the potential for active BMP signalling in these cells. It is also important to remember that WISH stains the cells that contain the mRNA of a gene of interest, and as BMP ligand protein is diffusible, the domains labelled in this experiment are likely an underestimate of the BMP ligand protein domain.

Western blot analysis of phosphorylated BMP R-Smad levels in wild-type dissected DLPs proved that BMP signalling is active in these cells, and therefore likely has a role in their genetic programming during development. As HSCs bud from the floor of the dorsal aorta, analysis of BMP R-Smad phosphorylation in these cells would indicate if BMP signalling is likely to play a role in this stage of HSC development. Unfortunately this type of analysis is not possible with regards to the *Xenopus* dorsal aorta. The stage 39 *Xenopus* dorsal aorta is approximately 4 cells in diameter and as such the manual dissection of the dorsal aorta would include a large proportion of non-haematopoietic tissue, preventing any conclusions regarding BMP R-Smad phosphorylation in the dorsal aorta itself. Fluorescence-activated cell sorting (FACS) is a method of isolating small numbers of cells with a high degree of accuracy. This technique relies on the fluorescent labelling of a target cell, which allows it to

be recognised and separated from non-labelled cells. Three factors prevent this technique from currently being used to isolate dorsal aorta cells in *Xenopus*. Firstly, this technique often uses antibodies to fluorescently label target cells. Few *Xenopus* specific antibodies have been designed and antibodies made for other organisms have thus far shown little cross-reactivity when tested in *Xenopus*. Secondly, an alternative to using antibodies to label the dorsal aorta is the use of a transgenic line, where a fluorescent protein is expressed under the control of a tissue-specific promoter (e.g. *runx1*:GFP). No such transgenic line currently exists in *Xenopus,* and as the genome sequence is incomplete, identifying, cloning and creating a transgenic line expressing a fluorescent tag in the dorsal aorta would require extensive work. Finally, FACS requires cells to be dissociated into single cells to allow them to pass through the sorter and to allow their separation. In our hands, dissociating stage 39 *Xenopus* embryos resulted in massive cell death, preventing cross-reacting antibodies from being tested (personal communication, Rachael Stephenson).

The small molecule inhibitor DMH1 has been shown to dorsalise zebrafish embryos and has been shown to inhibit BMP R-Smad phosphorylation *in vitro* in human embryonic kidney cells (HEK293 cells). Here we show that DMH1 inhibits BMP R-Smad phosphorylation in the developing *Xenopus* embryo *in vivo*. In addition, as the DLP was dissected when collecting these samples, we can say with confidence that DMH1 inhibits BMP signalling in the DLP, the precursors of the dorsal aorta and the adult HSCs. Furthermore DMH1 treatment of embryos prior to the completion of gastrulation results in the dorsalisation of the embryo. Altogether the data here show that DMH1 is able to inhibit BMP signalling at these later stages.

Although the heat-shock inducible noggin *Xenopus* transgenic line has been used by other groups to study the role of BMP signalling during tail regeneration, it was not used at the

stages or in the tissues examined here (Beck et al., 2001). The data presented here show that this transgenic line can be used to inhibit BMP signalling with accurate temporal control.

BMP signalling inhibition from stage 14 onwards did not affect the dorsal ventral patterning of the embryo. Expression pattern analysis of the mesodermal markers *foxf1, hoxc10* and *hand1* showed that mesoderm was normally patterned when BMP signalling was blocked post-gastrulation. A slight indentation can be seen in the developing tail from stage 24 onwards. BMP signalling is required for dorsal ventral patterning in a temporally progressive manner along the anterior-posterior axis during gastrulation (Tucker et al., 2008). As such, the tail organiser is formed at the end of gastrulation and disrupting BMP signalling at this stage results in this subtle tail defect.

When BMP signalling was blocked post-gastrulation, by DMH1 or by heat-shock induced *noggin* expression in the transgenic line, HSC markers were lost from the stage 39 dorsal aorta. This shows, by two methods of BMP signalling inhibition, looking at three HSC markers, that BMP signalling is required post-gastrulation for HSC development in *Xenopus*. It is not clear from this experiment when BMP signalling is required for HSC development, however it is clear that defective patterning of the embryo does not cause this defect.

Previous investigations, studying the role of BMP signalling in HSC programming have suffered due to a lack of suitable tools to allow temporal inhibition of BMP signalling. BMP signalling is required for mesodermal patterning and for convergence and extension movements during gastrulation (Tucker et al., 2008). The result of this requirement is that blocking BMP signalling during gastrulation results in gross morphological defects that prevent direct comparisons with control embryos.

Presented here is evidence that BMP signalling can be inhibited with temporal control during *Xenopus* embryonic development. Temporal inhibition of BMP signalling using the two

methods presented here, allows early patterning defects to be avoided so that for the first time, we demonstrate that BMP signalling is required, post-gastrulation for HSC ontogeny in *Xenopus*.

# Chapter 4

# Timing and target of BMP signalling in HSC development

# Chapter 4 Timing and target of BMP signalling in HSC development

## 4.1 Introduction

The data presented in the previous chapter indicate that BMP is required post-gastrulation for HSC programming during *Xenopus* embryonic development. BMP signalling could be required at multiple points prior to HSC formation in the dorsal aorta. It could be required to initiate the expression of haematopoietic genes in haemogenic endothelium, or BMP signalling could be required prior to dorsal aorta formation, i.e. during cell migration or programming events in the dorsal lateral plate. The identification of a specific time-point for the BMP signalling input to HSC programming will allow a more rigorous analysis to be performed, allowing the identification of the primary target of BMP signalling in this process.

Using the two independent methods of BMP inhibition established in the previous chapter, and our knowledge of the gene regulatory network required for HSC programming in the DLP (Figure 4-1), BMP signalling is shown here to be required for *gata2* expression in the dorsal lateral plate. Results from BMP overexpression experiments show that although *fli1* is BMP-independent in the DLP, BMP signalling is able to induce ectopic expression of both *gata2* and *fli1* in neural tissue. Analysis of the *Xenopus gata2* promoter suggests that the phosphorylated BMP R-Smad complex may bind to the *gata2* promoter and regulate its expression directly. Preliminary experiments to rescue post-gastrulation BMP signalling inhibition with an inducible *gata2* mRNA show promise and with further optimisation may determine whether *gata2* is the only gene in the DLP genetic regulatory network that requires BMP signalling. *Runx1* expression in the dorsal aorta is BMP-independent after stage 28.

This finding is inconsistent with previous analysis of BMP signalling in the zebrafish dorsal aorta (Wilkinson et al., 2009). This discrepancy will be discussed later.

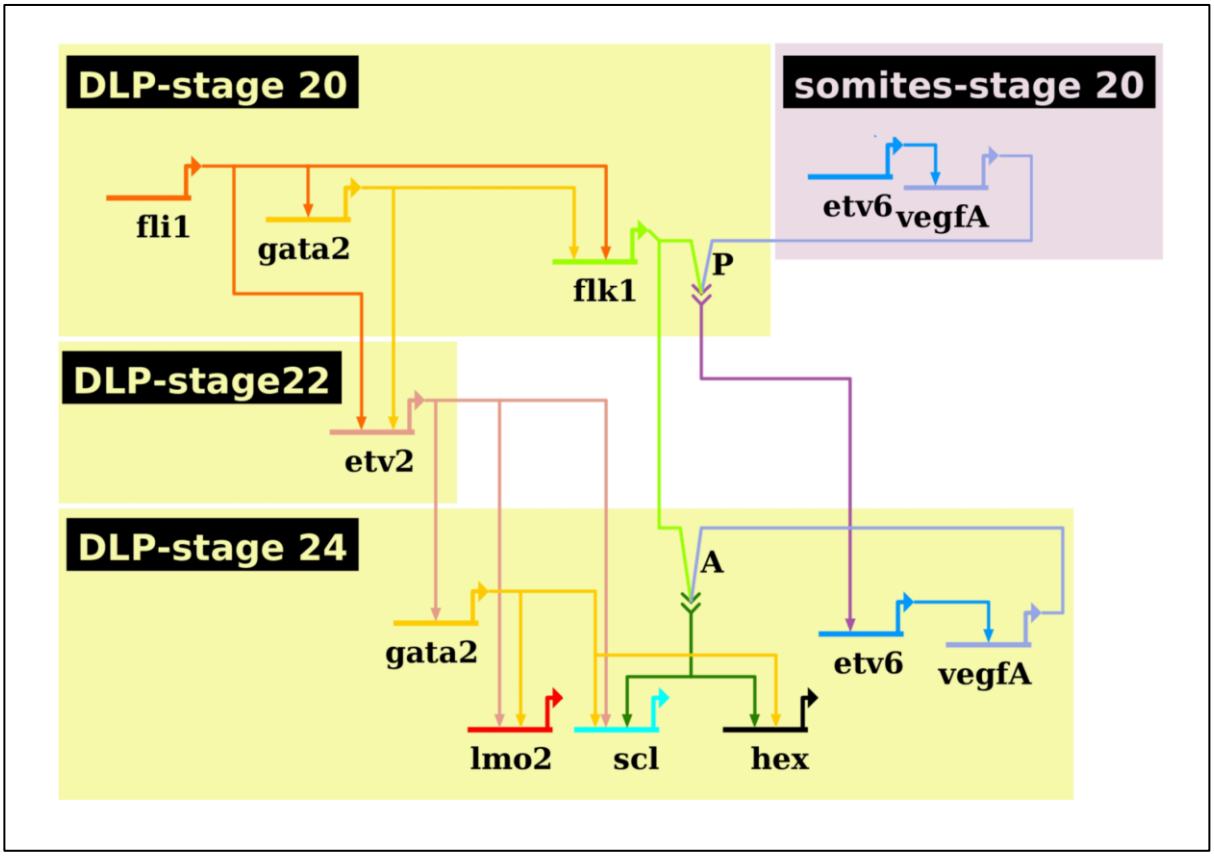



**Figure 4-1: Genetic network diagram summarising gene requirements in the DLP**

Genetic network diagram summarising the known requirements for programming in the dorsal lateral plate. The timing and tissue of expression is shown by the position of the gene name with gene inputs shown as arrows. Note that arrows do not necessarily signify direct interactions but indicate requirements for expression. Where two gene products physically interact for the expression of a target, their arrows converge on chevrons or a circle. Where a gene is shown twice indicates a change in its regulation (*gata2* is initiated by *fli1* but later requires *etv2* for maintenance). Image taken from (Ciau-Uitz et al., 2013).

### 4.2 *Runx1* expression in BMP signalling inhibition time course

To determine the stage at which BMP signalling is required for HSC programming, a time course of BMP inhibition was performed, using *runx1* expression in the dorsal aorta as a measure of HSC programming (Figure 4-2 and Figure 4-3). *Runx1* is a transcription factor, which is essential for HSC emergence from the floor of the dorsal aorta (North et al., 2002; Bee et al., 2009; Chen et al., 2009; Kissa and Herbomel, 2010a). BMP signalling was blocked by either Noggin protein, expressed by heat-shock in a transgenic *Xenopus* line, or by treatment of wild-type embryos with 100 μM of the DMH1 small molecule BMP inhibitor. The time points selected included key stages in haematopoietic programming during *Xenopus* embryonic development. Stage 14 is immediately post-gastrulation and is the earliest time point at which BMP can be inhibited without affecting gross morphology. Stage 20 marks the start of the DLP genetic regulatory network, when *fli1*, *gata2* and *flk1* are expressed and is when Vegfa protein signals to the DLP from the somites. *Etv2* initiates expression at stage 22, *scl* initiates expression at stage 24 and DLP cells begin to migrate to the midline at stage 28.

When BMP signalling was inhibited by *noggin* expression or DMH1 treatment from stage 14, to stage stage 24, *runx1* expression is absent in the dorsal aorta of stage 39 embryos (Figure 4-2 & Figure 4-3). This shows that BMP signalling is required post-gastrulation for initiation of *runx1* expression in the dosal aorta and therefore for HSC programming. When BMP signalling was inhibited from stage 26, embryos had low levels of *runx1* expression in the dorsal aorta at stage 39. Furthermore, inhibition of BMP signalling from stage 28 or stage 30 did not result in any loss of *runx1* expression (Figure 4-2 & Figure 4-3). This shows that BMP signalling is required between stages 14 and 26 but is dispensable for *runx1* expression in the dorsal aorta after stage 28.

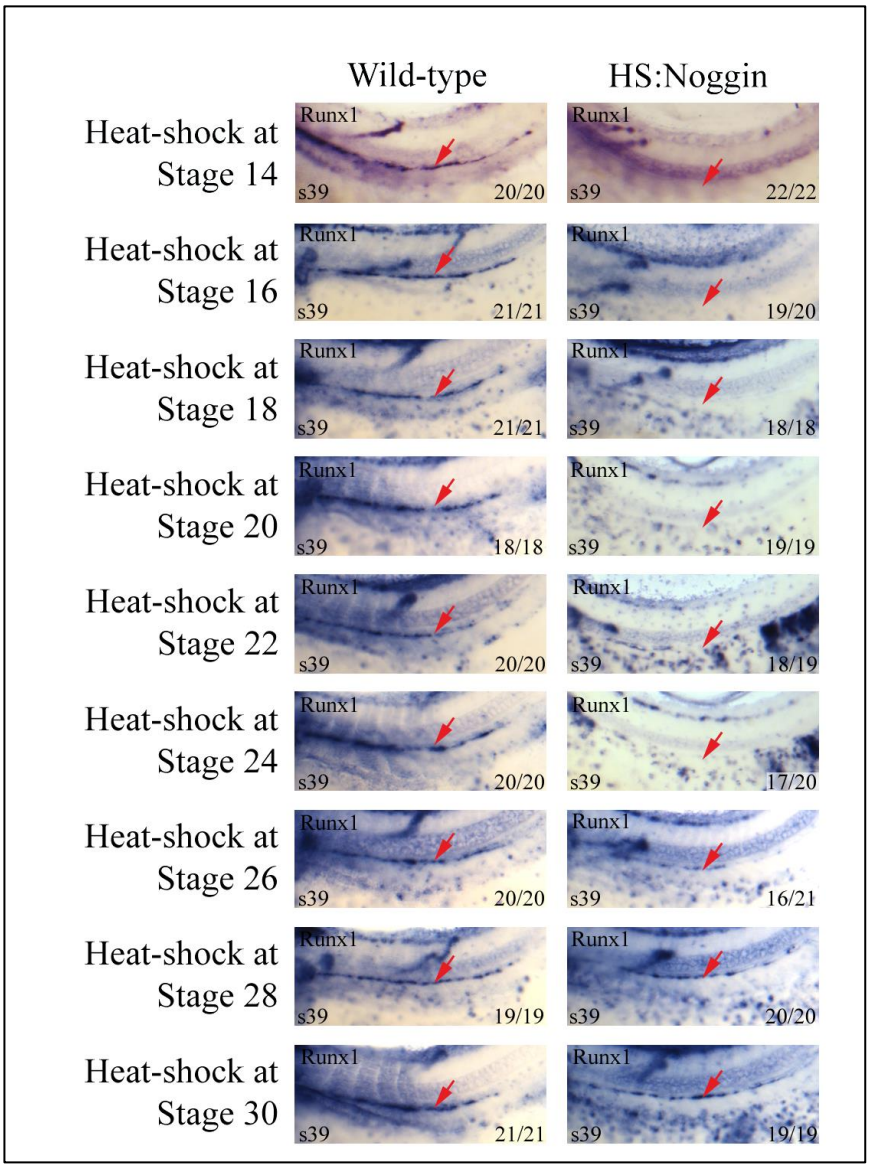



**Figure 4-2 *Runx1* whole-mount *in situ* hybridisation of heat-shock time course**

Embryos were heat shocked for 30 min at 35 °C at the stage shown and were heat shocked every 24 hr until stage 39, at which point the embryos were collected for analysis. *Runx1*-expressing cells were then stained by whole-mount *in situ* hybridisation and embryos were cleared with 2:1 BB:BA. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark the position of the dorsal aorta. Text on individual images shows the probe used (top left), the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates.

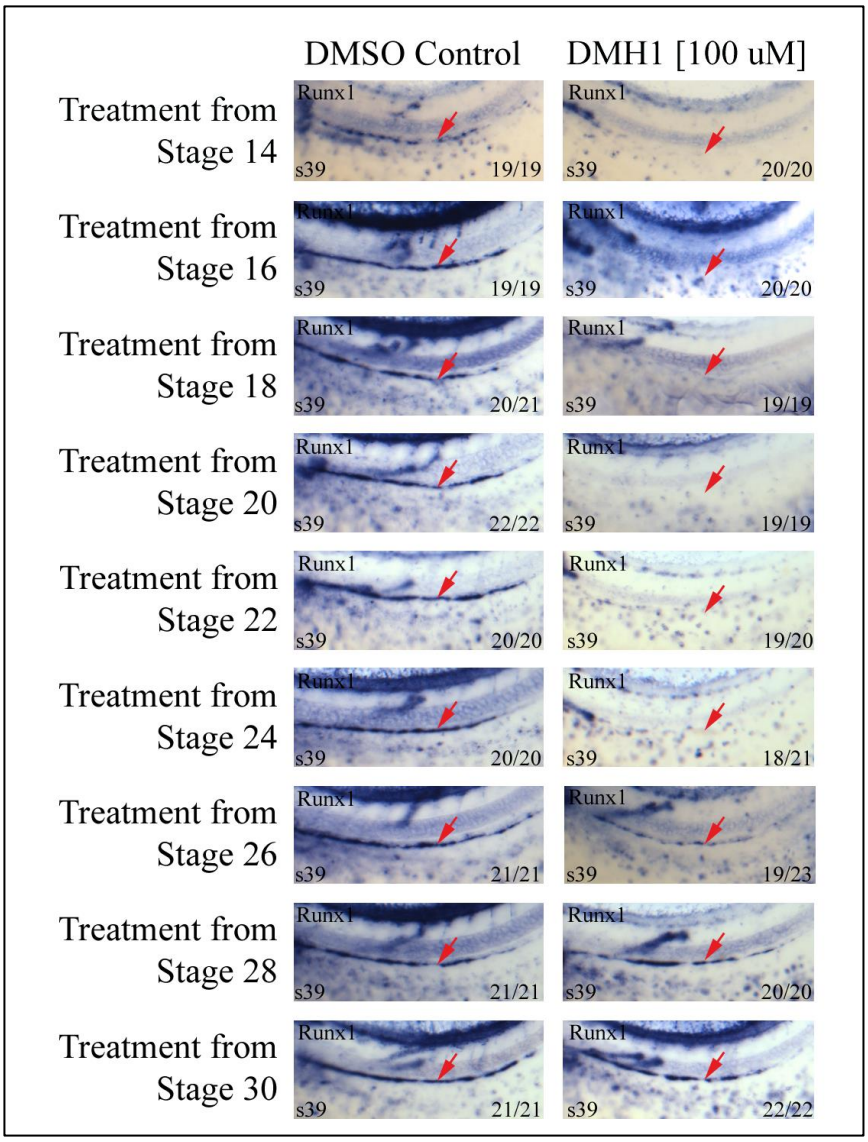



**Figure 4-3 *Runx1* whole-mount *in situ* hybridisation of DMH1 time course**

Embryos were washed into 0.1 X MBS containing 100 μM DMH1 at the stage shown and were cultured to stage 39, at which point the embryos were collected for analysis. *Runx1*-expressing cells were then stained by whole-mount *in situ* hybridisation and embryos were cleared with 2:1 BB:BA. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark the position of the dorsal aorta. Text on individual images shows the probe used (top left), the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates.

### 4.3 BMP signalling is dispensable from stage 28 for dorsal aorta formation and *runx1* expression

A requirement for BMP signalling in *runx1* initiation and maintenance in the zebrafish dorsal aorta has previously been reported (Wilkinson et al., 2009). The data shown here indicate that BMP signalling is not required past stage 28 in *Xenopus* for initiation of *runx1* expression in the dorsal aorta. To determine whether *runx1* maintenance requires BMP signalling, embryos were grown to stage 43, 2 to 3 days (depending on temperature) after *runx1* expression initiates in the dorsal aorta. At stage 43 the epidermis of the *Xenopus* embryo has thickened and whole-mount *in situ* hybridisation is not suitable for analysing gene expression in the dorsal aorta. For this reason *runx1* expression of stage 43 embryos was analysed by *in situ* hybridisation on wax sections taken through the trunk of the *Xenopus* embryo.

When BMP signalling was blocked at stage 28, prior to the formation of the dorsal aorta, *runx1* expression is not lost in embryos at stage 43 (Figure 4-4). Embryos sectioned at stage 43 show *runx1* expressing cells in the ventral wall of the dorsal aorta, as in wild-type embryos. Furthermore the dorsal aorta itself appears morphologically normal. This suggests that BMP signalling is not required after stage 28 for *runx1* initiation or maintenance in the dorsal aorta. BMP-inhibited embryos have other morphological defects, which act as internal controls for BMP signalling inhibition. Intestinal rotation begins at stage 42 and at stage 43 is clearly visible in wild-type embryos. When BMP signalling is inhibited, gut rotation does not occur (Walters et al., 2001). Heat-shock induced *noggin* expression and DMH1 treatment both resulted in a loss of gut rotation at stage 43. In addition, BMP inhibition has previously been reported to result in an irregular edge to the developing *Xenopus* tail (Walters et al., 2001). This is clearly seen using the two methods of BMP inhibition shown here at these stages. The irregular edge of the tail and the lack of intestinal rotation suggest that BMP signalling is inhibited in these embryos.

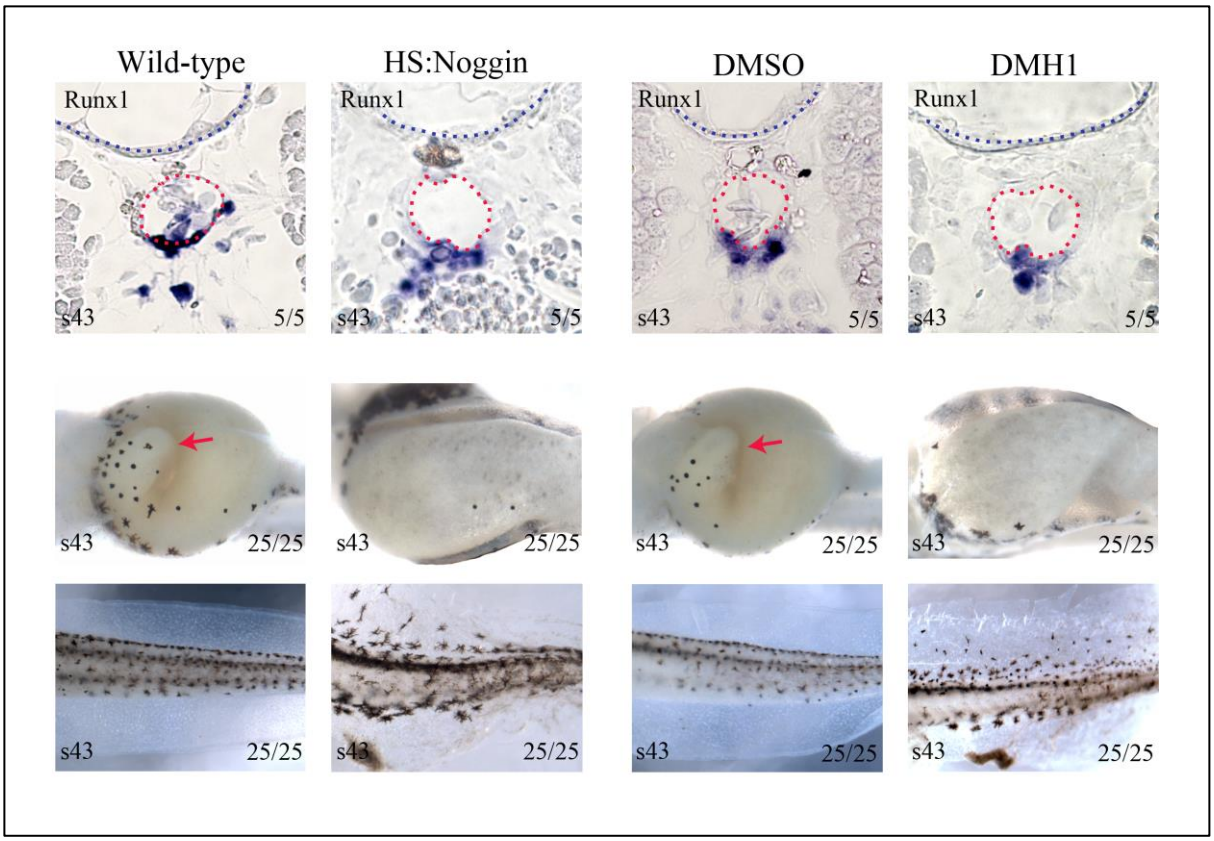



**Figure 4-4: BMP is not required for *runx1* maintenance in *Xenopus* dorsal aorta**

BMP was inhibited at stage 28 by either 100 μM DMH1 treatment or by *noggin* heat-shock induction. Embryos were collected at stage 43. The top panel shows *runx1*-expressing cells stained in purple by *in situ* hybridisation on wax sections. A dashed red line highlights the inner lumen of the dorsal aorta and a dashed blue line highlights the notochord. Sections are 10 μm transverse slices taken through the trunk of the embryo. The middle panel shows embryos visualised from the ventral aspect, with anterior to the left and left lateral to the top. Red arrows indicate normal intestinal torsion, which is absent in BMP-inhibited embryos. The bottom panel shows stage 43 embryo tails, visualised from the lateral view, with anterior to the left and dorsal to the top. BMP inhibited embryos have an irregular tail fin whilst control embryos have a smooth edge to the tail fin. Images and numbers are from one experiment and are representative of at least three biological replicates.

### 4.4 *Dll4* expression in BMP signalling inhibition time course

As definitive HSCs bud from the floor of the dorsal aorta, a time course of BMP signalling inhibition was performed, as above, and the expression of the dorsal aorta-specific gene *dll4* was analysed. When BMP signalling is inhibited from stage 14 through to stage 24, *runx1* expression is lost at the midline of stage 39 embryos. Although inhibition of BMP signalling from stage 28 does not disrupt either *runx1* expression or dorsal aorta formation (Figure 4-4), the loss of *runx1* at earlier stages could be linked to a programming defect in the dorsal aorta or an earlier defect, resulting in a loss of the dorsal aorta itself.

When BMP signalling was inhibited by *noggin* expression or DMH1 treatment from stages 14 through to stage 24, *dll4* expression is not seen at the midline of stage 39 embryos. This shows that either the artery itself has not formed or the artery no longer expresses arterial associated genes. In either scenario, it is likely that the primary defect in BMP inhibited embryos is prior to dorsal aorta formation and is likely due to a miss-patterning of precursors in the DLP.

As cells in the DLP begin to migrate to the midline at stage 28, and as stage 28 inhibition does not block either *runx1* expression or dorsal aorta formation, we can conclude that the requirement for BMP signalling is not during cell migration or in the dorsal aorta itself. This indicates that BMP signalling is required prior to stage 26 for both endothelial and haematopoietic programming in the DLP; i.e. BMP signalling is required to programme the adult haemangioblast in the DLP.

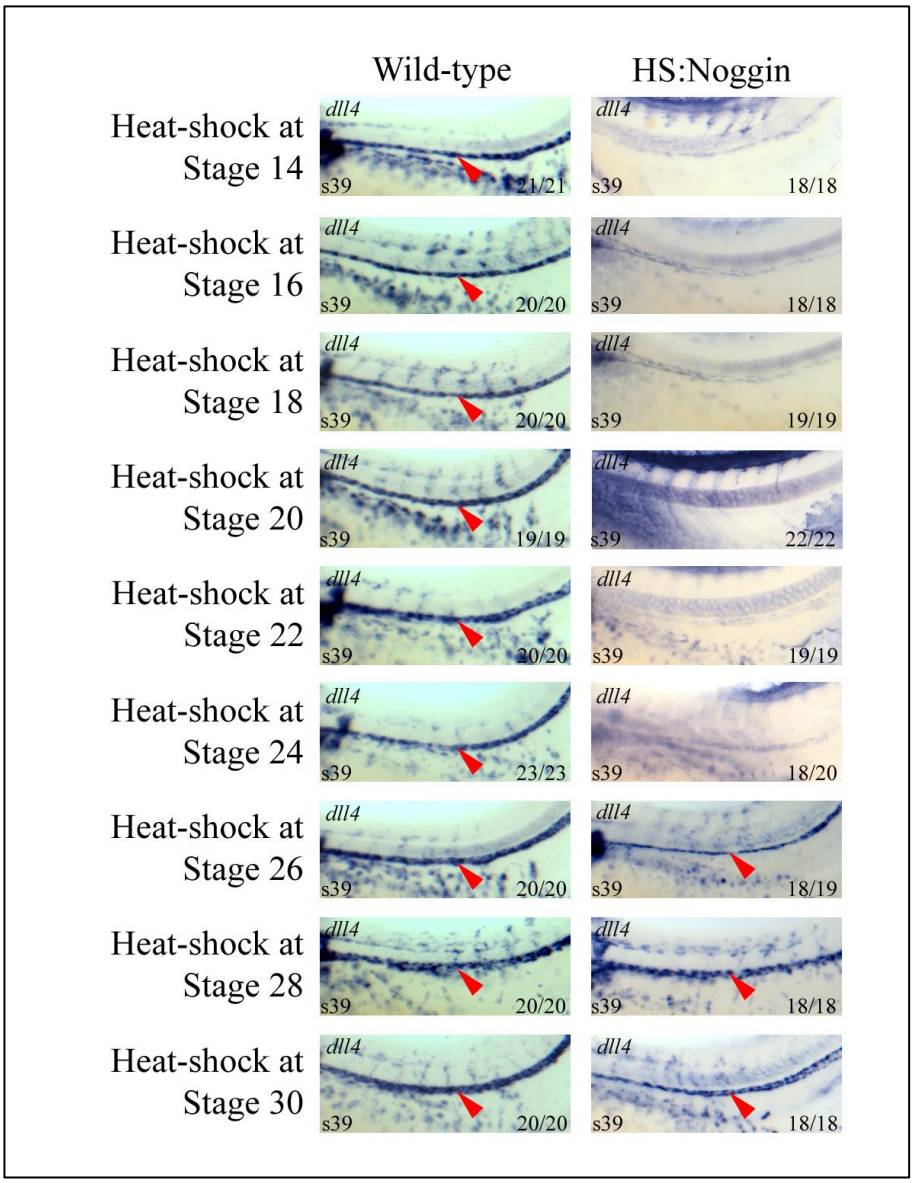



**Figure 4-5: *Dll4* whole-mount *in situ* hybridisation of heat-shock time course**

Embryos were heat-shocked for 30 min at 35 °C at the stage shown and were heat-shocked every 24 hr until stage 39, at which point the embryos were collected for analysis. *Dll4*-expressing cells were then stained by whole-mount *in situ* hybridisation and embryos were cleared with 2:1 BB:BA. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark the position of the dorsal aorta. Text on individual images shows the probe used (top left), the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates.

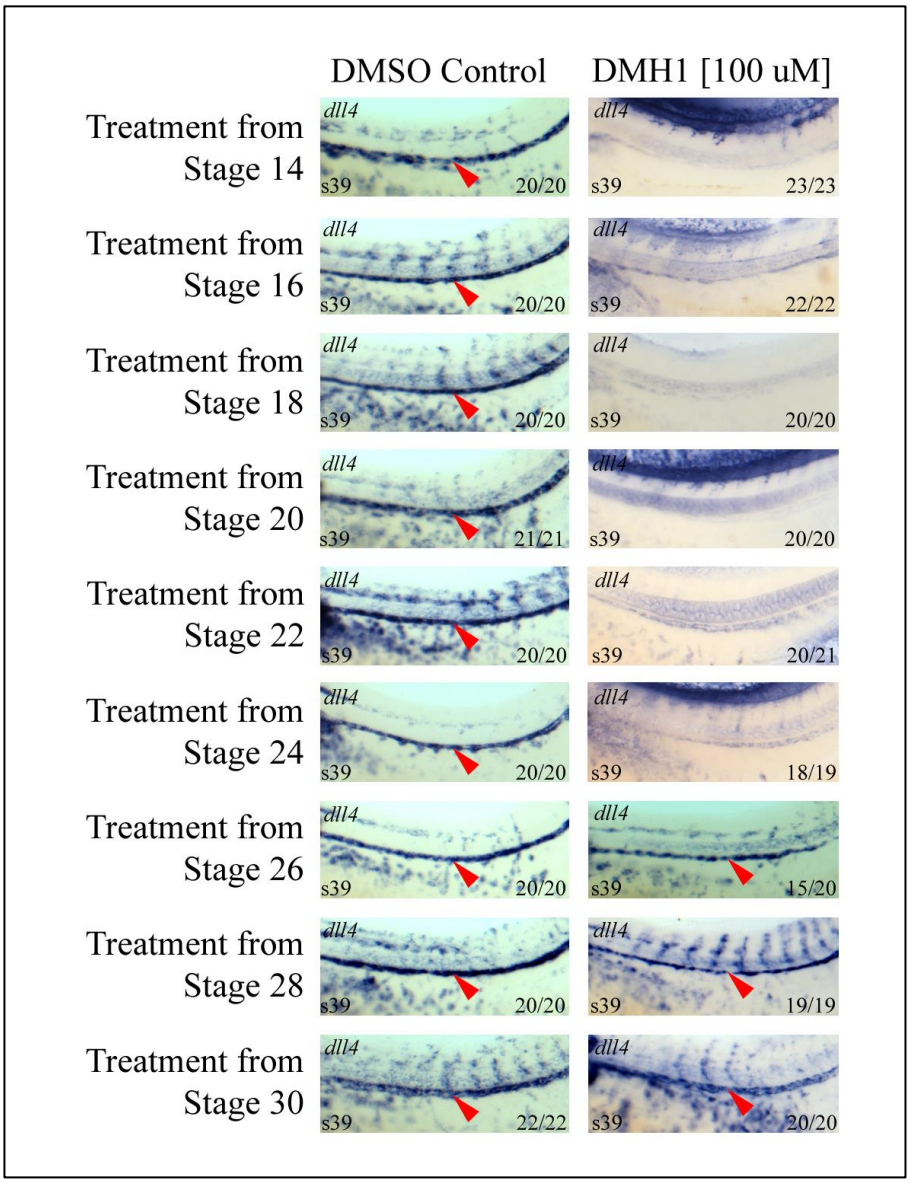



**Figure 4-6: *Dll4* whole-mount *in situ* hybridisation of DMH1 time course**

Embryos were washed into 0.1 X MBS containing 100 μM DMH1 at the stage shown and were cultured to stage 39, at which point the embryos were collected for analysis. *Dll4*-expressing cells were then stained by whole-mount *in situ* hybridisation and embryos were cleared with 2:1 BB:BA. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark the position of the dorsal aorta. Text on individual images shows the probe used (top left), the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates.

### 4.5 BMP signalling is required for *gata2* expression in the DLP

The gene regulatory network (GRN) required for programming cells in the DLP has been well studied by our group (Ciau-Uitz et al., 2000a; Liu et al., 2008; Ciau-Uitz et al., 2013). We know, for example, that *fli1* is required for both Vegfa-dependent and -independent pathways, which culminate with the initiation of *scl* expression at approximately stage 24 (Ciau-Uitz et al., 2013). Thus far, *fli1* is the master regulator of these cells and at the top of our known genetic hierarchy. Previous experiments that used a dominant-negative truncated BMP receptor suggested that although *fli1* expression in the VBI was BMP-independent, *fli1* expression in the DLP required BMP signalling (Walmsley et al., 2002a). As discussed previously, the gross morphology of those embryos was such that a loss of *fli1* could be an indirect patterning defect and required further, more targeted investigation. Building on the finding that BMP signalling is required post-gastrulation and prior to the formation of the dorsal aorta, the GRN of the DLP was examined in BMP inhibited embryos in order to identify the primary target of BMP signalling in this tissue. Stage 22 embryos were collected to examine the early establishment of the DLP GRN, whilst stage 26 was analysed as this is when the essential genes in the DLP are well established.

When BMP signalling was blocked by heat shock induction of Noggin protein in the transgenic line or by DMH1 treatment from stage 14, *fli1* expression was unaffected in the DLP at stage 22 or stage 26 (Figure 4-7, Figure 4-8, Figure 4-9 and Figure 4-10). This indicates that BMP signalling is not required for *fli1* initiation or maintenance in the DLP. In addition, as *fli1* expression in the DLP is normal at all stages analysed, any phenotype resulting from BMP inhibition is not a result of a loss of DLP cells via apoptosis or due to a gross patterning defect.

*Gata2* is immediately downstream of *fli1* in the *Xenopus* DLP GRN. When *gata2* expression was analysed in BMP-inhibited embryos, its expression was almost completely absent at both stage 22 and stage 26 (Figure 4-7, Figure 4-8, Figure 4-9 and Figure 4-10). This indicates a clear requirement for BMP signalling for *gata2* expression in the DLP. When *gata2* is knocked down by morpholino in *Xenopus* embryos, *flk1* expression is lost from the DLP, and *etv2* expression is reduced (Ciau-Uitz et al., 2013). Post-gastrulation BMP inhibition phenocopies these results (Figure 4-7, Figure 4-8, Figure 4-9 and Figure 4-10). Interestingly, although *etv2* expression is reduced in the DLP at stage 22, it is not completely lost. When *etv2* expression is visualised at stage 26, some recovery is observed. This indicates that although BMP signalling is required via *gata2* for normal expression of *etv2*, some other factor, likely to be *fli1*, is able to induce *etv2* expression in the DLP. When *fli1* is inhibited by morpholino, *etv2* expression is completely lost, rather than reduced as seen in *gata2* knockdown and post-gastrulation BMP inhibition. Altogether, this would suggest that both *fli1* and *gata2* are required for *etv2* expression in the DLP. Moreover, it has previously been shown that at stage 24 *gata2* expression becomes *fli1*-independent and switches to being maintained by *etv2* (Ciau-Uitz et al., 2013). Although *etv2* expression recovers in the DLP of BMP inhibited embryos, *gata2* expression does not, indicating a continued requirement for BMP signalling for maintaining *gata2* expression (Figure 4-7, Figure 4-8, Figure 4-9 and Figure 4-10).

Vegfa protein, secreted from the somites, is required to initiate *etv6* expression in the DLP and ultimately is required for programming the haemangioblast in the DLP. As the BMP type I receptors, *alk3* and *alk6*, the Type II receptor, *actrII*, and the BMP ligands, *bmp2* and *gdf6*, are all expressed in the somites during haemangioblast programming in the DLP (Figure 3-3), BMP signalling may also be required for *vegfa* signalling from the somites. When *vegfa* expression was analysed in the DLP, no significant difference in expression was observed

(Figure 4-7, Figure 4-8). Consistent with *gata2* morpholino knockdown (Ciau-Uitz et al., 2013), when *gata2* expression is absent in BMP signalling-inhibited embryos, *flk1*, *scl*, *lmo2* and *hex* expression is lost from the DLP (Figure 4-7, Figure 4-8, Figure 4-9 and Figure 4-10). Although the GRN required for haematopoietic programming in the VBI has not yet been established, the results presented here indicate that BMP signalling is required for *gata2, flk1, scl, lmo2* and *hex* expression in the VBI.

| Phenotype at stage 22 | Heat-shock at stage 14 | | Treatment from stage 14 | |
|---|---|---|---|---|
| | WT | Noggin | DMSO | DMH1 [100 μM] |
| *fli1* | ✓ | ✓ | ✓ | ✓ |
| *gata2* | ✓ | × | ✓ | × |
| *flk1* | ✓ | × | ✓ | × |
| *Etv2* | ✓ | ↓ | ✓ | ↓ |
| *vegfa* (somitic) | ✓ | ✓ | ✓ | ✓ |
| *vegfa (DLP)* | ✓ | ✓ | ✓ | ✓ |

**Table 4-1: Summary of DLP phenotype at stage 22 following stage 14 inhibition and analysing at stage 22**

Table summarises the expression of genes in the DLP at stage 22 following BMP inhibition from stage 14. ✓ indicates wild-type expression in the DLP. ↓ indicates decreased expression in the DLP. × indicates absence of expression in the DLP.

| Phenotype at stage 26 | Heat-shock at stage 14 | | Treatment from stage 14 | |
|---|---|---|---|---|
| | WT | Noggin | DMSO | DMH1 [100 μM] |
| *fli1* | ✓ | ✓ | ✓ | ✓ |
| *gata2* | ✓ | × | ✓ | × |
| *flk1* | ✓ | × | ✓ | × |
| *etv2* | ✓ | ↓ | ✓ | ↓ |
| *lmo2* | ✓ | × | ✓ | × |
| *scl* | ✓ | × | ✓ | × |
| *hex* | ✓ | × | ✓ | × |

**Table 4-2: Summary of DLP phenotype following stage 14 inhibition and analysing at stage 26**

Table summarises the expression of genes in the DLP at stage 26 following BMP inhibition from stage 14. ✓ indicates wild-type expression in the DLP. ↓ indicates decreased expression in the DLP. × indicates absence of expression in the DLP.

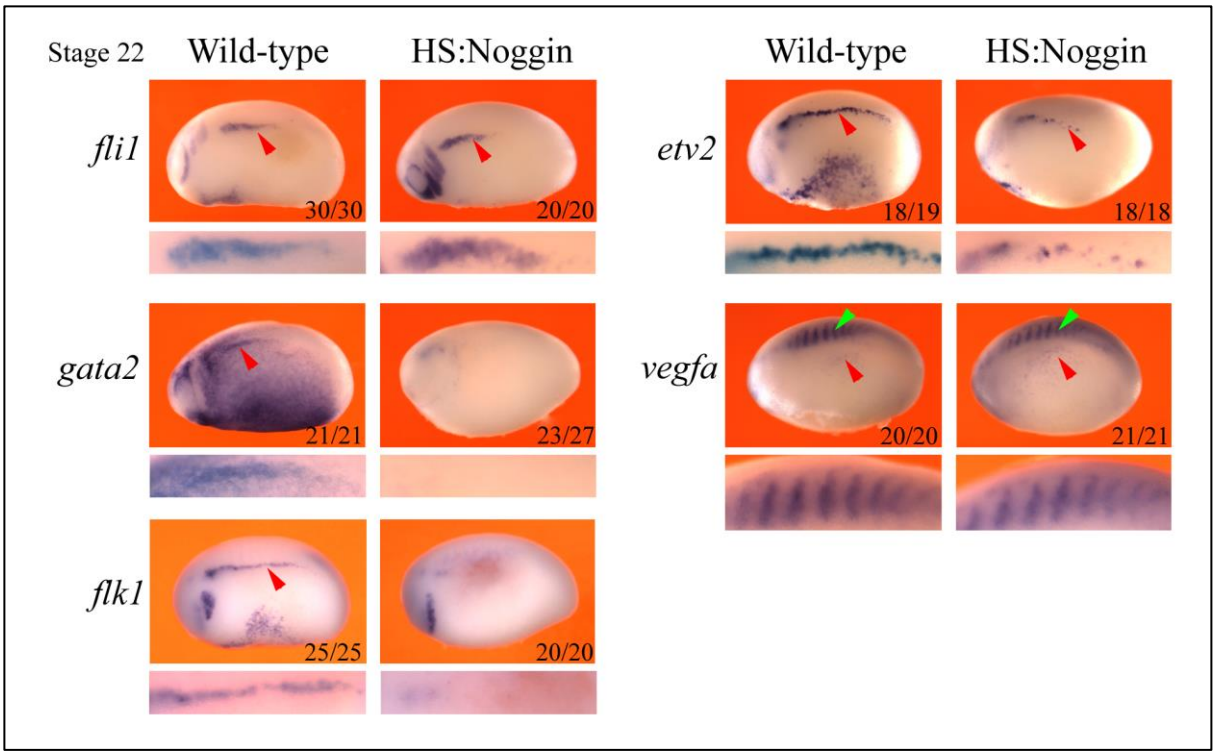



**Figure 4-7 Haematopoietic programming in the DLP, at stage 22, following heat shock-induced *noggin* expression at stage 14**

Embryos were heat-shocked for 30 min at 35 °C at the stage 14 and were collected for analysis at stage 22. Whole-mount *in situ* hybridisation was used to stain cells expressing genes that are essential to haematopoietic programming in the DLP; *fli1*, *gata2*, *flk1*, *etv2* and *vegfa*. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark staining in the DLP. Green arrowheads mark somatic staining. Text on individual images shows the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates. Panels underneath embryos show the DLP at increased magnification.

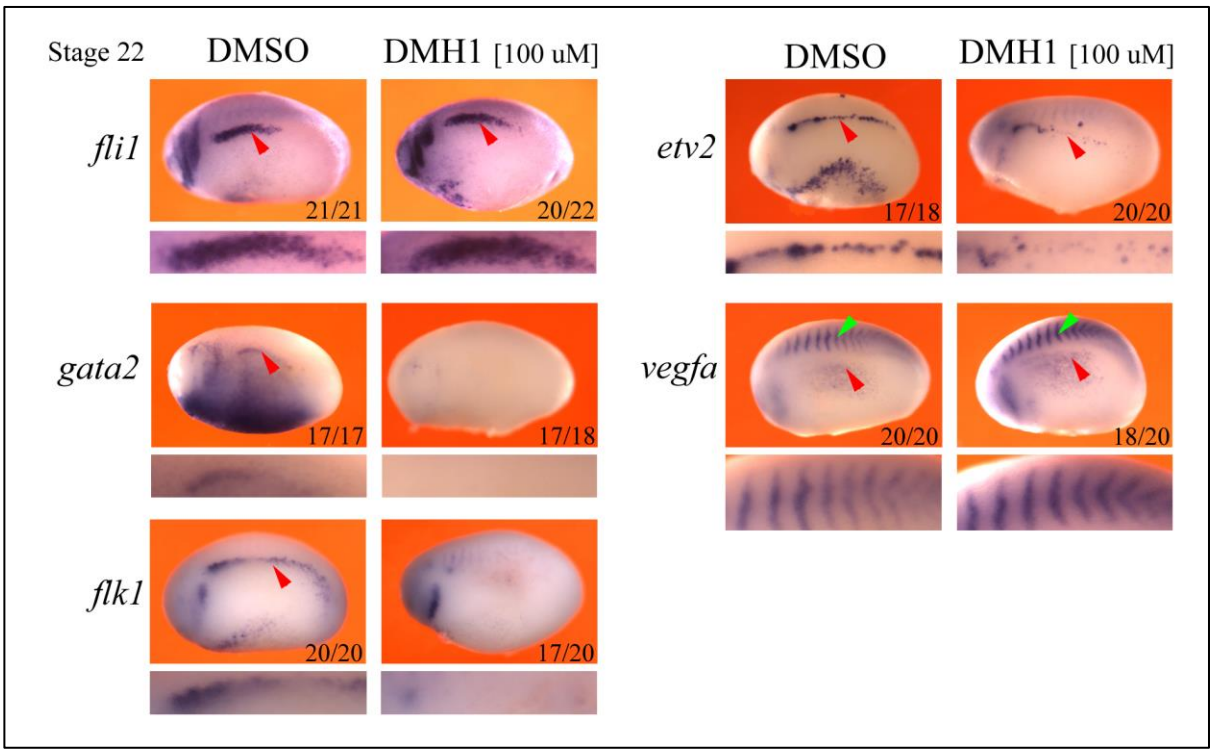



**Figure 4-8: Haematopoietic programming in the DLP, at stage 22, following DMH1 treatment at stage 14**

Embryos were treated with 100 μM DMH1 at the stage 14 and were collected for analysis at stage 22. Whole-mount *in situ* hybridisation was used to stain cells expressing genes that are essential to haematopoietic programming in the DLP; *fli1*, *gata2*, *flk1*, *etv2* and *vegfa*. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark staining in the DLP. Green arrowheads mark somitic staining. Text on individual images shows the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates. Panels underneath embryos show the DLP at increased magnification.

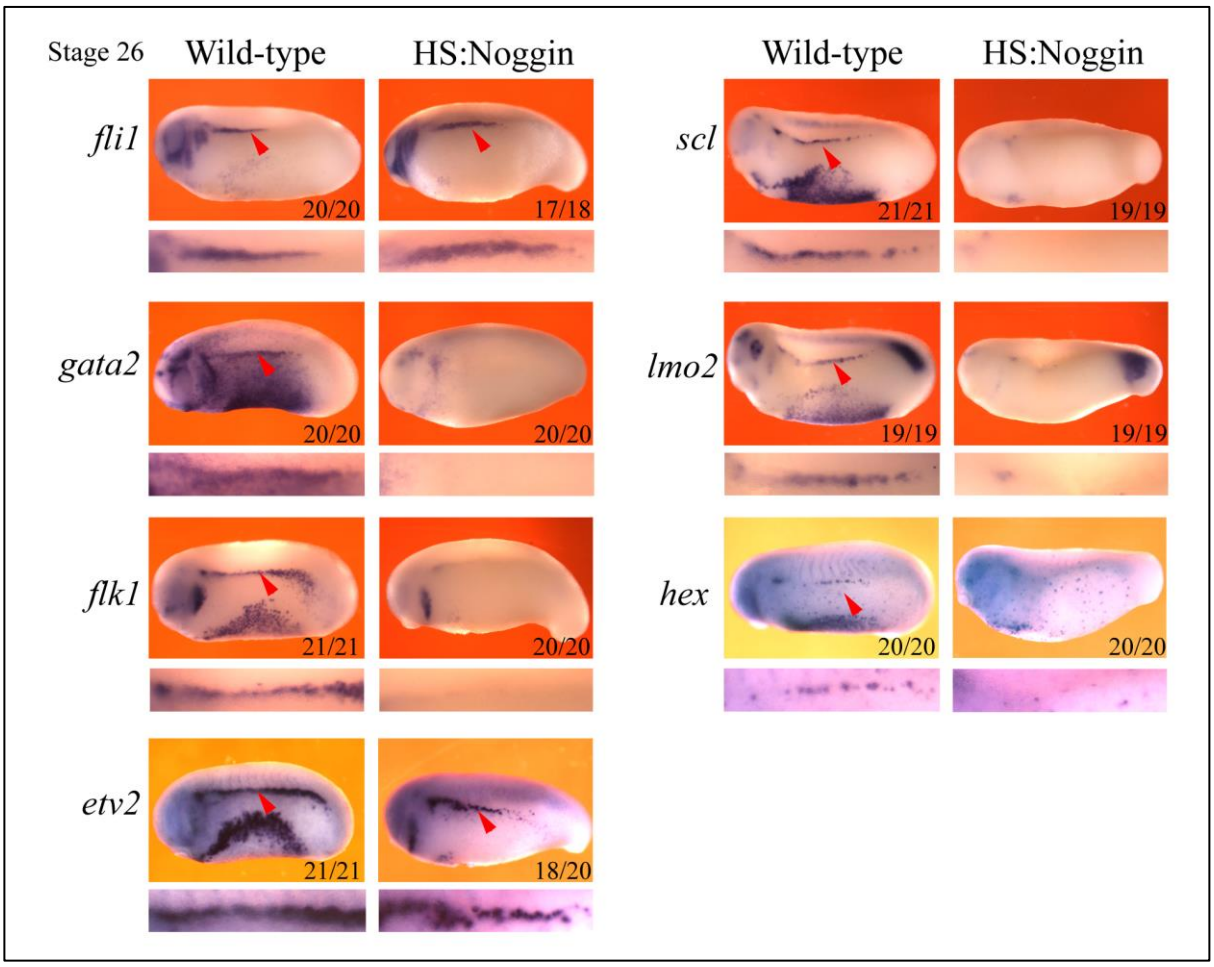



**Figure 4-9: Haematopoietic programming in the DLP, at stage 26, following heat shock-induced *noggin* expression at stage 14**

Embryos were heat-shocked for 30 min at 35 °C at the stage 14 and were collected for analysis at stage 26. Whole-mount *in situ* hybridisation was used to stain cells expressing genes that are essential to haematopoietic programming in the DLP; *fli1*, *gata2*, *flk1*, *etv2 scl*, *lmo2 and hex*. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark staining in the DLP. Text on individual images shows the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates. Panels underneath embryos show the DLP at increased magnification.

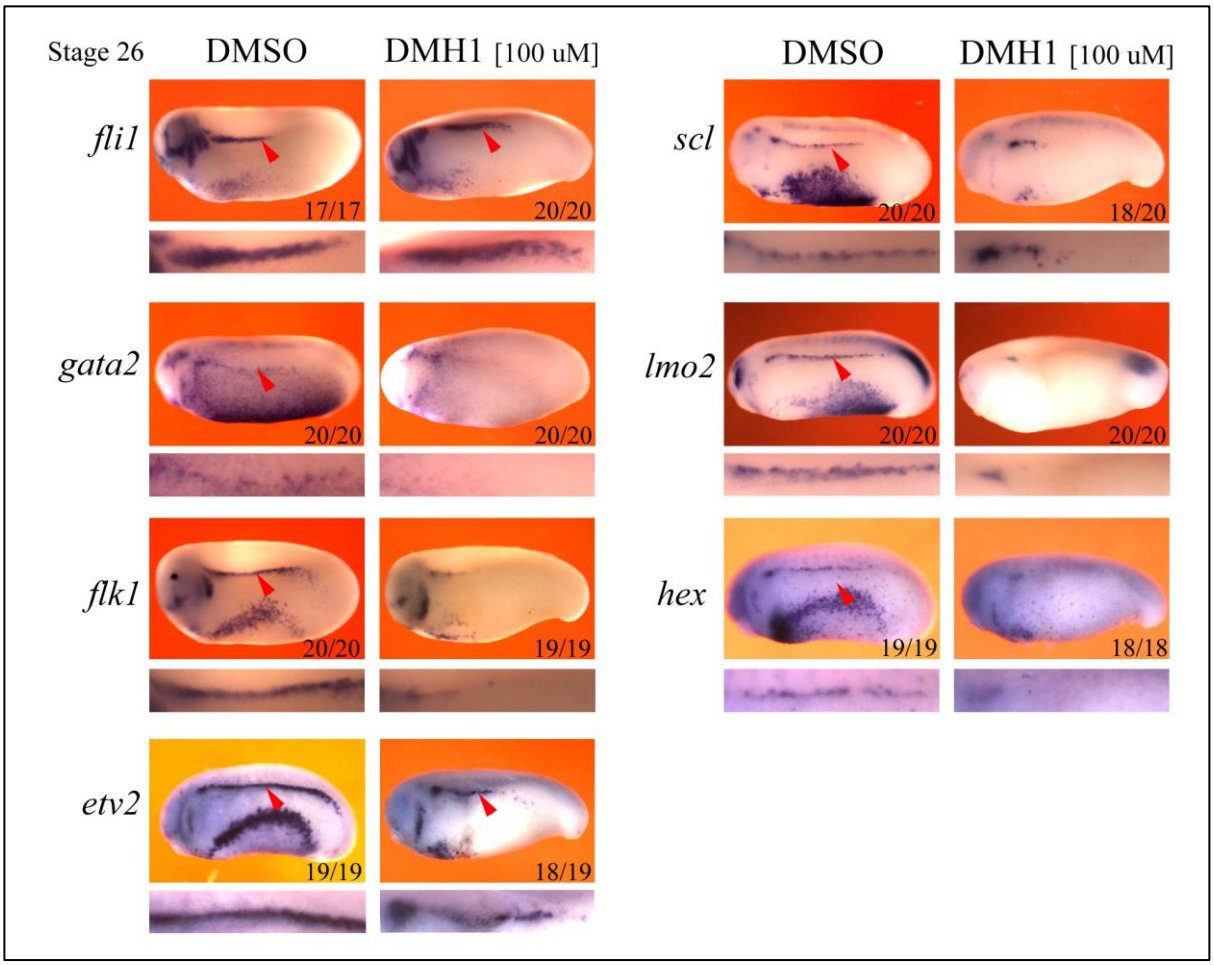



**Figure 4-10: Haematopoietic programming in the DLP, at stage 26, following DMH1 treatment at stage 14**

Embryos were treated with 100 μM DMH1 at the stage 14 and were collected for analysis at stage 26. Whole-mount *in situ* hybridisation was used to stain cells expressing genes that are essential to haematopoietic programming in the DLP; *fli1*, *gata2*, *flk1*, *etv2*, *scl lmo2* and *hex*. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark staining in the DLP. Text on individual images shows the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates. Panels underneath embryos show the DLP at increased magnification.

### 4.5.1 Q-PCR quantification of stage 26 dissected DLPs following BMP signalling inhibition

Whilst whole-mount *in situ* hybridisation gives excellent qualitative spatial information on gene expression changes, it is a poor measure of quantitative variation. To confirm the results seen from whole-mount *in situ* hybridisation and to obtain some quantitative measure of *gata2* loss, embryos were treated with 100 μM DMH1 or heat-shocked to induce *noggin* expression in the transgenic embryos at stage 14 and analysed at stage 26 by q-PCR. Three technical replicates were performed for each of the three biological replicates used to generate the results shown. Error bars represent standard error and significant difference was judged to be $p < 0.05$, as measured by Student's t-test.

The q-PCR analysis shown here clearly confirms that *fli1* is unaffected in BMP signalling-inhibited embryos whilst *gata2* and *flk1* levels are significantly down-regulated following BMP signalling inhibition (Figure 4-11 & Figure 4-12). The levels of BMP inhibition are consistent between DMH1-treated embryos and in the *noggin* transgenic line, further strengthening the validity of the results shown.

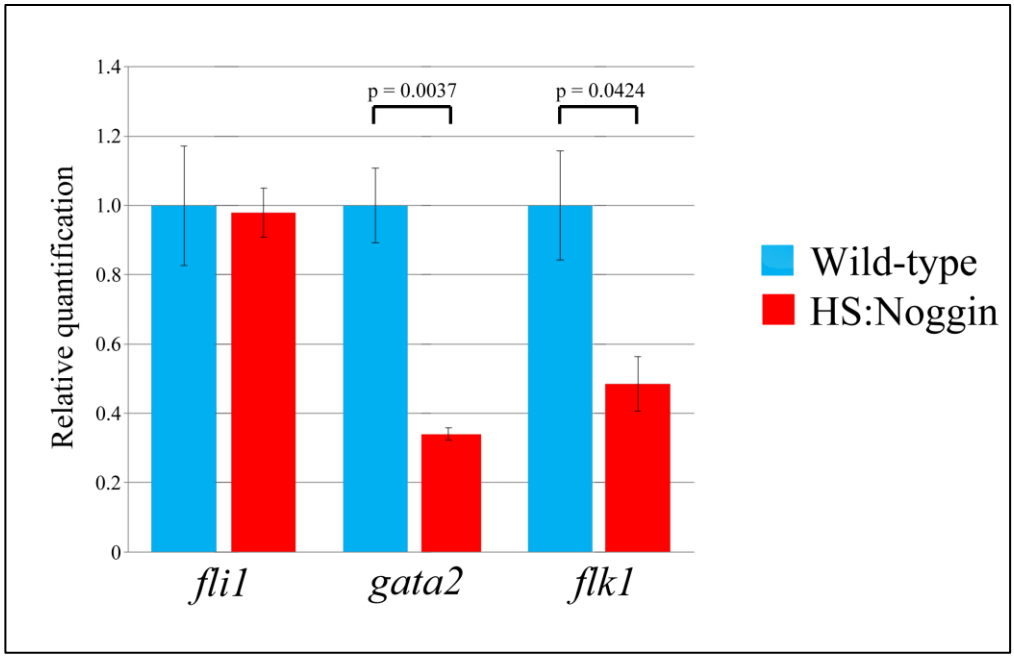



**Figure 4-11 Q-PCR analysis of stage 26 DLPs dissected from control and heat-shock *noggin* transgenic embryos**

Q-PCR analysis of *fli1*, *gata2* and *flk1* transcript levels in DLPs dissected from stage 26 *Xenopus* embryos. The mRNA transcript levels were normalized to ornithine decarboxylase (ODC) levels and fold changes were calculated using the ΔΔCt method (Heid et al., 1996). Error bars represent SEM and those treatments showing a significant change in mRNA levels relative to the controls are indicated, with their p value shown. The data shown summarises the results of 3 biological replicates for control and transgenic embryos, following a heat-shock at stage 14 and analyzing levels at stage 26.

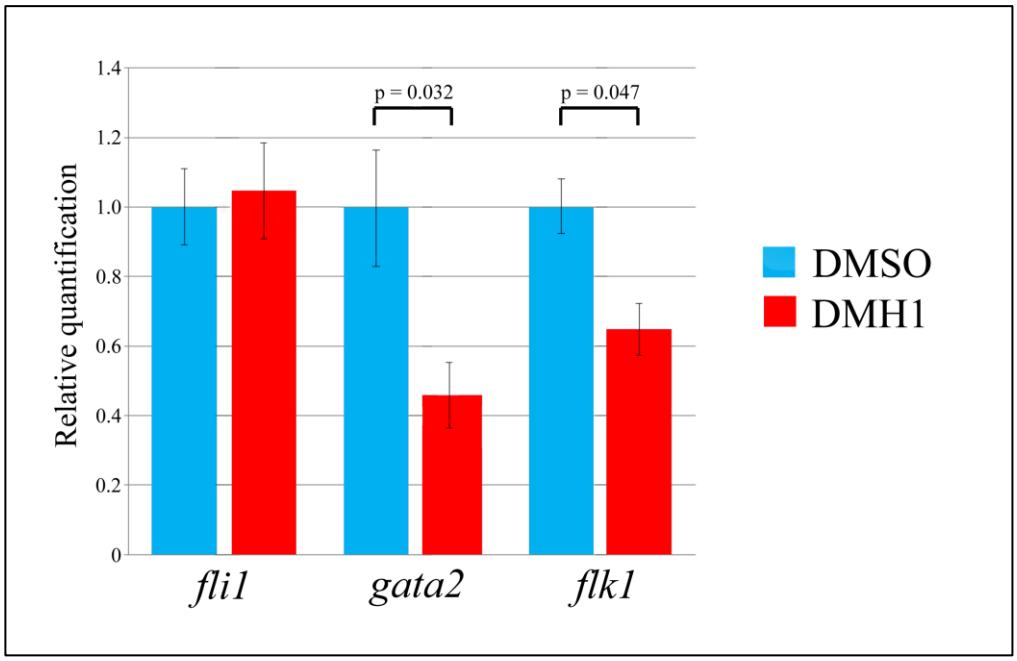



**Figure 4-12 Q-PCR analysis of stage 26 DLPs dissected from DMSO control and DMH1 treated embryos**

Q-PCR analysis of *fli1*, *gata2* and *flk1* transcript levels in DLPs dissected from stage 26 *Xenopus* embryos. The mRNA transcript levels were normalized to ornithine decarboxylase (ODC) levels and fold changes were calculated using the ΔΔCt method (Heid et al., 1996). Error bars represent SEM and those treatments showing a significant change in mRNA levels relative to the controls are indicated, with their p value shown. The data shown summarises the results of 3 biological replicates from embryos treated at stage 14 with DMSO or 100 μM DMH1.

### 4.6 The *Xenopus Gata2* promoter contains multiple Smad binding sites

The *Xenopus gata2* promoter sequence has been identified and published by our group (Brewer et al., 1995). Analysis of this promoter region shows that the sequence between nucleotides -66 and -241 relative to the ATG start site contains an element that is required for full *gata2* expression. Presented here is *in silico* analysis of this region identifying two 6 bp Smad binding elements and two 4 bp Smad binding elements (Figure 4-13). Statistically, a 6 bp sequence should occur every 4096 bp of random sequence. Therefore, the occurrence of two 6 bp Smad binding sequences in a 500 bp promoter region suggests that this Smad binding element is overrepresented in this sequence and is likely to be functional. Analysis of the 500 bp upstream of the transcription start site of human and mouse *gata2* and zebrafish *gata2a* and *gata2b* all show the presence of these Smad binding elements. The human 500 bp promoter region contains one 6 bp Smad binding element, two 5 bp Smad binding elements and five 4 bp binding elements. The mouse contains three 6 bp binding elements, two 5 bp binding elements and four 4 bp binding elements. Both zebrafish *gata2* promoters contain one 6 bp binding element and three 4 bp binding elements. This supports the hypothesis that BMP R-Smad complex binding to the *gata2* promoter is evolutionarily conserved.

The probability of a particular sequence occurring in a stretch of DNA relies on the assumption that the analysed region is inherently random, which we know to be a false assumption for promoters. Promoter regions have sequence bias and protein binding sites and therefore this sort of statistical evidence can only be used as supportive, and not definitive evidence for the functionality of these sites (Carninci et al., 2006).

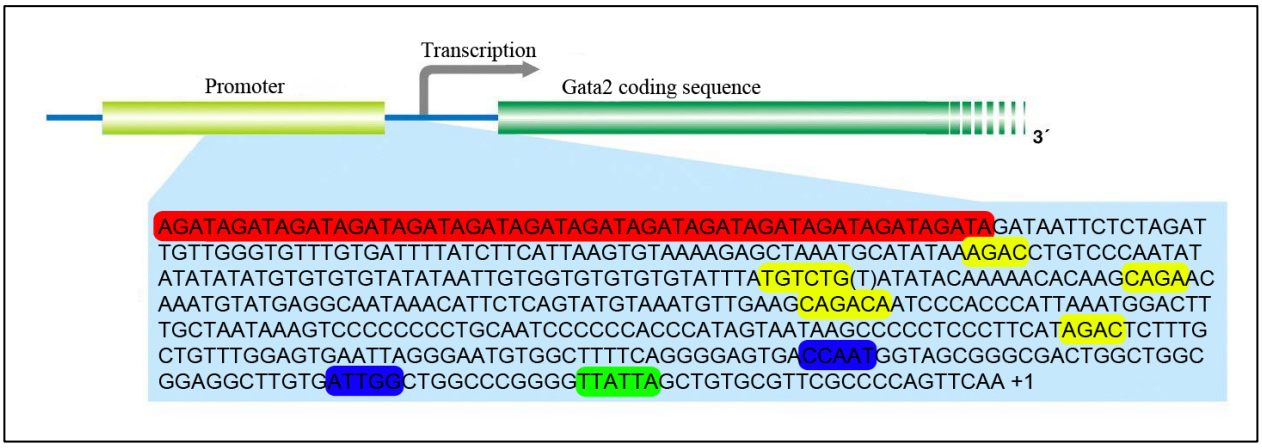



**Figure 4-13: The *Xenopus gata2* promoter**

A schematic representation of the *Xenopus* gata2 promoter including 491 bp of sequence data. This region contains a TATA box (green), 2 CAT boxes (blue), multiple GATA tetranucleotide repeats (red), and 5 potential Smad binding sites (yellow). The "(T)" at position -292 was present in a preliminary version of the *Xenopus* genome sequence, kindly given to us by Dr. R Harland, but was not present in the *gata2* promoter sequence generated in our own lab and published in (Brewer et al., 1995).

### 4.7 Injection and induction of mRNA for a constitutively active, inducible smad1

If BMP signalling is required for direct activation of *gata2* expression, then up-regulation of BMP signalling may result in the up-regulation of *gata2* expression. To test this possibility, an inducible and constitutively active Smad1 was used to up-regulate BMP signalling during *Xenopus* development.

The site of BMP-dependent Smad1 phosphorylation has been mapped to the last two serine residues of the MH2 protein-binding domain, at the conserved SSXS sequence (S: Serine, X: any amino acid) (Abdollah et al., 1997; Souchelnytskyi et al., 1997). Phosphorylation of these serines gives a negative electrostatic charge, affecting tertiary structure of the protein and allowing it to bind to Smad4 and activate BMP signalling. Glutamic acid (E) substitutions can be used to mimic serine phosphorylation (Fuentealba et al., 2007). As such, mutating the SSVS amino acid sequence of *Xenopus* Smad1 to SEVE results in a constitutively active BMP-independent phospho-mimetic Smad1 (CA-Smad1) (Fuentealba et al., 2007).

This constitutively active Smad1 was cloned and fused to the glucocorticoid receptor, which sequesters the Smad1 protein in the cytoplasm, blocking its activity (Arif Kirmizitas, unpublished data). Upon addition of the hormone dexamethasone, the GR-CA-Smad1 fusion protein translocates to the nucleus to affect gene expression. The mRNA for this GR-CA-Smad was injected into embryos and activated at stage 14 by dexamethasone treatment.

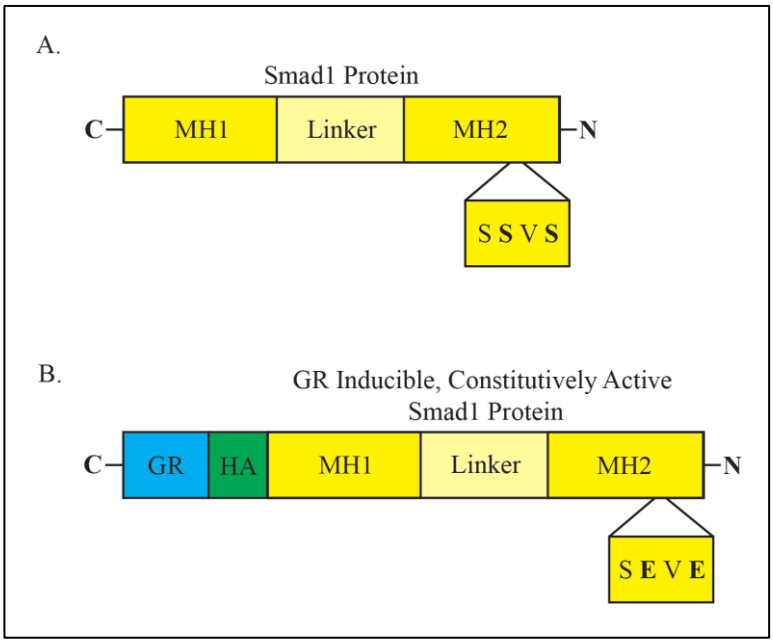



**Figure 4-14: Wild-type and Dex-inducible constitutively active Smad1 protein structure**

A) Smad1 protein contains a DNA binding MH1 domain and a protein binding MH2 domain. The MH2 protein-binding domain contains a conserved SSXS sequence (S: Serine, X: any amino acid), the last two serine residues of which are phosphorylated following BMP ligand-receptor binding. B) The substitution of the last two serine residues in the SSXS sequence with glutamic acid (E) results in constitutive activation of the Smad1 protein. This constitutively active Smad1 sequence was then fused to an HA tag and the glucocorticoid receptor, which sequesters the Smad1 protein in the cytoplasm, blocking its activity. Treatment with the hormone dexamethasone allows the Smad1 protein to translocate to the nucleus to affect gene expression.

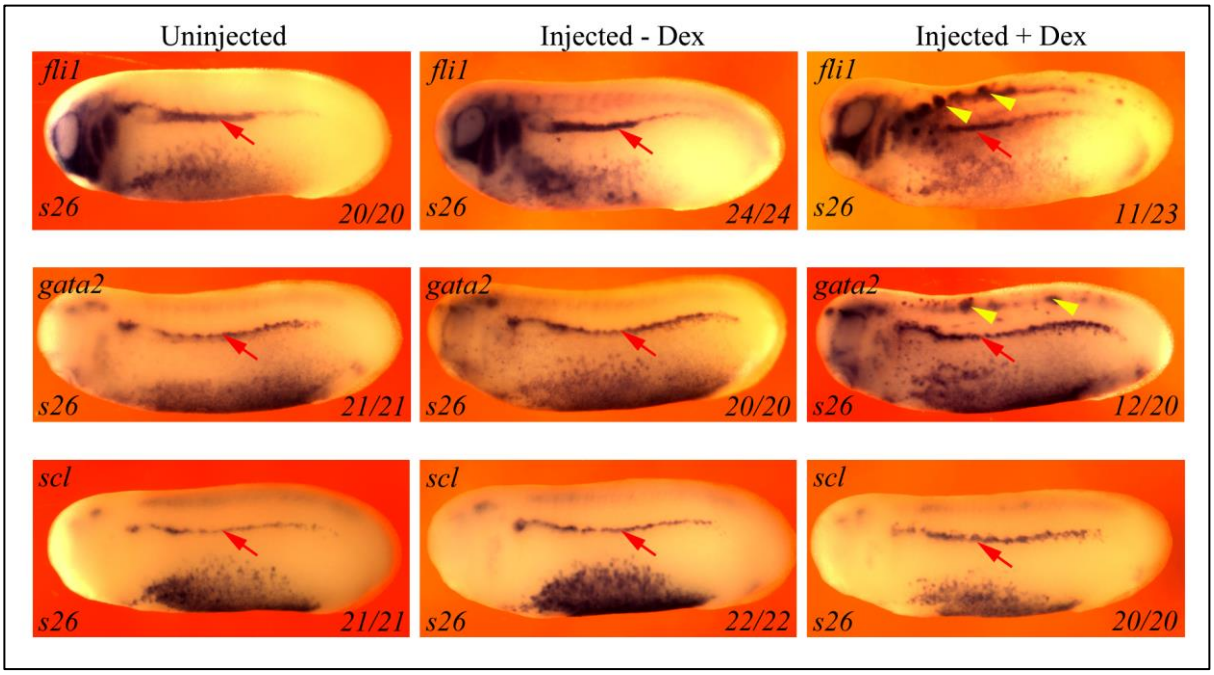



**Figure 4-15 Whole-mount *in situ* hybridization analysis of post-gastrulation BMP stimulation**

Whole-mount *in situ* hybridisation was used to stain cells expressing genes that are essential to haematopoietic programming in the DLP; *fli1*, *gata2* and *scl*. Embryos were injected with 500 pg GR-CA-Smad1 at the two cell stage and treated with 30 μM dexamethasone from stage 14. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark staining in the DLP, yellow arrowheads mark ectopic expression. Text on individual images shows the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of two biological replicates.

The injection of 500 pg of GR-CA-Smad1 resulted in a slight up-regulation of *fli1*, *gata2* and *scl* levels in the absence of the hormone dexamethasone (Figure 4-15). This suggests that at this concentration, fusion to the glucocorticoid receptor is not 100 % efficient at sequestering the constitutively active Smad1 protein in the cytoplasm. Dexamethasone treatment at stage 14 resulted in increased levels of *fli1*, *gata2* and *scl* in the DLP and VBI at stage 26, however ectopic expression of *gata2* and *fli1* is additionally observed in neural tissue (Figure 4-15). This indicates that although BMP is not required for *fli1* expression in the DLP, BMP signalling is able to induce the expression of *fli1* ectopically and may be required for *fli1* regulation in other tissues and at other stages in development. Ectopic expression of *gata2* is consistent with BMP signalling being sufficient to induce *gata2* expression.

### 4.8 Rescue of post-gastrulation BMP inhibition by injection and post-gastrulation induction of an inducible *gata2*

If *gata2* is the only direct target of BMP signalling in the DLP then haematopoietic programming should be rescued if *gata2* expression is restored. Conversely if BMP signalling has multiple targets that are required for programming in the DLP then restoration of *gata2* expression will not rescue the expression of haematopoietic genes in the DLP.

To test which of these scenarios is true, mRNA coding for an inducible *gata2* was injected into embryos and induced simultaneously with BMP inhibition by DMH1 treatment. The *gata2* coding sequence was fused to the glucocorticoid receptor, which sequesters Gata2 protein in the cytoplasm (Aldo Ciau-Uitz, unpublished data). Upon addition of the hormone dexamethasone, the Gata2 fusion protein will translocate to the nucleus to affect gene expression.

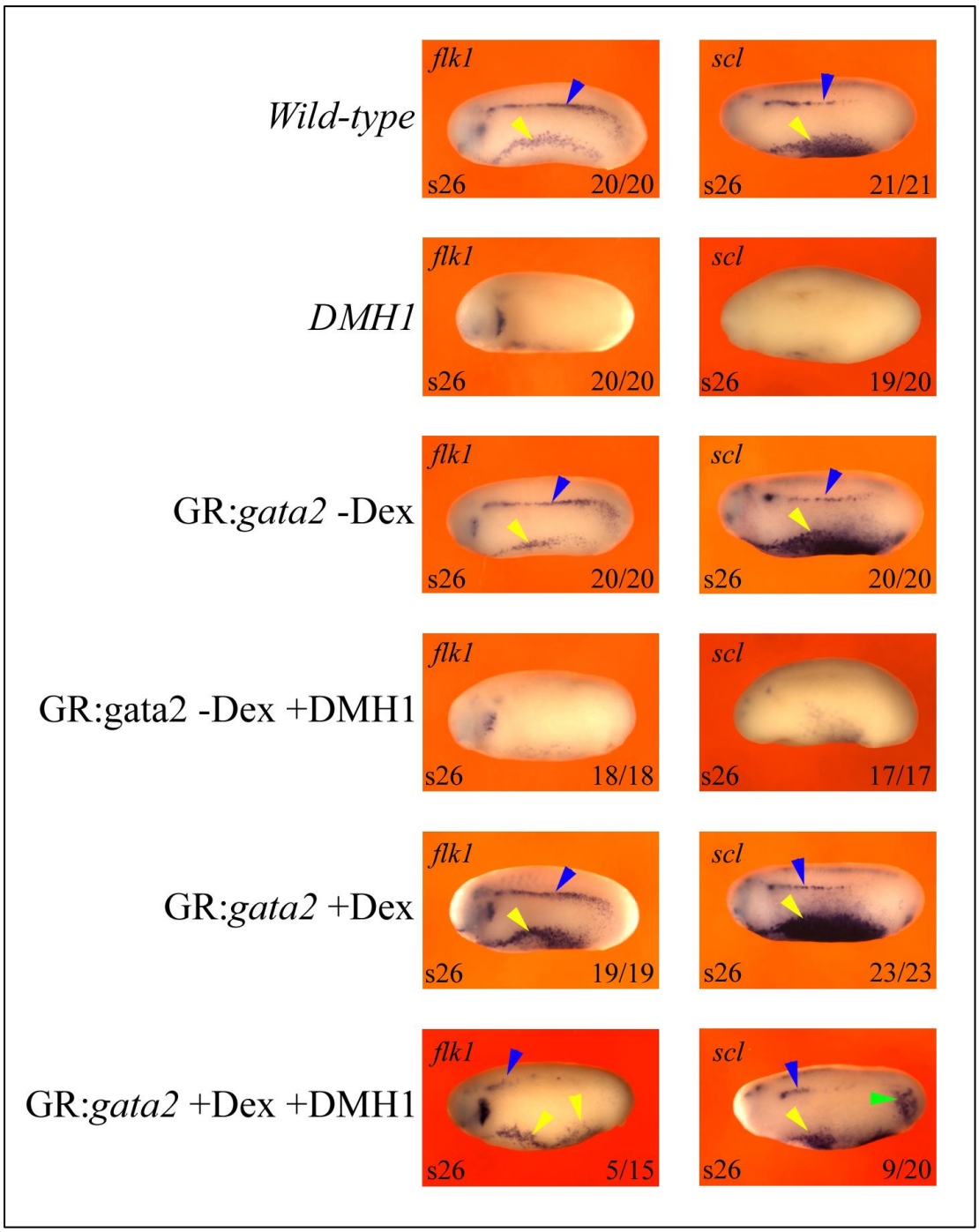

Wild-type
flk1
s26
20/20
scl
s26
21/21
DMH1
flk1
s26
20/20
scl
s26
19/20
GR:gata2 -Dex
flk1
s26
20/20
scl
s26
20/20
GR:gata2 -Dex +DMH1
flk1
s26
18/18
scl
s26
17/17
GR:gata2 +Dex
flk1
s26
19/19
scl
s26
23/23
GR:gata2 +Dex +DMH1
flk1
s26
5/15
scl
s26
9/20

**Figure 4-16 Whole-mount *in situ* hybridisation analysis of DMH1 rescue experiment using an inducible Gata2**

Whole-mount *in situ* hybridisation was used to stain cells expressing *flk1* and *scl*. Embryos were treated with 100 μM DMH1, injected with 500 pg of GR:*gata2* mRNA and/or treated with 30 μM dexamethasone from stage 14. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Blue arrowheads mark staining in the DLP, yellow arrowheads mark expression in the VBI. Green arrowheads mark ectopic expression. Text on individual images shows the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment.

Preliminary results show that the induction of *gata2* expression appears to partially rescue haematopoietic programming in the VBI. This suggests that *gata2* may be the only gene that is affected directly by BMP signalling in the VBI. The recovery of expression of *flk1* and *scl* expression in the DLP was restricted to the anterior DLP and was observed in ⅓ (for *flk1*) and ½ (for *scl*) of the embryos. This suggests that, with some optimization, *gata2* mRNA injection may be able to rescue the DLP GRN in BMP-inhibited embryos. Varying GR:*gata2* mRNA concentration, targeting the injection more efficiently and increasing the number of embryos analysed may provide evidence that *gata2* is the only target of BMP signalling in the known DLP network.

### 4.8.1 Conclusions and discussion

Both DMH1-treated embryos and heat shock-induced *noggin* transgenic embryos showed the same temporal requirement for BMP signalling for *runx1* expression in the dorsal aorta; between stages 14 and stage 26. Analysis of *dll4* expression following BMP signalling inhibition indicates that blocking BMP signalling prior to stage 26 inhibits dorsal aorta formation. As cells in the DLP begin to migrate to the midline at stage 28, and stage 28 BMP inhibition does not result in a loss of *runx1* or *dll4* expression, we can conclude that BMP signalling is not required during cell migration. This suggests that BMP signalling is required in the DLP, prior to dorsal aorta formation.

Our lab has studied the gene regulatory network required for haematopoietic stem cell programming in the *Xenopus* DLP (Ciau-Uitz et al., 2000a; Liu et al., 2008; Ciau-Uitz et al., 2013). This work has culminated in the publication of Vegfa-dependent and -independent pathways that are required in the DLP for HSC ontogeny (Ciau-Uitz et al., 2013). By analysing the expression of these genes in BMP-inhibited embryos it is shown here, by whole-mount *in situ* hybridisation and Q-PCR analysis, using two independent methods of

BMP signalling inhibition, that *gata2* is likely the primary target for BMP signalling in the DLP and this is required for HSC programming. Analysis of the *gata2* promoter suggests that the active BMP R-Smad complex is able to bind to and directly regulate *gata2* expression. ChIP PCR using the phosphorylated BMP R-Smad antibody would be required to prove that BMP signalling in the DLP directly targets the *gata2* promoter.

This finding is in contrast with findings reported by Schmerer and Evans 2003. In this publication the estrogen receptor ligand-binding domain was fused to the BMP I-Smad, Smad6 (Schmerer and Evans, 2003). When *gata2* levels were assessed by northern blot after Smad6 induction at stage 13 and collected at stage 22, no difference was observed when compared to un-induced controls. This is in stark contrast to the whole-mount *in situ* hybridisation data presented here where BMP inhibition from stage 14 results in a dramatic loss of *gata2* expression at stage 22.

However, this publication is missing controls and also misinterprets several findings. Firstly, the western blot shown for the ER-Smad6 fusion protein made from injected mRNA appears to show degradation from stage 13 onwards despite their claims that the protein is stable until stage 23. This western blot also lacks a loading control making it impossible to make confident conclusions regarding quantification. When Smad6 is induced from stage 10.5, during gastrulation, no effect on dorsal ventral patterning was observed. This contradicts our own findings that show that BMP inhibition from stage 13 is sufficient to cause dramatic dorsalisation of the embryo. This suggests that, for some reason, their construct is no longer able to inhibit BMP from this stage 13 onwards. As such, results showing no change in *gata2* levels by northern blot after Smad6 induction at stage 13 require further examination. In addition, northern blotting is a poor quantitative analysis of gene expression and gives no spatial information of gene expression patterns. The whole-mount *in situ* hybridisation and

Q-PCR data presented here, following two separate methods of BMP signalling inhibition, is a more robust analysis of the requirement for BMP signalling.

Time course experiments of BMP signalling inhibition showed that BMP signalling inhibition from stage 28 did not result in any loss of *runx1* expression in the dorsal aorta of stage 39 or stage 43 embryos. Our lab has previously published evidence that BMP signalling is required for *runx1* expression in the zebrafish dorsal aorta (Wilkinson et al., 2009). In these experiments expressing the dominant negative BMP receptor using a heat-shock promoter, immediately prior to *runx1* expression in the zebrafish dorsal aorta, blocked *runx1* expression. The results presented here suggest that BMP signalling is not required in *Xenopus* for *runx1* expression in the dorsal aorta from stage 28 onwards. One explanation for this discrepancy is a shift, over evolutionary time, in the temporal requirement for BMP signalling. Another potential explanation for this discrepancy is that in zebrafish, the overexpression of a dominant-negative truncated BMP receptor is inhibiting more than BMP signalling. Tgf-β signalling is a potential off-target candidate as inhibition of Tgf-β results in a loss of *runx1* expression in the zebrafish dorsal aorta (Personal communication Rui Monteiro). As BMP signalling is closely related to Tgf-β signalling, the overexpression of a truncated BMP receptor may block Tgf-β receptors, resulting in a loss of *runx1* expression via a BMP-independent mechanism. Evidence supporting this hypothesis comes from preliminary experiments performed by Rui Monteiro in our lab. Using a range of concentrations, DMH1 treatment at the same stages as those reported previously does not result in a loss of *runx1* expression. Although these experiments are preliminary and conclusions must be judged with caution, this suggests that BMP signalling is not required for *runx1* expression in the dorsal aorta.

The injection of an inducible constitutively active Smad1 resulted in increased levels of haematopoietic gene expression in the DLP. In addition ectopic expression of both *fli1* and

*gata2* is observed in neural tissue following GR-CA-Smad1 induction. This suggests that although *fli1* does not require BMP signalling for expression in the DLP, BMP signalling may regulate *fli1* expression in other tissues and at other time points. The ectopic expression of *fli1* and *gata2* is observed in discrete locations in neural tissue and GR-CA-Smad1 induction does not result in a diffuse ectopic expression of either *fli1* or *gata2*. This may be due to the epigenetic repression of the *fli1* and *gata2* locus in specific tissues or may be due to a gene(s), required for BMP to initiate *fli1* and *gata2* expression, which is found in neural, VBI and DLP tissue and not elsewhere.

Attempts to rescue post-gastrulation BMP signalling inhibition with an inducible *gata2* mRNA are preliminary yet promising. As this experiment has not been replicated and as only one concentration and time point of *gata2* mRNA induction was attempted, no firm conclusions can be drawn from this experiment. Nevertheless, induction of *gata2* expression appears to rescue haematopoietic programming in the VBI. This suggests that *gata2* is a major target for BMP signalling in the VBI. The recovery of expression of *flk1* and *scl* expression in the DLP was restricted to the anterior DLP and was observed in ⅓ (for *flk1*) and ½ (for *scl*) of the embryos. This suggests that, with some optimisation, *gata2* mRNA injection may be able to rescue the DLP GRN in BMP-inhibited embryos. Varying GR:*gata2* mRNA concentration, targeting the injection more efficiently and increasing the number of embryos analysed may provide evidence that *gata2* is the only target of BMP signalling in the DLP.

# Chapter 5
# Evi3. A haematopoietic BMP cofactor

# Chapter 5 Evi3. A haematopoietic BMP cofactor

## 5.1 Introduction.

*Evi3* is a BMP cofactor that is potentially involved in programming the HSC. The first suggestion of this came from its identification as a retroviral integration site, often identified as causing B-cell lymphoma in the inbred mouse line AKXD27 (Gilbert et al., 1993; Warming et al., 2004). These integrations caused an overexpression of Evi3 in B-cell precursors and blocked their differentiation. Similar findings were also identified in a transgenic mouse line expressing the oncogenic fusion E2A:HLF (Yamasaki et al., 2010). Along with Evi3, the haematopoietic transcription factors Gfi1 and Ikaros were also identified as secondary hits, leading to leukaemia in this screen. As Gfi1 and Ikaros have long been implicated in HSC programming (Willett et al., 2001; Wei et al., 2008), this observation suggests that Evi3 may have a similar role. Evi3 is expressed in $CD34^+$ human haematopoietic progenitor cells (Bond et al., 2004) and is down regulated during differentiation (Bond et al., 2008), consistent with a role in HSCs.

Evi3 contains 30 C2H2 zinc fingers (Pabo et al., 2001). Zinc fingers 2 to 8 are required for DNA binding whilst zinc fingers 14 to 19 are necessary for Evi3 to bind the Smad1/4 active complex (Hata et al., 2000). Evi3 has also been shown to bind the histone deacetylase complex (Bond et al., 2004) and has been co-immunoprecipitated with the histone acetyltransferase, P300 (Kamiya et al., 2011a), thus Evi3 is able to recruit either positive or negative histone modifiers to gene targets.

### 5.2 Evi3 protein is highly conserved from humans to *Xenopus*

As *evi3* has not previously been studied in *Xenopus*, and its current annotation on the database 'Xenbase' is only provisionally given as *evi3*, a protein alignment was performed, comparing the human, mouse and *Xenopus* Evi3 protein sequences. Conservation of sequence, especially covering functional domains, is a strong indicator of conserved function across species. As the use of *Xenopus* as a model organism is ultimately to learn more about the genetic regulation of human haematopoiesis, a high level of conservation between humans and *Xenopus* is desirable when wishing to extrapolate findings from one organism to another.

Protein alignment between human, mouse and *Xenopus* Evi3, shows high levels of conservation throughout (Figure 5-1). This striking level of homology is potentially due to the distribution of the 30 zinc finger domains throughout the protein. The sequence conservation between these species suggests that these zinc fingers are required for Evi3 function across vertebrates and so little amino acid variation has been permitted across evolutionary time.

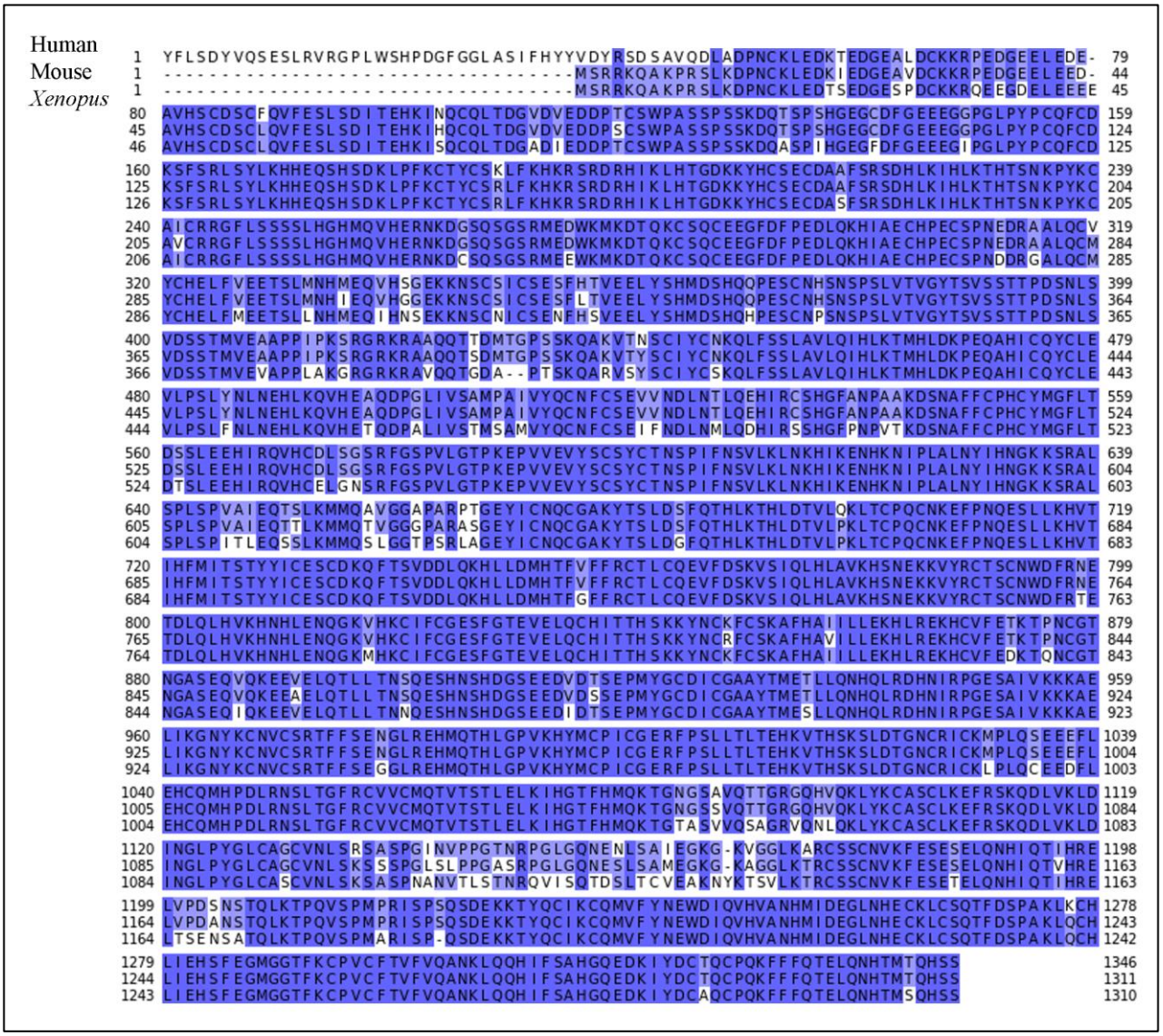



**Figure 5-1: Evi3 protein alignment**

Human, mouse and *Xenopus* Evi3 protein alignment. Complete conservation is shown in dark blue, partial conservation in light blue and amino acids with no conservation are shown in white. Numbers indicate amino acid positions. Human Evi3 is shown on the top, mouse Evi3 in the middle and *Xenopus* Evi3 at the bottom.

### 5.3 Evi3 is expressed in haematopoietic tissues during *Xenopus* embryonic development

If *evi3* plays a role in haematopoietic programming in the developing *Xenopus* embryo, it may be expected to be expressed in haematopoietic sites. To test this hypothesis, wild-type embryos were collected from a range of stages during haematopoietic development and *evi3* expression was analysed by whole-mount *in situ* hybridisation (Figure 5-2).

*Evi3* is expressed in the DLP from stage 20, when programming in the DLP initiates, and is present until DLP cells migrate to the midline at stage 30 (DLP: red arrows). The expression of *evi3* in the DLP during key stages of haematopoietic programming suggests that it may play a role in programming cells in the DLP. *Evi3* is also expressed in the VBI from stage 20 to stage 32 (VBI: yellow arrow heads). This suggests that *evi3* may also play a role in primitive haematopoiesis. A high level of neural expression was observed at all stages during development. This is consistent with previous reports that Evi3 is essential for neural differentiation of mouse ES cells (Kamiya et al., 2011b) and suggests that *evi3* has a role in neural development in *Xenopus*. At stage 39, no staining is observed in the dorsal aorta. This suggests that *evi3* is not involved in HSC emergence from the dorsal aorta.

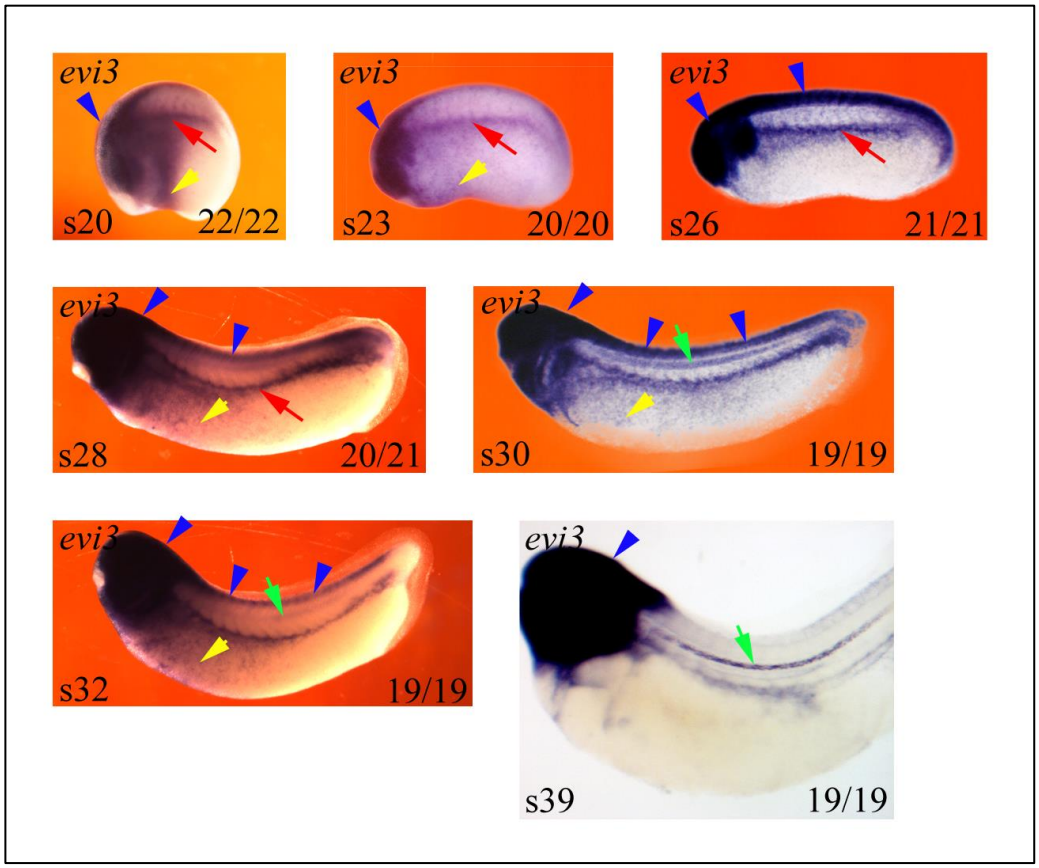



**Figure 5-2: Whole-mount *in situ* analysis of wild-type *evi3* expression during development**

*Evi3*-expressing cells were stained by whole-mount *in situ* hybridisation. Stage 39 embryos were cleared with 2:1 BB:BA. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark staining in the DLP. Staining in the VBI is annotated with a yellow arrow. Blue arrowheads indicate neural staining and the notochord is labelled with a green arrow. Text on individual images shows the probe used (top left), the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates.

## 5.4 *Evi3* morpholino design

A knockdown strategy was used to test the role of *evi3* during *Xenopus* HSC programming. Two separate morpholinos were designed to target *evi3* mRNA. One morpholino targeted the 5' UTR in order to block translation. The other morpholino was designed to block splicing.

### 5.4.1 *Evi3* translation-blocking morpholino

A 25 bp morpholino was designed to target the 5' UTR of *evi3*, to prevent ribosome binding and inhibit translation. Care was taken to ensure that this 25 bp sequence was not complementary to any sequence other than the *evi3* 5' UTR target. This was done using BLAST (Basic Local Alignment Search Tool) and the *Xenopus* expressed sequence tag database. No significant off-target homology was identified, which suggests that this morpholino should only bind to the 5' UTR of *evi3* and not any other mRNA sequence.

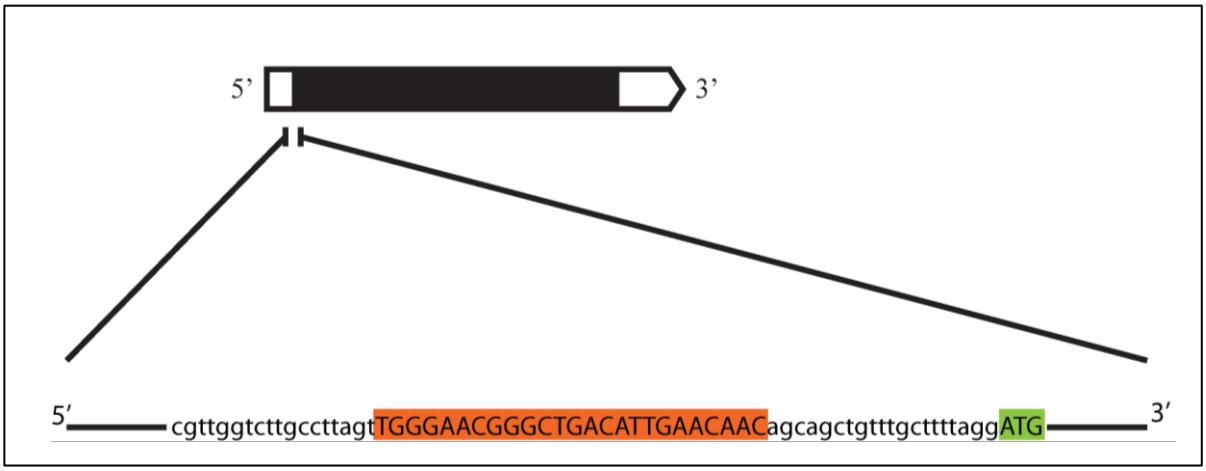



**Figure 5-3: *Xenopus evi3* translation-blocking morpholino**

Schematic of *Xenopus evi3* mRNA and the translation-blocking morpholino target sequence. The *evi3* translation-blocking morpholino target sequence is highlighted in orange and the start codon is shown in green.

### 5.4.2 *Evi3* splice-blocking morpholino

Splice-blocking morpholinos are designed to bind the intron-exon boundary of target pre-mRNAs. It is therefore necessary to know the sequence of pre-spliced mRNA. As the *Xenopus laevis* genomic sequence is not yet complete or annotated, the coding sequence for *evi3* was blasted against the genomic scaffold sequences kindly given to us by Dr. R Harland in order to identify the intronic sequences. Overlap between these scaffolds was identified and used to generate four contiguous sequences, containing all of the *Xenopus evi3* coding sequence. This was then used to reconstruct the sequence of pre-spliced *evi3* mRNA (Figure 5-4).

*Xenopus evi3* is encoded by 5 exons with 4 introns. The majority of *evi3* protein coding sequence is contained in the fourth exon (1117 amino acids of the total 1311 amino acids). Three sections of sequence were incomplete: part of the second intron, the third exon-intron boundary and the last intron-exon boundary (shown as a space flanked by red lines in Figure 5-4). As the intron-exon boundary for the fourth exon was identified, and as it encoded the majority of the *evi3* coding sequence, a splice morpholino was designed to target the splicing of this exon (Figure 5-5).

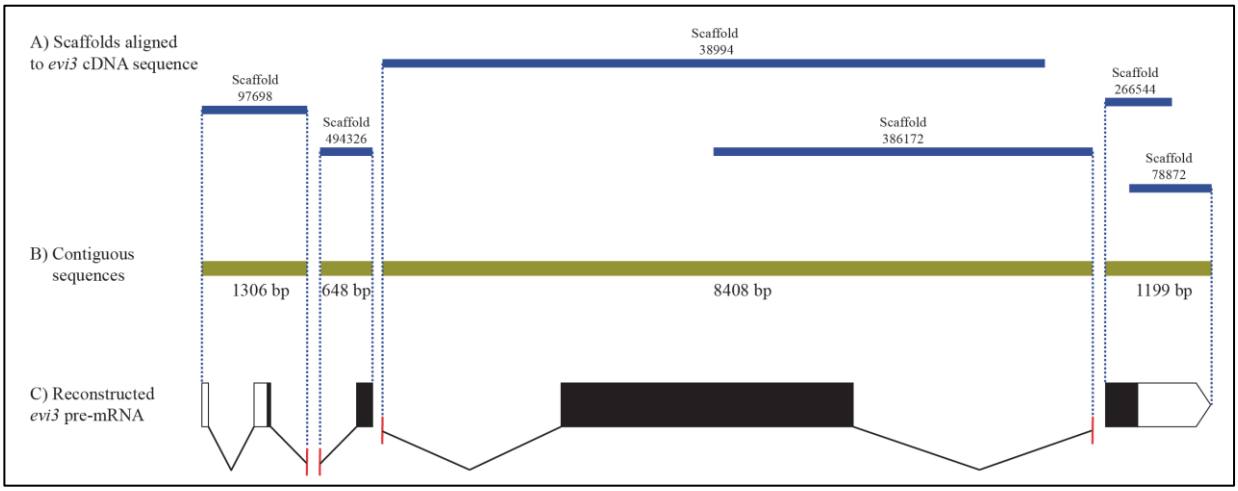



**Figure 5-4: *In silico* reconstruction of the *Xenopus evi3* pre-mRNA from genomic sequencing scaffolds**

*A) Xenopus evi3* coding sequence was blasted against the genomic scaffold sequences kindly given to us by Dr. R Harland. B) Overlapping sequences were joined to form four contiguous sequences using the *evi3* coding sequence as a guide. C) This allowed for the reconstruction of *evi3* pre-mRNA sequence and the identification of intron-exon boundaries. Vertical red lines show gaps in the known sequence.

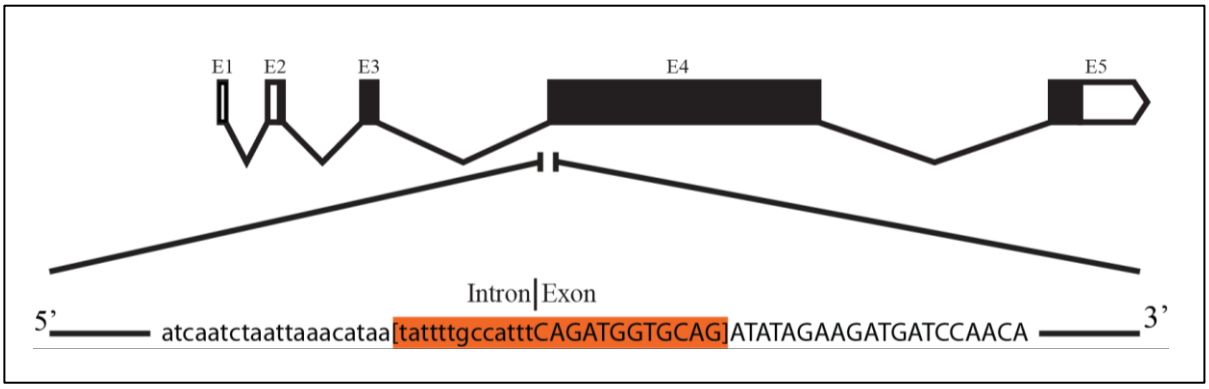



**Figure 5-5: Evi3 pre-mRNA and splice morpholino target sequence**

Schematic of *Xenopus evi3* mRNA and splice-blocking morpholino target sequence. The *evi3* translation-blocking morpholino target sequence is highlighted in orange. Intronic sequence is shown in lowercase lettering and exonic sequence is shown as capitals.

To confirm that this morpholino was able to alter the normal splicing of the *evi3* mRNA, PCR primers were designed to span the morpholino target site and amplify this region from cDNA. Total RNA was collected from stage 25 embryos that were either un-injected wild-type controls or embryos that had been injected with 50 ng of *evi3* splice morpholino at the two-cell stage. This RNA was then reverse transcribed to cDNA and used as template for PCR. Both primer pairs used the same forward primer, which bound to the start of the coding sequence in exon two (Figure 5-6). Two separate reverse primers were used to amplify across the morpholino binding site. The first reverse primer was designed to bind to exon 4 and produce an amplicon of 1641 bp in normally-spliced mRNA. The second reverse primer was designed to bind to exon 5 and produce a 5492 bp amplicon in normally-spliced mRNA. Primers for the housekeeping gene ornithine decarboxylase 1 (*odc*) were used as a control for cDNA quality and these primers produced an amplicon of 386 bp.

The first primer pair only produced the expected 1641 bp amplicon from wild-type cDNA. PCR using this first primer pair and cDNA made from injected embryos did not produce any bands. This suggests that the exonic sequence targeted by the morpholino and bound by the first reverse primer was not spliced into the processed *evi3* mRNA and indicates that *evi3* splicing has been effectively targeted.

The second primer pair was designed to amplify a large 5492 bp amplicon in wild-type spliced *evi3* mRNA and a shorter 1479 bp sequence if exon 4 was skipped in morpholino injected embryos. Despite several attempts in varying PCR conditions, the large 5492 bp amplicon was not amplified from either control or morphant cDNA. This is not entirely surprising given its large size. Morphant cDNA however produced additional bands corresponding to amplification from *evi3* cDNA that does not contain exon 4. This again is an indication of efficient targeting of *evi3* splicing.

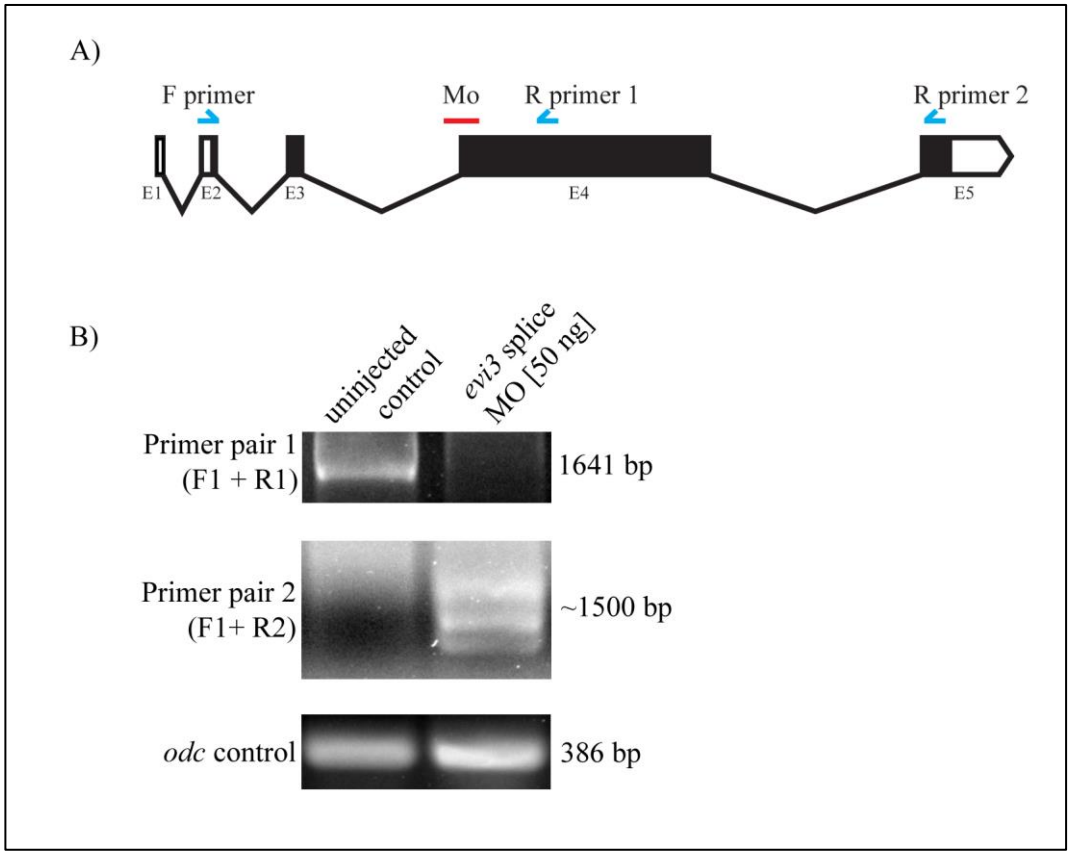



**Figure 5-6: PCR analysis of the splice targeting evi3 morpholino**

A) Schematic of *evi3* mRNA showing morpholino target site as a red line and primer binding sites in blue. B) Photograph of an agarose gel showing bands from PCR amplification across the *evi3* splice boundary. Amplification of ornithine decarboxylase 1 (*odc*) was used as a control for cDNA quality. Images show results from one experiment, which is representative of two biological replicates.

### 5.5 *Evi3* is required for HSC programming during *Xenopus* embryonic development

In order to test if *evi3* is required during development for HSC programming, embryos were injected with 25 ng of *evi3* translation-blocking morpholino and were grown to stage 39. HSC programming was analysed by whole-mount *in situ* hybridisation staining for the haematopoietic transcription factors *runx1*, *spib*, *scl* and *lmo2*. *Scl* and *lmo2* are expressed by circulating primitive erythrocytes at stage 39, and can be used as a measure of primitive haematopoiesis. Unfortunately time constraints prevented the use of the splice-blocking morpholino described above and so only results obtained from embryos injected with the translation blocking morpholino are shown below.

Knockdown of *evi3* mRNA via morpholino resulted in a complete loss of *runx1, spib, scl* and *lmo2* expression at stage 39 (Figure 5-7). This indicates that *evi3* is required for HSC programming during *Xenopus* development. To test if *evi3* morphants have normal dorsal aorta specification, or if, like BMP inhibited embryos, the primary defect is prior to dorsal aorta formation, embryos were stained for dorsal aorta marker *dll4* and pan-endothelial markers *ephrinb2* and *erg1* (Figure 5-8). All *dll4, ephrinb2* and *erg1* expression is lost from the midline of stage 39 embryos, indicating an arterial defect. Either the artery itself has not formed or the artery no longer expresses arterial associated genes. In either scenario, it is likely that the primary defect in *evi3* morphant embryos is prior to dorsal aorta formation and is likely due to a miss-patterning of precursors in the DLP.

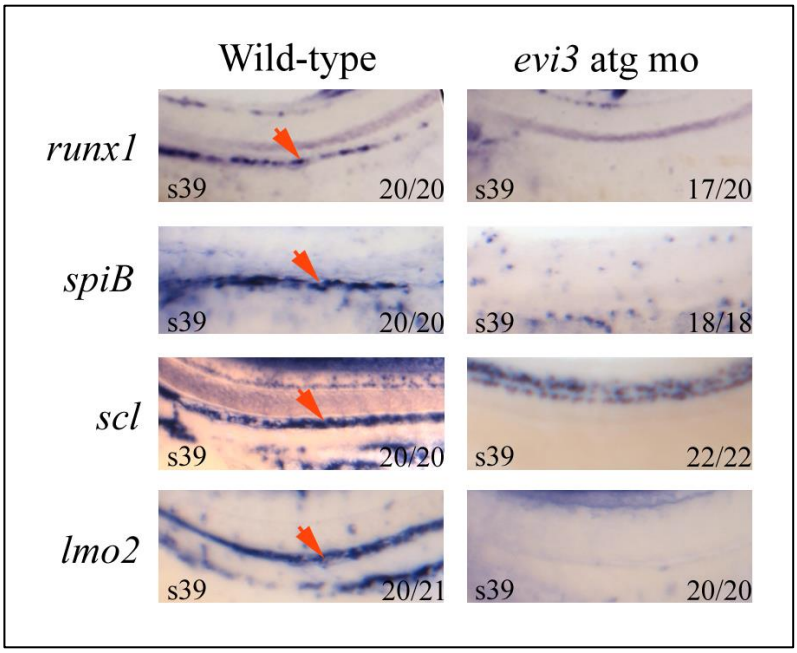



**Figure 5-7: Analysis of HSC programming at stage 39 following *evi3* knockdown**

Embryos were injected with 25 ng *evi3* translation-blocking morpholino and were cultured to stage 39, at which point the embryos were collected for analysis. *Runx1-, spib-, scl- and lmo2*-expressing cells were then stained by whole-mount *in situ* hybridisation and embryos were cleared with 2:1 BB:BA. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark the position of the dorsal aorta. Text on individual images shows the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates.

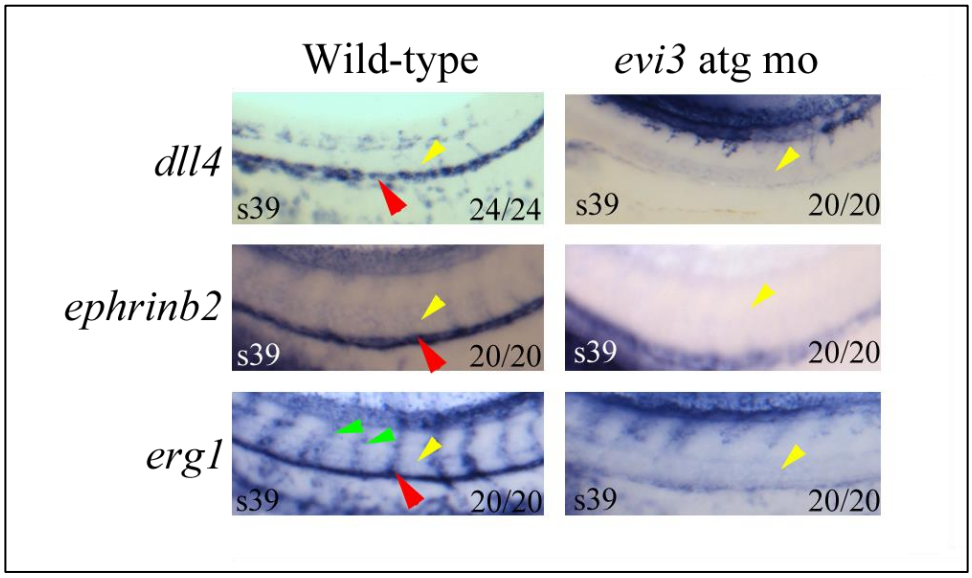



**Figure 5-8: Analysis of arterial programming at stage 39 following *evi3* knockdown**

Embryos were injected with 25 ng *evi3* translation-blocking morpholino and were cultured to stage 39, at which point the embryos were collected for analysis. *Dll4-, ephrinb2- and erg1*-expressing cells were then stained by whole-mount *in situ* hybridisation and embryos were cleared with 2:1 BB:BA. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark the position of the dorsal aorta. Yellow arrowheads mark the notochord whilst green arrowheads mark the intersomitic vessels. Text on individual images shows the stage imaged (bottom left), and the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of three biological replicates.

### 5.6 *Evi3* is required for *gata2* expression in the DLP

The above results indicate that *evi3* is required prior to dorsal aorta formation for HSC programming. The most likely site of this requirement is in the DLP, where *evi3* is expressed and HSCs are programmed during development. To test this hypothesis, *Xenopus* embryos were injected with 25 ng of *evi3* translation-blocking morpholino and were cultured to stage 22. Embryos were then analysed by whole-mount *in situ* hybridisation for *fli1*, *gata2*, *flk1* and *etv2*; genes known to be crucial for HSC programming in the DLP (Figure 5-9).

*Fli1* appears to be normally expressed in the DLP of both control and morphant embryos. Expression of *gata2* however is lost in morphant embryos, as is expression of *flk1* and *etv2*, which require *gata2* (Figure 5-9). This suggests that *evi3* is required for *gata2* initiation in the dorsal lateral plate mesoderm. Expression of *gata2*, *flk1* and *etv2* in the VBI is also lost in morphant embryos. This shows that *evi3* is also required for haematopoietic programming in the primitive haematopoiesis in the VBI.

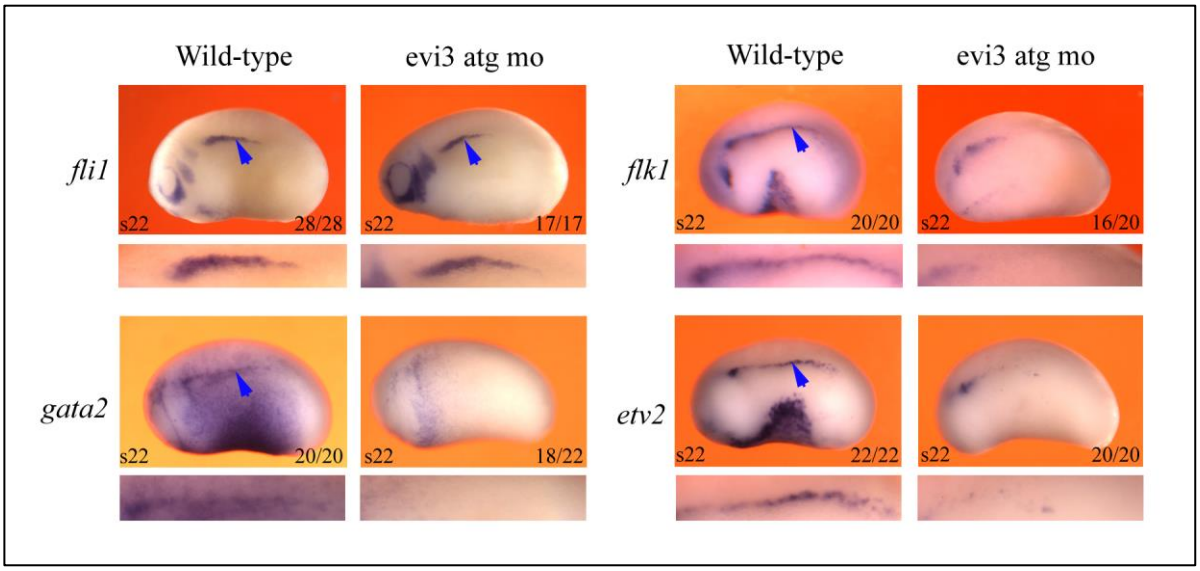



**Figure 5-9: Analysis of DLP gene expression at stage 22 following *evi3* knockdown**

Embryos were injected with 25 ng *evi3* translation-blocking morpholino and were collected for analysis at stage 22. Whole-mount *in situ* hybridisation was used to stain cells expressing genes that are essential to haematopoietic programming in the DLP; *fli1*, *gata2*, *flk1*, and *etv2*. Stained embryos were visualised from the lateral view, with anterior to the left and dorsal to the top. Red arrowheads mark staining in the DLP. Text on individual images shows the proportion of embryos represented by that image (bottom right). Images and numbers are from one experiment and are representative of at least three biological replicates. Panels underneath embryos show the DLP at increased magnification.

### 5.7 Conclusions and discussions

The high levels of Evi3 protein conservation between human, mouse and *Xenopus* suggests that its function is well conserved across vertebrates. Whole-mount *in situ* hybridisation of *evi3* shows that it is expressed in the DLP and VBI during development and morpholino knockdown has shown that this expression is required for HSC programming in the DLP and for primitive blood programming in the VBI. Our knowledge of the gene regulatory network programming cells in the DLP allows us to pinpoint *gata2* as the first gene expressed in the DLP that requires *evi3* for expression. *Gata2*, *flk1* and *etv2* expression in the VBI also require *evi3*, however as we currently do not know the epistatic relationship of these genes in the VBI we cannot currently identify the primary target of BMP signalling in the VBI.

Bioinformatic analysis of preliminary genomic sequencing scaffolds was used to identify several intron-exon boundaries of *Xenopus evi3*. This information was used to design a splice morpholino, which successfully inhibits-splicing according to PCR analysis. Further work on *evi3* will include the characterisation of the phenotype resulting from the use of this splice-blocking morpholino.

The phenotype resulting from *evi3* knockdown phenocopies post-gastrulation BMP signalling inhibition. The primary defect in both cases is a loss of *gata2* expression in the DLP, resulting in a block in HSC programming. As *evi3* has been shown to bind to the BMP R-Smad protein complex in cell culture and as *evi3* also binds histone modifying cofactors, a likely hypothesis is that *evi3* is required to initiate expression of genes required for HSC programming in the DLP. Morpholino knockdown of *evi3* does not result in any gross morphological defects. This allows us to conclude that *evi3* is not required for all BMP signalling, making *evi3* a BMP signalling cofactor that is required specifically for post-gastrulation BMP signalling in the DLP and VBI.

# Chapter 6

# Summary and future work

# Chapter 6 Summary and future work

BMP is known to be crucially important for haematopoietic stem cell programming during mesodermal patterning (Walmsley et al., 2002a). However the post-gastrulation role of BMP signalling in HSC ontogeny has not previously been explored in *Xenopus,* where the physical separation of the primitive and definitive blood lineages allows unique questions to be addressed. Understanding the post-gastrulation role for BMP signalling in HSC programming may be used to improve HSC *in vitro* culture, their generation from embryonic stem cells or induced pluripotent stem cells, and their potential therapeutic application.

The finding that BMP ligands and both type I and type II BMP receptors are all expressed in sites of HSC programming (namely the DLP and dorsal aorta) (Figure 3-3) supported the hypothesis that BMP signalling plays a role in post-gastrulation programming of the HSC. In order to investigate a potential requirement for BMP signalling in post-gastrulation HSC programming, two independent methods of BMP inhibition were used that allowed for temporal control of BMP signalling inhibition.

The use of small molecule BMP inhibitor DMH1 had previously been reported in zebrafish and in cell culture (Hao et al., 2010), however it had not previously been used in any other model organism. Furthermore, BMP R-Smad phosphorylation (an indicator of active BMP signalling) had not been analysed in an *in vivo* model. Presented here is evidence that DMH1 can be used to effectively inhibit BMP R-Smad phosphorylation *in vivo* in the developing *Xenopus* embryo. BMP R-Smad phosphorylation was blocked to a level undetectable by western blot, and pre-gastrulation DMH1 treatment induced dorsalisation of the embryo, which is consistent with previous reports following BMP inhibition.

A *Xenopus* transgenic line that expresses the BMP antagonist *noggin* has previously been used to study the role of BMP signalling in tail regeneration from stage 49 onwards (Beck et al., 2003). The results shown here indicate that this line can be used to block BMP signalling from as early as stage 13. Beck et al. used *noggin* mRNA quantification as a measure of transgene induction. Here, western blotting was used to measure the rate of induction and stability of Noggin protein levels. Furthermore, BMP R-Smad phosphorylation levels are inhibited to undetectable levels following heat shock. This control was not presented in previous publications.

Use of the small molecule inhibitor DMH1, and the heat shock *noggin* transgenic line, allowed BMP signalling to be temporally inhibited, post-gastrulation. This avoided the early patterning defects that have prevented previous investigations into the role of BMP signalling at later stages of development. The inhibition of BMP signalling at various time points during haematopoietic programming in the *Xenopus* embryo identified a post-gastrulation requirement for BMP signalling and for the transcription factor *gata2,* as the target of BMP signalling in this process.

Analysis of the *Xenopus gata2* promoter region identified multiple potential Smad binding sites, which suggest that the requirement for BMP signalling for *gata2* expression is direct. A ChIP assay, using the phosphorylated BMP R-Smad antibody, could be used to prove that BMP signalling acts directly on the *gata2* promoter in the *Xenopus* dorsal lateral plate (DLP). Primer pairs spanning the potential Smad binding elements identified in the *gata2* promoter were designed and tested, however time limitations prevented the completion of this experiment prior to the submission of this thesis. Results from this experiment will give an indication as to whether the BMP active R-Smad complex is bound to the *gata2* promoter region and thus whether *gata2* is directly targeted by BMP signalling.

Preliminary data attempting to rescue haematopoietic programming in BMP-inhibited embryos by injection of an inducible *gata2* mRNA show promise. Further optimisation of mRNA targeting, injected inducible *gata2* mRNA concentrations, timing of induction and repetition may clarify if *gata2* expression is able to rescue post-gastrulation BMP inhibition. If *gata2* is sufficient to rescue post-gastrulation BMP signalling inhibition then this would indicate that *gata2* is the primary target for BMP signalling in the DLP gene regulatory network (GRN). However, if *gata2* is not sufficient to rescue the BMP signalling inhibition phenotype, then further analysis of other genes in the GRN may be required to identify multiple inputs from BMP signalling.

The finding that BMP signalling is not required beyond stage 28 for *runx1* expression or maintenance in the *Xenopus* dorsal aorta contradicts data presented in the zebrafish, where BMP inhibition by heat-shock induced expression of the dominant negative BMP receptor (tBR) and resulted in a loss of *runx1* expression in the dorsal aorta (Wilkinson et al., 2009). This discrepancy may be due to a shift, over evolutionary time, of the requirement for BMP signalling to a different temporal window in *Xenopus*. Another explanation is that overexpression of tBR may also inhibit the Tgf-β receptor and block Tgf-β signalling. Inhibiting Tgf- β results in a loss of *runx1* expression in the zebrafish dorsal aorta (personal communication, Rui Monteiro) and as BMP signalling is closely related to Tgf-β signalling, the overexpression of tBR may block Tgf-β receptors, resulting in a loss of *runx1* expression via a BMP-independent mechanism. Some preliminary data supporting this hypothesis comes from experiments performed by Rui Monteiro in our lab. To address this, DMH1 treatment was carried out at a range of concentrations prior to *runx1* expression in the dorsal aorta. The preliminary results generated by Rui Monteiro, indicate that DMH1 treatment at these stages does not result in a loss of *runx1* expression. Although these results are preliminary and conclusions must be judged with caution, they suggest that BMP signalling is not required for

*runx1* expression in the dorsal aorta. Two simple experiments logically follow on from this hypothesis. Firstly, Tgf-β R-Smad phosphorylation should be analysed by western blot in tBR-injected embryos to determine whether Tgf-β signalling is perturbed. Secondly the Tgf-β small molecule inhibitor sb431542 could be used in *Xenopus* from stage 28 onwards to determine whether Tgf-β signalling is required in the *Xenopus* dorsal aorta for *runx1* expression and HSC emergence.

Although *runx1* expression appeared normal in embryos where BMP signalling was blocked from stage 28, it would have been interesting to analyse long-term HSC function in these embryos. This would indicate whether BMP signalling is required post EHT for HSC differentiation. B-cells could be used to measure HSC function as they are only made during definitive haematopoiesis in *Xenopus* (Chen and Turpen, 1995). This would require culturing embryos to at least 10 days and analysing the rearrangement of heavy and light chain immunoglobulin genes by PCR (Mussmann et al., 1998).

*Evi3* has been shown here to be required for HSC programming in *Xenopus*. This is the first report of the requirement for *evi3* in HSC development in an *in vivo* model organism. The use of *Xenopus* has allowed the identification of the primary defect in *evi3*-knockdown to be a loss of *gata2* expression in the DLP mesoderm. Previous reports showing *evi3* binding to the phosphorylated BMP R-Smad complex as well as other cofactors, suggest that *evi3* is a BMP-specific cofactor; binding with the Smad complex at target genes, recruiting other cofactors and altering gene expression. This is supported by the phenocopying of post-gastrulation BMP inhibition in *evi3*-knockdown embryos. The expression pattern of *evi3* does not encompass all of the tissues where BMP signalling is active, and so it is not a universally essential member of the BMP signalling pathway. Evidence supporting this comes from the lack of dorsalisation following morpholino knockdown of *evi3*. The morpholino used to

generate the data shown in this thesis binds to the 5' UTR of *evi3* mRNA and blocks its translation. To ensure that the resulting phenotype is a result of *evi3* mRNA inhibition and not an unintentional secondary target, a second morpholino should be used. A second, splice morpholino was designed here, which can be used to confirm the phenotype resulting from the translation-blocking morpholino.

As *evi3* has only been shown to bind to the BMP R-Smad complex in cell culture, a pull-down experiment using phosphorylated BMP R-Smad antibody and probes for *evi3* could prove that this interaction is occurring in *Xenopus*. Furthermore, this interaction could be tested in the specific tissue where we hypothesise *evi3* is participating in HSC programming by using dissected DLP.

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