

Elucidating the input of *notch* ligand
delta-like 4 (dll4) in zebrafish blood stem cell ontogeny

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Table of contents

Table of contents	i
Table of figures.....	v
Table of tables.....	viii
Abbreviations.....	ix
 Abstract	 xi
 Acknowledgements	 xiii
 Research declaration	 xviii
 Chapter 1 Introduction	 1
1.1 What is a haematopoietic stem cell?	2
1.2 Current and future clinical applications for HSCs.....	5
1.3 A brief overview of developmental haematopoiesis	6
1.4 HSC specification and emergence	8
1.5 On the origin of HSCs	11
1.5.1 Haemangioblasts generate primitive blood and endothelial cells.....	11
1.5.2 Endothelial cells are specified as arterial and venous through Notch signalling.....	13
1.5.3 Haemogenic endothelium is specified from the ventral side of the artery and gives rise to HSCs	23
1.6 The Notch pathway in haematopoiesis.....	29
1.7 The zebrafish as a model for HSC research.....	34
1.8 Zebrafish haematopoiesis.....	37
1.9 Overview and objectives	43
 Chapter 2 Materials and Methods	 45
2.1 Genes and their nomenclature	46
2.2 Materials.....	50
2.2.1 Solutions.....	50
2.2.2 Morpholinos	52
2.2.3 Chemical inhibitors	53
2.2.4 Primers	53
2.2.5 Plasmids	55
2.3 Zebrafish husbandry and manipulation of embryos	60
2.3.1 Zebrafish maintenance	60
2.3.2 Fin-clipping zebrafish	60
2.3.3 Breeding, embryo collection and maintenance.....	60
2.3.4 Micro-injections of zebrafish embryos.....	61

2.3.5	Dechoriation and fixation of zebrafish embryos	61
2.3.6	Dissection of trunks and tails	62
2.3.7	Zebrafish lines	62
2.3.8	Isolation of single cells and fluorescence-activated cell sorting	63
2.4	Extraction, preparation and manipulation of DNA.....	64
2.4.1	Transformation of chemically competent <i>Escherichia coli</i>	64
2.4.2	Growing bacterial cultures for DNA harvests.....	65
2.4.3	Isolation and extraction of plasmid DNA.....	65
2.4.4	Genomic DNA extraction from zebrafish embryos	66
2.4.5	Genomic DNA extraction from fin-clips	66
2.4.6	Agarose gel electrophoresis	66
2.4.7	Determining nucleic acid concentration.....	67
2.4.8	Restriction enzyme digest.....	67
2.4.9	Polymerase chain reaction (PCR)	67
2.4.10	Cloning of PCR products.....	68
2.4.11	DNA sequencing.....	69
2.5	Extraction, preparation and manipulation of RNA.....	69
2.5.1	RNA isolation from tissue samples.....	69
2.5.2	Reverse transcription of RNA.....	71
2.5.3	<i>In vitro</i> synthesis of capped mRNA for injection into embryos	71
2.5.4	<i>In vitro</i> synthesis of antisense RNA-probes.....	72
2.5.5	Whole mount <i>in situ</i> hybridisation.....	72
2.5.6	Quantitative Polymerase chain reaction (qPCR).....	74
2.5.7	RNA sample preparation for RNA sequencing and quality/quantity determination	74
2.5.8	Nanostring.....	75
2.6	Microscopy, imaging and statistical analysis	75
2.6.1	Still digital photography.....	75
2.6.2	Wide-field fluorescence imaging.....	76
2.6.3	Image analysis and representation	76
2.7	Bioinformatic tools	77
2.7.1	Genomic, transcript, and protein sequence information.....	77
2.7.2	Analysis of homology, identity, and synteny	77
2.7.3	Predictions of conserved protein domains.....	77
Chapter 3 The role of Notch ligand <i>dll4</i> in definitive haematopoiesis		78
3.1	Introduction.....	79
3.2	Expression pattern of Notch ligands around the time of haematopoietic stem cell emergence in the zebrafish embryo.....	80
3.3	Choosing and titrating <i>dll4</i> morpholinos	88
3.4	<i>Dll4</i> is required for definitive haematopoiesis in the zebrafish embryo	90
3.5	<i>Dll4</i> is not required for vascular specification in the zebrafish embryo	95
3.6	Loss of <i>dll4</i> results in reduction of all HSC-derived lineages.....	100

3.7	Discussion	105
3.7.1	Overview	105
3.7.2	<i>Dll4</i> is the best Notch ligand candidate for a role in HSC specification in the zebrafish.....	106
3.7.3	How severe is the vascular phenotype and why does it differ from the mouse?	110
3.7.4	<i>Dll4</i> is required for HSC emergence	113
3.7.5	Conclusions and directions.....	117
Chapter 4 Establishing <i>dll4</i> targets		118
4.1	Introduction	119
4.2	Comparative RNA-seq to establish <i>dll4</i> targets upstream of (or parallel to) <i>runx1</i>	121
4.3	Preparing and choosing samples for RNA-seq analysis	124
4.4	Quality control and differential gene expression analysis (DE) of RNA-seq output	127
4.5	Validation of the RNA-seq data	129
4.5.1	Some genes described as <i>dll4</i> targets in other models and developmental timepoints are also represented in the RNA-seq dataset.....	132
4.5.2	Validation of the RNA-seq experiment using nanostring.....	133
4.6	Identifying best <i>dll4</i> candidate targets for a role in HSC specification	135
4.6.1	Comparative analysis of the two RNA-seq datasets reveals potential <i>dll4</i> target genes upstream of <i>runx1</i>	135
4.6.2	Identification of <i>dll4</i> target genes with likely transcription factor and signalling activity.....	136
4.6.3	Expression profiling reveals best candidate genes for a role in HSC specification	138
4.7	Discussion	145
4.7.1	Overview	145
4.7.2	Not all <i>runx1</i> targets are regulated by <i>dll4</i>	146
4.7.3	Dissection of trunk/tail tissues causes high dilution of HSC precursors within the samples	147
4.7.4	Approaches for gene prioritisation.....	150
4.7.5	The expression screen revealed more spatially restricted genes than expected	153
4.7.6	Conclusions and directions.....	156
Chapter 5 Functional assessment of candidate <i>dll4</i> targets.....		157
5.1	Introduction.....	158
5.2	Designing a “perfect” morpholino screen.....	159
5.3	Validating best candidate <i>dll4</i> target genes	161
5.4	<i>Claudin5b</i> is required for arterial specification and consequently haematopoietic stem cell emergence	163
5.5	<i>Hypoxia-inducible factor 1 alpha (hif1ab)</i> is required for HSC cell emergence and vascular specification	165
5.6	<i>Zinc finger and AT hook domain containing (zfat)</i> gene is required for HSC emergence.....	167
5.7	<i>Forkhead box D1 (foxd1)</i> is required for arterio-venous specification and haematopoietic stem cell emergence	169

5.8	<i>Hes6</i> and <i>her8a</i> are required for HSC emergence	172
5.8.1	<i>Hes6</i> is required for HSC development.....	172
5.8.2	<i>Her8a</i> is required for HSC development.....	177
5.9	<i>Forkhead box C1b</i> (<i>foxc1b</i>) is required for haematopoietic stem cell emergence but not vascular specification	181
5.10	Discussion.....	183
5.10.1	Overview.....	183
5.10.2	Choosing the best reagent for a functional screen.....	184
5.10.3	All genes included in the MO screen are involved in haematopoiesis.....	186
5.10.4	Conclusions and directions.....	191
Chapter 6 <i>Foxc1b</i> is required for HSC emergence		192
6.1	Introduction.....	193
6.2	<i>Foxc1b</i> is expressed in the sclerotome and the vasculature	194
6.3	<i>Foxc1b</i> is dispensable for vascular development.....	198
6.4	<i>Foxc1b</i> is required for HSC specification.....	202
6.5	<i>Foxc1b</i> also has a <i>dll4</i> -independent role in HSC development.....	206
6.6	Do <i>cmyb</i> ^{h_h3} mutants show a similar phenotype to <i>foxc1b</i> morphants?.....	214
6.7	Discussion.....	217
6.7.1	Overview.....	217
6.7.2	The role of <i>foxc1b</i> downstream of <i>dll4</i> in HSC specification.....	219
6.7.3	Speculative mechanisms for the action of <i>foxc1b</i> in Phase II	222
6.7.4	Speculative non-cell autonomous models of <i>dll4</i> -independent regulation of <i>foxc1b</i>	226
6.7.5	Conclusions and directions.....	229
Chapter 7 Discussion		230
Chapter 8 Appendices		234
8.1	Appendix to Chapter 3 - The role of the Notch ligand <i>dll4</i> in definitive haematopoiesis.....	239
8.2	Appendix to Chapter 4 - Establishing <i>dll4</i> targets.....	246
8.3	Appendix to Chapter 5 - Functional assessment of candidate <i>dll4</i> targets.....	258
8.4	Appendix to Chapter 6 - <i>Foxc1b</i> is required for HSC emergence	262
Chapter 9 Bibliography.....		265

Table of figures

Chapter 1 - Introduction

Figure 1-1.	Model of a definitive haematopoietic lineage tree.....	4
Figure 1-2.	Cellular ontogeny of definitive haematopoiesis.....	7
Figure 1-3.	Schematic representation of the major pathways establishing arterial identity.....	18
Figure 1-4.	Haemogenic endothelium and HSC emergence across model species.	25
Figure 1-5.	Schematic representation of Notch signalling between neighbouring cells.	31
Figure 1-6.	Comparative developmental haematopoiesis of mouse and zebrafish.	35
Figure 1-7.	Tissues and markers of zebrafish definitive haematopoiesis.....	41

Chapter 3 - The role of the Notch ligand *dll4* in definitive haematopoiesis

Figure 3-1.	Expression of the <i>jagged</i> family of Notch ligands in the zebrafish embryo around the early stages of HSC development.....	83
Figure 3-2.	Expression of the <i>delta</i> family of Notch ligands in the zebrafish embryo around the early stages of HSC development.....	85
Figure 3-3.	<i>Dll4</i> expression in the developing zebrafish embryo.	87
Figure 3-4.	<i>Dll4</i> morpholino design and testing of specificity.....	89
Figure 3-5.	<i>Runx1</i> positive cells are reduced in the DA in <i>dll4</i> morphants.	91
Figure 3-6.	The loss of <i>runx1</i> in <i>dll4</i> morphants represents a loss of HSCs.....	92
Figure 3-7.	The <i>dll4</i> HSC phenotype is rescued by <i>runx1</i> overexpression but <i>dll4</i> is not affected in <i>runx1</i> morphants.....	94
Figure 3-8.	Loss of <i>dll4</i> activity affects arterial patterning in a minor way at the time of HSC emergence.....	97
Figure 3-9.	The venous programme is not affected by loss of <i>dll4</i> activity at the time of HSC emergence.....	98
Figure 3-10.	The endothelial programme is established appropriately in <i>dll4</i> morphants.....	99
Figure 3-11.	Number of HS(P)Cs are reduced in the CHT of <i>dll4</i> morphants.	101
Figure 3-12.	The erythroid and myeloid lineages are severely reduced in <i>dll4</i> morphants.	103
Figure 3-13.	Loss of <i>dll4</i> causes severe reduction in HSC-derived lymphocytes.	104

Chapter 4 - Establishing *dll4* targets

Figure 4-1.	Aims and experimental approach taken in Chapter 4.....	120
Figure 4-2.	Schematic representation of the comparative RNA-seq approach.....	123
Figure 4-3.	Establishing <i>dll4</i> targets in the zebrafish.....	125
Figure 4-4.	WISH to assess knockdown quality for samples submitted for RNA-seq	126
Figure 4-5.	Gene count and clustering analyses on RNA-seq samples.	128
Figure 4-6.	Gene expression boxplots.....	131

Figure 4-7.	Nanostring analysis reveals differentially expressed genes similar to RNA-seq.....	134
Figure 4-8.	Defining the RNA-seq data subset specific to <i>dll4</i> MO-vs-wt.....	136
Figure 4-9.	Identifying the most promising RNA-seq data subset by molecular function.....	137
Figure 4-10.	Expression patterns of <i>dll4</i> targets	142-144
Figure 4-11.	A succession of filters prioritises the RNA-seq dataset systematically.....	151
Figure 4-12.	Summary of the expression screen by gene classification.....	155

Chapter 5 - Functional assessment of candidate *dll4* targets

Figure 5-1.	Aims and experimental approach taken in Chapter 5	159
Figure 5-2.	Morpholino screen design	161
Figure 5-3.	Validation of candidate <i>dll4</i> target genes by qPCR	162
Figure 5-4.	<i>Cldn5b</i> is required for appropriate arterial and HSC specification	164
Figure 5-5.	MO screen to assess the potential role of <i>hif1ab</i> in HSC development	166
Figure 5-6.	MO screen to assess the potential role of <i>zfat</i> in HSC development	168
Figure 5-7.	MO screen to assess the potential role of <i>foxd1</i> in HSC development.....	170
Figure 5-8.	MO screen to assess the potential role of <i>hes6</i> in HSC development.....	173
Figure 5-9.	Extended phenotypic analysis of <i>hes6</i> morphants.....	175
Figure 5-10.	MO screen to assess the potential role of <i>her8a</i> in HSC development.....	178
Figure 5-11.	Extended phenotypic analysis of <i>her8a</i> morphant.....	180
Figure 5-12.	MO screen to assess the potential role of <i>foxc1b</i> in HSC development.....	182

Chapter 6 - *Foxc1b* is required for HSC emergence

Figure 6-1.	<i>Foxc1b</i> is expressed in the <i>dll4</i> -gfp positive and negative fractions of the zebrafish trunk/tail region	195
Figure 6-2.	<i>Dll4</i> is required for <i>foxc1b</i> expression which is required for <i>runx1</i> expression at 26 hpf	197
Figure 6-3.	<i>Foxc1b</i> is not required for the endothelial programme at the time of HSC emergence	199
Figure 6-4.	Arterial programming is normal at the time of HSC emergence in <i>foxc1b</i> morphants.....	200
Figure 6-5.	The venous programme is not majorly affected in the absence of <i>foxc1b</i>	201
Figure 6-6.	<i>Foxc1b</i> is required for HSC emergence and HSC-derived T-cell maturation.....	203
Figure 6-7.	Cells of the definitive blood lineage are reduced in the CHT of <i>foxc1b</i> morphants.....	205
Figure 6-8.	<i>Foxc1b</i> morphants show increased expression of HSC markers at 36 hpf	207
Figure 6-9.	Timecourse of <i>runx1</i> expression in the DA and CHT in <i>foxc1b</i> morphants.....	208-209
Figure 6-10.	Percentage of <i>foxc1b</i> MO embryos with changed <i>runx1</i> expression in the DA and CHT over time.....	212
Figure 6-11.	<i>Runx1</i> expression timecourse in <i>cmyb^{hkHz3}</i> mutants	216
Figure 6-12.	Overview of the <i>dll4</i> , <i>foxc1b</i> and <i>cmyb^{hkHz3}</i> phenotypes	218
Figure 6-13.	Speculative genetic regulatory model for Phase I	220
Figure 6-14.	Speculative genetic regulatory mechanism for Phase II.....	224

Chapter 8 - Appendices

Appendix Figure 8-1.	Expression of Notch receptors in the zebrafish embryo around early stages of HSC development.....	243
Appendix Figure 8-2.	Homology analyses for vertebrate <i>Dll4</i> genes	244
Appendix Figure 8-3.	Quality of RNA submitted for sequencing and read assignment by category	246
Appendix Figure 8-4.	Gene count and heatmap analyses on RNA-seq samples.....	247
Appendix Figure 8-5.	Expression vs fold change plots	248
Appendix Figure 8-6.	Titration of MOs included in the expression screen	258
Appendix Figure 8-7.	<i>Zfat</i> MO design and testing MO efficacy.....	261
Appendix Figure 8-8.	<i>Hes6</i> and <i>her8a</i> morphants show an additive effect on HSCs and their derivative	261
Appendix Figure 8-9.	Homology analyses for vertebrate <i>foxc1</i> genes.....	262
Appendix Figure 8-10.	Regulation of <i>dll4</i> by <i>foxc1b</i> is highly dynamic	263
Appendix Figure 8-11.	Characterising <i>cmyb</i> ^{hkz3} mutants	264
Appendix Figure 8-12.	<i>Vegfaa</i> expression is repressed by <i>foxc1b</i> at 36 hpf but not 26 hpf	264

Table of tables

Abbreviations	ix
Acknowledgement and overview of sources of data, figures and tables	xix

Chapter 2 - Materials and Methods

Table 2-1.	Comparative nomenclature across model organisms	46
Table 2-2.	Zebrafish genes referred to in this thesis and their symbols, IDs and previous names.....	47
Table 2-3.	Solutions.....	50
Table 2-4.	Morpholinos used for knockdown analyses.....	52
Table 2-5.	Chemical inhibitors used in this thesis.....	53
Table 2-6.	QPCR primer pairs used in this thesis	54
Table 2-7.	Primer sets for testing MO and mutant efficiency	54
Table 2-8.	Primer pairs for general WISH probes of interest.....	55
Table 2-9.	General haematopoiesis-related antisense probes used in this thesis and their sources	55
Table 2-10.	Primers, plasmids, restriction enzymes and polymerases used for each gene included in the expression screen conducted in Chapter 4.....	57
Table 2-11.	Plasmids for injection	59
Table 2-12.	Fish lines used in this thesis	62

Chapter 3 - The role of the Notch ligand *dll4* in definitive haematopoiesis

Table 3-1.	Notch receptor and ligand orthologues.	81
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Chapter 5 - Functional assessment of candidate *dll4* targets

Table 5-1.	MO screen summary	188
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Chapter 8 - Appendices

Appendix Table 8-1.	Breakdown of MO phenotypes represented in Figures.....	235
Appendix Table 8-2.	Schematic representation of the expression of Notch receptors and ligands during zebrafish embryonic development.....	240
Appendix Table 8-3.	Nanostring analysis.....	250
Appendix Table 8-4.	Significantly differentially expressed genes in <i>dll4</i> MO-vs-wt but not <i>runx1</i> MO-vs-wt (<i>dll4</i> ⁺ / <i>runx1</i> ⁻).....	253

Abbreviations

Abbreviation	Description
adjp	adjusted p-value
AGM	aorta/gonad/mesonephros
ALM	anterior lateral plate mesoderm
ANS	anus
bp	base pair
CHT	caudal hematopoietic tissue (<i>Danio rerio</i>), analogous to foetal liver
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CNS	central nervous system
d	day
<i>D. rerio</i>	<i>Danio rerio</i> , zebrafish
DA	dorsal aorta
DE	differential expression
DLAV	dorsal longitudinal anastomotic vessel
<i>dll4</i> ⁻ / <i>runx1</i> ⁺	<i>runx1</i> but not <i>dll4</i> targets
<i>dll4</i> ⁺ / <i>runx1</i> ⁻	<i>dll4</i> but not <i>runx1</i> targets
<i>dll4</i> ⁺ / <i>runx1</i> ⁺	common <i>dll4</i> and <i>runx1</i> targets
<i>dll4</i> MO-vs-wt	differentially expressed genes between <i>dll4</i> MO and wt
dpf	days post fertilisation
E	embryonic day (<i>M. musculus</i>)
ECT	ectoderm
EHT	endothelial-to-haematopoietic transition
EMP	erythro-myeloid progenitor
ES(C)	embryonic stem (cell)
FACS	fluorescence-activated cell sorting
FL	foetal liver (<i>Mus musculus</i>)
<i>G. gallus</i>	<i>Gallus gallus</i> , chicken
GMP	granulocyte-macrophage progenitor
GO	gene ontology
h	hour
<i>H. sapiens</i>	<i>Homo sapiens</i> , human
hESC	human embryonic stem cell
HPC	haematopoietic progenitor cell
hpf	hours post fertilisation
HS(P)C	Haematopoietic stem (progenitor) cell
HSC	haematopoietic stem cell
HUVEC	human umbilical vein endothelial cell
ICM	intermediate cell mass (<i>Danio rerio</i>)
iPS cells	induced pluripotent stem cells
ISV	intersomitic / intersegmental vessels

Abbreviation	Description
IT-HSC	intermediate-term repopulating HSC
MOkbp	kilo base pair
LIV	liver
LL	lateral line (<i>Danio rerio</i>)
LMPP	lymphoid-primed multi-lineage progenitor
log2	binary logarithm
LT-HSC	long-term repopulating HSC
LTR	long-term repopulation assay
<i>M. musculus</i>	<i>Mus musculus</i> , mouse
MEP	megakaryocyte erythroid progenitor
MO	morpholino
MPP	multipotent progenitor
n	number of samples
n/a	not applicable
NC	notochord
NECD	notch extracellular domain
NICD	notch intracellular domain
NT	neural tube
PBI	posterior blood island (<i>Danio rerio</i>), analogous to early foetal liver
PCA	principal component analysis
PCR	polymerase chain reaction
PCV	posterior cardinal vein (axial vein, <i>Danio rerio</i>)
PE (sequencing)	paired end (sequencing)
PLM	posterior lateral plate mesoderm
PND	pronephric duct
qRT-PCR	quantitative reverse transcriptase PCR
<i>R. norvegicus</i>	<i>Rattus norvegicus</i> , rat
RT	room temperature
runx1MO-vs-wt	list of differentially expressed genes between <i>runx1MOe6</i> and wt
SEM	standard error of the mean
SOM	somites
<i>spp.</i>	<i>species pluralis</i> , multiple species
ST-HSC	short-term repopulating HSC
TB	tailbud
TF	transcription factor
THY	thymus
trunk/tail	the trunk and tail region of <i>D. rerio</i> , gained by dissection
WISH	wholemount <i>in situ</i> hybridisation
wt	wild type
<i>X. laevis</i>	<i>Xenopus laevis</i> , xenopus
<i>X. tropicalis</i>	<i>Xenopus tropicalis</i> , xenopus
YS	yolk sac (<i>Mus musculus</i> , <i>Gallus gallus</i>)

Abstract

Multipotent haematopoietic stem cells (HSCs) supply the organism with mature blood cells of all lineages throughout adult life. These cells first originate in the dorsal aorta (DA) of the vertebrate embryo, and a multitude of signalling pathways regulate their specification in the embryo. The emergence of HSCs is dependent on appropriate arterial specification and vessel maturation, processes which are heavily dependent on Notch signalling. This arterial involvement of Notch obscures its later roles in HSC specification. The Notch ligand *dll4* is crucially involved in arterial development in the mammalian embryo, while zebrafish embryos deficient for *dll4* activity only exhibit minor arterial defects at the time of HSC emergence. Here, the zebrafish model has been exploited to reveal the first specific evidence for a role of *dll4* in HSC specification.

Dll4 is required for the expression of *runx1*, a transcription factor (TF) required for HSC specification, prior to any observed effects on vascular development. HSCs and all their derivatives are depleted in *dll4* morphants. To disentangle the genetic regulatory cascade downstream of *dll4* and upstream of *runx1*, RNA-seq was employed to discover downstream effectors of this signalling. Expression and functional screening of best candidate genes revealed seven genes with novel roles in HSC development. *Foxc1b* is a *dll4* target predominantly mirroring the *dll4* phenotype, and is thus likely to be the downstream effector of *dll4*, upstream of *runx1*. Interestingly, *foxc1b* also has a later *dll4*-independent role in HSC development, remarkably similar to that of *cmyb*.

Taken together I show here for the first time a requirement of *dll4* upstream of *runx1* in HSC specification, mediated by *foxc1b*, followed by a later *dll4*-independent phase in HSC development.

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“When I was One,
I had just begun.

When I was Two,
I was nearly new.

When I was Three
I was hardly me.

When I was Four,
I was not much more.

When I was Five, I was just alive.

But now I am Six, I'm as clever as clever,
So I think I'll be six now for ever and ever.”

— A. A. Milne, *Now We Are Six*

Research declaration

All work presented in this thesis is my own, unless indicated otherwise. Any text, data and representation that is not my own work are indicated as such, acknowledged and referenced accordingly.

- Experimental design and sample generation for the RNA-seq experiment in Chapter 4 have been carried out in collaboration with Dr. Philip Pinheiro and Dr. Tessa Peterkin (WIMM, Oxford)
- RNA sequencing in Chapter 4 was carried out by the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics (WTCHG, Churchill Hospital, Oxford) and all bioinformatic analysis of the RNA-seq output was carried out by Dr. Helen Lockstone (WTCHG, Oxford).
- GO-Term analysis in Chapter 4 was carried out by Dr. Jie Zuo (WIMM, Oxford)
- Cell-dissociation of samples for FACS sorting and RNA processing in Chapters 5 and 6 was conducted in collaboration with Dr. Tessa Peterkin and FACS sorting of dissociated cells was carried out by Kevin Clark (WIMM, Oxford)
- *runx1* cDNA and *tol2* mRNA constructs for the *dll4* rescue experiment in Chapter 3 were kindly provided by Dr. Philip Pinheiro

Acknowledgement and overview of sources of data, figures and tables.

Figure 1-1	Model of a definitive haematopoietic lineage tree.	p. 4	generated collating data from Adolfsson et al., 2005, Buza-Vidas et al., 2007, Blank et al., 2008, Doulatov et al., 2012 and Jagannathan-Bogdan and Zon, 2013.
Figure 1-4-A&B	Haemogenic endothelium and HSC emergence across model species.	p. 25	adapted from Jaffredo et al. 2005 (Figure 2)
Figure 1-6	Comparative developmental haematopoiesis of mouse and zebrafish.	p. 36	adapted from Ciau-Uitz et al., 2014 (Figure 1)
Table 3-1	Notch receptor and ligand orthologues.	p. 81	orthologue assignment carried out using ensembl (Flicek et al., 2014)
Figure 4-4	WISH to assess knockdown quality for samples submitted for RNA-seq.	p. 126	data generated in collaboration with Dr. Tessa Peterkin and Dr. Philip Pinheiro (WIMM, Oxford)
Figure 4-5	Gene count and clustering analyses on RNA-seq samples.	p. 128	kindly provided by Dr. Helen Lockstone (WTCHG, Oxford), adapted
Figure 4-6	Gene expression boxplots.	p. 131	kindly provided by Dr. Helen Lockstone (WTCHG, Oxford), adapted
Figure 4-7	Nanostring analysis reveals differentially expressed genes similar to RNA-seq.	p. 134	data generated in collaboration with Dr. Tessa Peterkin (WIMM, Oxford)
Figure 4-8	Defining the RNA-seq data subset specific to dll4MO-vs-wt.	p. 136	experimental data generated in collaboration with Dr. Tessa Peterkin and Dr. Philip Pinheiro (WIMM, Oxford), bioinformatic analysis provided by Dr. Helen Lockstone (WTCHG, Oxford),
Figure 4-9	Identifying the most promising RNA-seq data subset by molecular function.	p. 137	partial generation of data by Dr. Jie Zuo, (WIMM, Oxford)
Figure 6-1-A	<i>foxc1b</i> is expressed in the dll4-gfp positive and negative fractions of the zebrafish trunk/tail region.	p. 195	10ss embryo flat-mounted and photographed by Dr. Jana Koth (WIMM, Oxford)
Figure 6-1-C	<i>foxc1b</i> is expressed in the dll4-gfp positive and negative fractions of the zebrafish trunk/tail region.	p. 195	taken from Skarie and Link, 2009 (Figure 1-D)
Appendix Figure 8-3-C&D	Quality of RNA submitted for sequencing and Read assignment by category.	p. 246	kindly provided by Dr. Helen Lockstone (WTCHG, Oxford), adapted
Appendix Figure 8-4	Gene count and heatmap analyses on RNA-seq samples.	p. 247	kindly provided by Dr. Helen Lockstone (WTCHG, Oxford), adapted
Appendix Figure 8-5	Expression vs fold change plots.	p. 248	kindly provided by Dr. Helen Lockstone (WTCHG, Oxford), adapted
Appendix Table 8-4	Significantly differentially expressed genes in dll4MO-vs-wt but not runx1MO-vs-wt (dll4+/runx1-).	p. 252	differential expression analysis conducted by Dr. Helen Lockstone (WTCHG, Oxford), GO term analysis conducted by Dr. Jie Zuo (WIMM, Oxford), CAGE values provided by Prof. Adrian Harris (WIMM, Oxford)

Chapter 1

Introduction

1.1 What is a haematopoietic stem cell?

The blood system is made up of a variety of different cell types circulating through the body via the vasculature. Each haematopoietic cell type has a distinctive and essential function, ranging from tissue oxygenation to immune defence. These mature blood cells have a limited lifespan and thus have to be continuously replaced throughout life, a process known as haematopoiesis.

Haematopoiesis is made possible through two characteristics of the haematopoietic stem cell (HSC): self-renewal and multipotency. Self-renewal is the ability of HSCs to undergo cell division without differentiation, allowing for the maintenance of the HSC pool. Multipotency of HSCs describes the capacity of a single cell to give rise to differentiated progeny of all haematopoietic lineages. Self-renewal and multipotency of HSCs can be assessed by *in vivo* long-term reconstitution (LTR) experiments and *in vitro* clonal differentiation protocols, respectively (reviewed in Cumano and Godin, 2007). These HSCs reside in the bone marrow of most adult vertebrates and the kidney marrow of zebrafish. Bone marrow transplants have been used as a treatment for patients with blood-related disorders since the late 1950s and early 1960s, and have provided the basis for HSC transplantations ever since (Till and McCulloch, 1961; Thomas et al., 1957, Mathé et al., 1959, Mathé et al., 1963).

Using the transplantation assay, several distinct HSC populations have been identified (Figure 1-1): short-term (ST-) and intermediate-term (IT-) repopulating HSCs are capable of reconstitution for 3-6 months with limited serial reconstitution capacity, while long-term repopulating HSCs (LT-HSCs) are capable of reconstituting the host for a minimum of six months, and have full serial reconstitution capacity (Morrison and Weissman, 1994, Adolfsson et al., 2001, Adolfsson et al., 2005, Doulatov et al., 2012).

Therefore it is the exceedingly rare LT-HSCs that are the cells of interest for bone marrow transplants. These cells comprise as little as 0.00001% of all cells in the bone marrow, creating a major challenge for the use of HSCs in clinical treatments (Harrison et al., 1988, Harrison et al., 1993).

The concept of a single cell type in the bone marrow being the origin of all blood lineages was first independently proposed by Neumann and Maximow in the early 20th century, derived from microscopic observation. The first experimental proof of this theory was provided by the bone marrow transplantation experiments in irradiated mice, conducted by Till and McCulloch in 1961 (reviewed in Boisset and Robin, 2012). The ability of single, labelled, murine, foetal liver cells to provide long-term reconstitution of the whole haematopoietic system of irradiated mice further contributed to the evidence for the HSC (Jordan and Lemischka, 1990, Jordan et al., 1990, Keller and Snodgrass, 1990, Lemischka, 1991, Harrison et al., 1993). HSCs can be enriched using a combination of several surface markers for fluorescence-activated cell sorting (FACS). In the absence of a single unique marker, HSCs can be defined as the LSK-population (Lin-/Sca1+/c-Kit+) (Greaves et al., 1981, Spangrude et al., 1988, Baum et al., 1992, Okada et al., 1992). Nevertheless, it remains difficult to create *in vitro* conditions that allow HSC expansion without differentiation, despite our continuously improving understanding of the intricate regulation of these cells, indicating that major parts of the regulatory landscape are still unknown (reviewed in Hofmeister and Zhang, 2007).

The bone (or kidney) marrow is seeded by HSCs that were generated during embryogenesis, and thus understanding of the mechanisms that underlie the *de novo* generation of HSCs in the embryo are vital to make novel approaches to the treatment

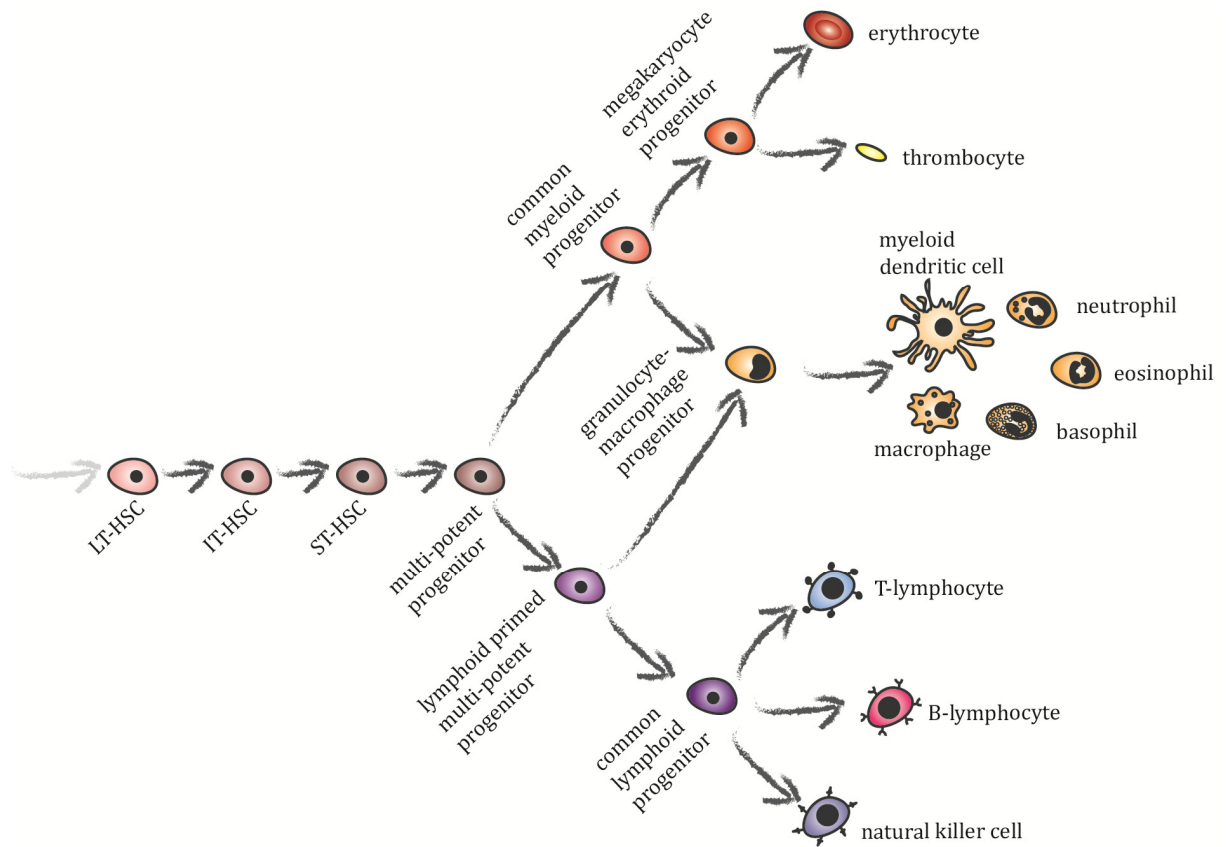


Figure 1-1. Model of a definitive haematopoietic lineage tree.

A simplified version of the definitive haematopoietic lineage tree, showing differentiated blood cells and their known multipotent progenitor cells. HSCs are the root of the tree and can be split into long-term (LT-), intermediate (IT-) and short-term (ST-) HSCs, based on their repopulating potential. A multipotent progenitor (MPP) gives rise to a common myeloid progenitor (CMP) and a lymphoid-primed multi-lineage progenitor (LMPP). The CMP gives rise to two distinct progenitor populations: the megakaryocyte erythroid progenitor (MEP) and the granulocyte-macrophage progenitor (GMP). The LMPP gives rise to the lymphoid cells via a common lymphoid progenitor (CLP) and, independently of the CLP to the GMP. Inferred lineage relationships are indicated by arrows, progenitors are indicated by angled labelling. This scheme was generated collating data from Adolfsson et al., 2005, Buza-Vidas et al., 2007, Blank et al., 2008, Doulatov et al., 2012 and Jagannathan-Bogdan and Zon, 2013.

of diseases of the blood system possible.

Many signalling pathways are required for HSC specification and maintenance, Notch being one of them. The Notch pathway is particularly interesting as, although it has been implicated in HSC specification, it has been difficult to address this role due to the major developmental abnormalities caused in mouse mutants and their embryonic lethality at around the time of HSC specification.

1.2 Current and future clinical applications for HSCs

Much of the interest in understanding HSCs comes from their established and potential clinical applications: HSCs are the only tissue stem cells in routine clinical therapies for malignant and non-malignant diseases, nevertheless there is still an unmet clinical need to expand the HSPC pool prior to transplantation and to culture patient's own HSCs prior to treatment (reviewed in Staal et al., 2011). The potential of induced pluripotent stem cells (iPS cells) from the patient's non-haematopoietic tissues to induce HSCs would avoid having to find an immune-compatible donor and acquiring sufficient numbers of LT-HSCs, both of which are bottlenecks for traditional bone marrow transplants (Bordignon, 2006, Irion et al., 2008, Murry and Keller, 2008). Embryonic stem cells (ES cells) and iPS cells can generate cells of the different haematopoietic lineages *in vitro*, including erythrocytes, myelocytes and megakaryocytes. Despite their great clinical potential, generating HSCs from ES or iPS cells remains very limited (Kyba et al., 2002, Burt et al., 2004). To date it has not been possible to generate iPS-derived HSCs that are capable of long-term self-renewal and produce a normally balanced set of mature blood cell lineages (reviewed in Peters et al., 2010 and Sakamoto et al., 2010). Remarkably, human skin-derived fibroblasts can be re-programmed to become multi-lineage haematopoietic progenitors through expression of haematopoietic transcription

factors (Takahashi and Yamanaka, 2006, Takahashi et al., 2007, Szabo et al., 2010, Pereira et al., 2013), providing a new promising way of *in vitro* generation of HSCs.

The failure to produce fully functional HSCs *in vitro* for clinical treatment indicates that crucial mechanisms of HSC development and function are still not understood and it remains a major goal to elucidate the complex cellular and molecular network governing the generation of HSCs. Studies *in vivo* can aid the understanding of those mechanisms, by elucidating HSC generation during embryogenesis. Understanding the genetics underlying stem cell generation will further support studies aiming to understand the causes of congenital diseases linked to bone marrow failure, such as aplastic anaemias and congenital neutropenia (Chung and Kim, 2014, Clements and Traver, 2013).

1.3 A brief overview of developmental haematopoiesis

Development of the vertebrate blood system occurs in three distinct waves in the embryo: initially, the primitive wave takes place in the yolk sac blood islands of the mouse and the anterior and posterior lateral mesoderm of the zebrafish embryo. This transient wave produces a population of erythrocytes and myeloid cells in both organisms. The subsequent second wave occurs from haemogenic endothelium in the yolk sac of the mouse, and the posterior blood island of the zebrafish. The cells produced in this wave are multipotent progenitor cells which migrate to the foetal liver of the mouse and the equivalent tissue in the zebrafish, where they differentiate into erythro-myeloid and lymphoid lineages (reviewed in Ciau-Uitz et al., 2014 and Carroll and North, 2014).

In all vertebrate species studied to date, *bona fide* HSCs are generated in the definitive wave from the haemogenic endothelium (HE) of the ventral dorsal aorta (DA). After emerging from the DA through an endothelial-to-haematopoietic transition (EHT), HSCs enter the circulation and populate the haematopoietic tissues where they give rise to cells of all haematopoietic lineages (reviewed in Swiers et al., 2013b and Orkin and Zon, 2008).

Lineage tracing experiments indicate that zebrafish HSCs emerge in the region between the DA and the axial (posterior cardinal) vein. Cells that have emerged from the DA of Tg(*itga2b:gfp*) transgenic embryos begin expressing low levels of the *itga2b* (*CD41*) transgene and initiate migration to the caudal haematopoietic tissue (CHT), indicating their haematopoietic character (Murayama et al., 2006, Kissa et al., 2008).

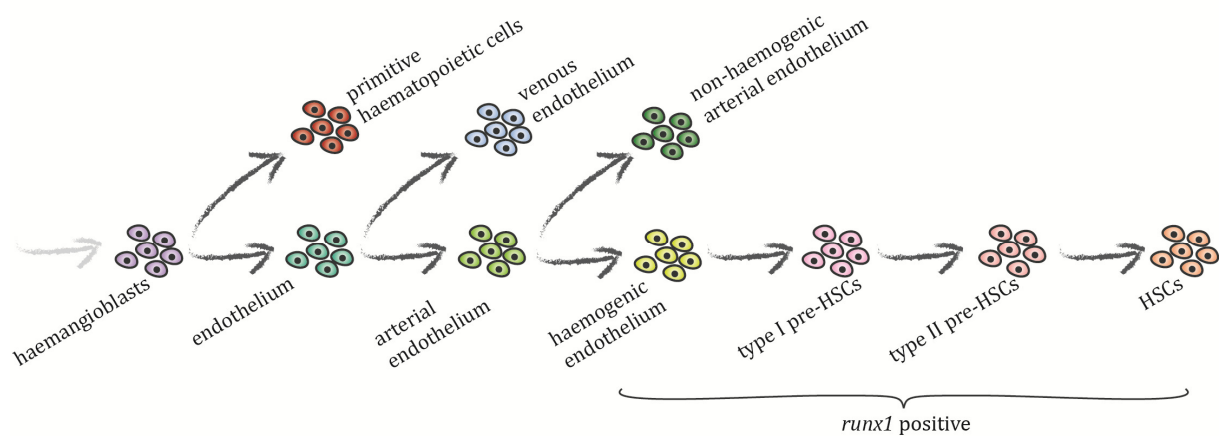


Figure 1-2. Cellular ontogeny of definitive haematopoiesis.

A highly simplified representation of the ontogeny of HSCs: the population of haemangioblasts gives rise to primitive haematopoietic cells and endothelium which undergoes arterio-venous specification. Haemogenic endothelial cells arise from arterial endothelial cells and in turn give rise to HSCs via type I and type II pre-HSCs. Note that this model represents cell populations and not individual cells, and simplifies the relationship between primitive and definitive blood.

1.4 HSC specification and emergence

Definitive haematopoiesis initiates with genetic changes in the haemogenic endothelium of the DA in the mouse, followed by the emergence of haematopoietic clusters, and the emergence of HSCs from these clusters (Swiers et al., 2013a, Swiers et al., 2013b, Ciaua-Uitz et al., 2014). HSCs start to emerge from the ventral wall of the DA at E10.5 in the mouse and 30 hpf in the zebrafish (Müller et al., 1994, Medvinsky and Dzierzak, 1996, de Bruijn et al., 2000, Kumaravelu et al., 2002, Bertrand et al., 2010a, Kissa and Herbomel, 2010). At E10.5 HSCs are also present in the vitelline and umbilical arteries in the mouse, and by E11/E11.5 HSCs are also apparent in other highly vascularized tissues: the yolk sac, placenta and the foetal liver (FL) (de Bruijn et al., 2000, Kumaravelu et al., 2002, Gekas et al., 2005, Ottersbach and Dzierzak, 2005). While HSCs are present in single digits in the mouse at and before E11, by E12 their numbers increase immensely in the placenta and foetal liver (Kumaravelu et al., 2002, Gekas et al., 2005). The main site of haematopoiesis switches to the foetal liver at mid-gestation in the mouse and HSCs begin the colonization of the bone marrow, the site of adult haematopoiesis, at E17.

Recently, two distinct pre-HSC cell types, embryonic ancestors of definitive HSCs, were identified: type I and type II pre-HSCs both giving rise to HSCs (Taoudi et al., 2008, Rybtsov et al., 2011). Characteristic marker expression distinguished type I (a VE-Cadherin⁺CD41^{lo}CD45⁻) from type II (VE-Cadherin⁺CD41^{lo}CD45⁺). Both lack the ability to form endothelial tubules and sheets in culture, indicating the loss of vascular characteristics and progressive haematopoietic fate of these cell populations. Pre-HSC as well as HSC activity appears to be restricted to the arterial but not venous compartment (De Bruijn et al., 2000, Gordon-Keylock et al., 2013). While this observation can be explained by up-

regulation of CD45 in the endothelial lineage, integration of an external precursor, from the mesenchyme for example, into the endothelial lining cannot be ruled out (Taoudi, 2008). This hypothesis is further supported by the observation that pre-HSCs are not exclusively localised to the floor of the DA, but are also present in the underlying mesenchyme (Gordon-Keylock et al., 2013, Taoudi et al., 2008).

Gordon-Keylock and colleagues have further shown that extra-embryonic umbilical chord and vitelline vessels contain type I pre-HSCs, despite the lack of autonomous HSC stimulatory capacity of these arteries. This appears to be caused by *Interleukin 3 (IL-3)* deficiency in these vessels, and HSCs can be induced in explants in the presence of exogenous IL-3 (Gordon-Keylock et al., 2013). This extra-embryonic generation of pre-HSCs is unlikely to be a secondary event caused by seeding through the circulation, as no pre-HSCs have yet been observed in the peripheral blood (Gordon-Keylock et al., 2013).

Taken together these results reaffirm the notion that HSCs arise from endothelial cells. However, rather than being either endothelial or haematopoietic in nature, cells undergo a gradual loss of endothelial potential while the haematopoietic molecular programme is taking over (Swiers et al., 2013, Gordon-Keylock et al., 2013, Rybtsov et al., 201, Taoudi et al., 2008)

Clinical transplantation treatments currently rely heavily on bone marrow and umbilical cord blood-derived HSCs, with an inadequate supply of suitable donors. Bone marrow HSCs are different in character, behaviour and self-renewal capacity to developmentally younger HSCs (Liu et al. 2010, Ivanovs et al., 2011, Ivanovs et al., 2014). While reliable protocols exist for purification of bone marrow and umbilical cord blood-derived HSCs, it remains more difficult to enrich for those initial HSCs that emerge in the embryo (Osawa et al., 1996, Taoudi et al., 2005, Purton and Scadden, 2007). This is most likely at

least partially due to adult HSC markers used for isolation not being expressed by developmentally younger HSCs, due to varying surface marker expression throughout development and between species and strains (McKinney-Freeman et al., 2009, Robin et al., 2011). One marker that has been proposed to be required for and expressed by murine embryonic and neonatal but not adult HSCs is Sox17 (Kim et al., 2007). In addition to their distinct expression pattern, HSCs also behave inherently differently in the embryo than in the adult, as they transit through several different anatomical niches and are actively self-renewing, making them particularly interesting for clinical applications (Morrison et al., 1995, Bowie et al., 2006). Developmentally younger HSCs in the mouse model have higher self-renewal capacity compared to adult bone marrow HSCs (Copley et al., 2013, Pawliuk et al., 1996). HSCs in the human AGM also possess very high self-renewal potential, reflected by the high level of haematopoietic repopulation of NOD scid gamma (NSD) mice transplanted with a single human AGM HSC (Ivanovs et al., 2011). This self-renewal potential of developmentally younger HSCs in the human is significantly higher than that of umbilical cord blood HSCs, indicating their clinical potential and the need for further study of the genetic pathways regulating their specification (Liu et al., 2010, Ivanovs et al., 2011).

A major limitation in the study of the role of signalling pathways required for HSC specification is the requirement for many of them in preceding arterial development. This is the case for Notch signalling: many *Notch* mutants exhibit embryonic lethality due to vascular defects (Hrabě de Angelis et al., 1997, Xue et al., 1999, Krebs et al., 2000, Krebs et al., 2004, Limbourg et al., 2005). Even in the absence of embryonic lethality, the major effects on arterial development observed in most of these mutants have precluded the direct analysis of definitive haematopoiesis. Nevertheless, some advances have been

made focussing on those *notch* pathway components that are not essential for arterial development.

1.5 On the origin of HSCs

1.5.1 Haemangioblasts generate primitive blood and endothelial cells

The population of haemangioblasts harbours the potential to give rise to haematopoietic and endothelial lineages. These cells are believed to derive from brachyury-positive (Bry⁺) mesoderm in the posterior primitive streak of the mouse and the ventral mesoderm of the zebrafish (reviewed in Xiong, 2008). This tissue is set up by transforming growth factor beta (TGF β), Wingless-related integration site (Wnt) and fibroblast growth factor (FGF) signalling in zebrafish and *Xenopus* (Mathieu et al., 2004, Schier and Talbot, 2005, Walmsley et al., 2008). Bone morphogenic protein (BMP), a member of the TGF β family, and its antagonists, play a pronounced role in mesodermal patterning, in a gradient-dependent manner in *Xenopus* (Harland and Gerhart, 1997, Ben-Zvi et al., 2008). Low BMP specifies dorsal axial mesoderm and paraxial mesoderm, while high levels are required for the lateral plate mesoderm from which blood and vascular precursors arise in the zebrafish (Neave et al., 1997). Within the lateral plate of the zebrafish, the posterior (PLM) is set up by higher levels of BMP, which reduce along a gradient to specify the heart and the anterior (ALM) regions (Lieschke et al., 2002). Shh-regulated Notch signalling regulates the proliferation of *fli1* (*fli*) and *etv2* (*etsrp*) positive haemangioblasts in the lateral plate mesoderm in fish (Sumanas et al., 2005, Jin et al., 2007, Liu et al., 2008, Sumanas et al., 2008, Ren et al., 2010, Chun et al., 2011).

Primitive blood cells arise at least partially from the population of haemangioblasts and precede the emergence of HSCs in earlier “waves” of developmental haematopoiesis. Here, transient haematopoietic populations that lack the potential to give rise to all haematopoietic lineages in the absence of an identified self-renewing stem cell are considered as primitive blood. Blood derived from a stem cell with full multi-lineage potential is here considered as definitive.

At least three distinct waves with increasing multi-lineage potential are thus considered primitive here (Figure 1-6): the first haematopoietic cells in the mouse are primitive erythrocyte progenitors; observed around embryonic day 7.5 (E7.5) in the extra-embryonic yolk sac (YS) of the mouse, and can be directly identified under the microscope by their characteristic morphology and by surface markers (Barker, 1968, Ferkowicz and Yoder, 2005). These erythrocytes arise in a belt around the YS alongside the surrounding endothelial layer from the extra-embryonic mesodermal layer rather than a true HSC, as these have not been identified at this early stage (Müller et al., 1994, Medvinsky and Dzierzak, 1996, Ferkowicz and Yoder, 2005). The zebrafish has equivalent primitive erythrocytes which arise in the intermediate cell mass (ICM) in the embryo proper (Davidson and Zon, 2004). Shortly afterwards, macrophages, megakaryocytes and mast cells are also observed in the mouse YS and the zebrafish ICM (Palis et al., 1999, Davidson and Zon, 2004). There is no evidence for HSC activity during these waves, indicating their primitive nature (Müller et al., 1994, Medvinsky and Dzierzak, 1996, Davidson and Zon, 2004). A second YS wave in the mouse (also known as transient definitive wave) generates erythro-myeloid progenitors (EMPs) that have some limited multi-lineage potential, mainly producing erythrocytes, megakaryocytes and myeloid cells (Palis et al., 1999, Bertrand et al., 2007, Yokomizo et al., 2007, Chen et

al., 2011b, McGrath et al., 2011). These cells have adult-like morphology and are generated in several different embryonic haematopoietic sites, including the yolk sac, vitelline and umbilical arteries, placenta, heart and the aorta-gonad-mesonephros (AGM) region (reviewed in Dzierzak and Speck, 2008 and Medvinsky et al., 2011). As these EMPs emerge in the absence of an adult HSC, they are considered transient and primitive in nature, although some cells of the myeloid and lymphoid lineage might persist into adulthood (Yoshimoto et al., 2011, Schulz et al., 2012, Yoshimoto et al., 2012). The majority of the haematopoietic progenitor cells that colonise the murine foetal liver (FL) at E10 migrate from the yolk sac and differentiate into the first erythro-myeloid cells circulating in the embryo (Lux et al., 2008, Chen et al., 2011b). EMPs and multipotent progenitors in the mouse express similar cell surface markers but differ in their differentiation and clonogenic potential (Bertrand et al., 2005). These progenitors colonise the blood stream of the embryo and the foetal liver, coexisting with AGM-derived haematopoietic cells and their progeny. An equivalent wave of EMP generation is observed in the posterior blood island in the zebrafish (Bertrand et al., 2007).

1.5.2 Endothelial cells are specified as arterial and venous through Notch signalling

With HSCs and haemogenic endothelium having a close developmental relationship with the vasculature, appropriate formation of the vascular system is not only necessary for embryonic survival but also for definitive haematopoiesis. The vasculature develops from angioblasts that arise from the haemangioblast population or directly from mesoderm (reviewed in Park et al., 2005).

In vitro, the blast colony-forming cell (BL-CFC) population, which is considered to be equivalent to the haemangioblast population in vivo, contains clonal haematopoietic as well as endothelial cell precursors (Kennedy et al., 1997, Choi et al., 1998). In the population of haemangioblasts in the yolk sac blood islands of the chick and mouse and the posterior lateral mesoderm of the zebrafish, expression of *Etv2* (*Etsrp*) appears to drive endothelial fate (reviewed in Xiong, 2008 and De Val and Black, 2009). In the zebrafish, *etsrp* expression is restricted to vascular endothelial lineage, and loss or reduction of *etsrp* activity causes severe deficiencies in vascular development, while forced expression of *Etsrp* or mouse *Etv2* in zebrafish leads to expansion of both hemangioblast and vascular endothelial lineages (Sumanas et al., 2005, Sumanas and Lin, 2006, Pham et al., 2007, Sumanas et al., 2008).

The first identifiable angioblasts occur in association with the endoderm in mouse and chick, a process driven by vascular endothelial growth factor (VEGF) (Flamme et al., 1995, Damert et al., 2002). These angioblasts then progress inwards towards the midline, where they form the early dorsal aorta.

The vascular system is established in the embryo through two distinct mechanisms: vasculogenesis and angiogenesis. Vasculogenesis describes the *de novo* formation of vascular vessels from mesodermal cells, mainly driven by recruitment of undifferentiated mesodermal cells to the endothelial lineage and their assembly into blood vessels. Angiogenesis is the generation of new blood vessels from endothelial cells of existing blood vessels, driven by proliferation and allowing extensive growth (reviewed in Drake, 2003). These mechanisms allow the creation of a circulatory system in the developing embryo that meets the demands of tissue oxygenation, waste removal

and nutrient delivery, immune response and temperature regulation in mammalian and avian embryos.

In the mouse, vasculogenesis initiates just after gastrulation, at E 8.5 in the embryonic mesoderm, yolk sac, head mesenchyme, allantois and placenta, generating the axial vasculature and primitive vascular plexuses. Highly motile angioblasts generate a primitive vascular network through proliferation, migration, coalescence and differentiation into endothelial cells (reviewed in Drake, 2003). These angioblast precursors arise from a subset of Brachyury-positive cells in the primitive streak and their initial formation is dependent on fibroblast growth factor (Fgf), hedgehog (HH) and vascular endothelial growth factor (Vegf) signalling (Cox and Poole, 2000, Rossant and Howard, 2002, Vokes and Krieg, 2002, Vokes et al., 2004, Coultas et al., 2005). Soon after, the Vegf receptors, *Kdr* (*Flk1/Vegfr2*) and *Flt1* (*Vegfr1*), are expressed by these cells, indicating the instructive role of Vegf signalling in the formation of the vasculature (Millauer et al., 1993, Yamaguchi et al., 1993). *Kdr* null embryos are embryonic lethal at E.9, caused by failure of vascular and haematopoietic development, similar to embryos lacking a single *VegfA* allele (Shalaby et al., 1995, Carmeliet et al., 1996, Ferrara et al., 1996, Shalaby et al., 1997). *Flt1* (*vegfr1*) null embryos die prematurely with excessive endothelial progenitors, suggesting that Flt1 may negatively regulate VegfA signalling (Fong et al., 1999). The crucial role of Vegf in early endothelial development is further extended by studies in *Xenopus*, showing that localized ectopic *Vegf* overexpression causes aberrant migration of angioblasts towards the midline, implicating Vegf in the migratory behavior of these cells (Cleaver and Krieg, 1998). Knockdown of Vegf in zebrafish similarly results in fewer angioblasts while their migratory behavior appears unaffected, suggesting redundancy in the fish or a functional divergence across species

(Jin et al., 2005). Roles for neuropilins and Tgf β signalling in vasculogenesis are beginning to emerge (Patel-Hett, 2011), and a *cis*-acting FOX:ETS enhancer motif found in many endothelial enhancers is capable of driving endothelial gene expression (De Val et al., 2008). This enhancer motif is bound by Forkhead and Ets transcription factors, and the combinatorial overexpression of *Foxc2* and *Etv2* can induce vascular expression ectopically, while their depletion severely disrupts vascular development (De Val et al., 2008)

Angioblasts in the posterior lateral plate mesoderm (PLM) give rise to the dorsal aorta, forming bilateral paired dorsal aortae in the chick and the mouse; which lumenise before they fuse at the midline (Wasteson et al., 2008, Garriock et al., 2010, Zovein et al., 2010, Chun et al., 2011). This fusion is Bmp-dependent and occurs prior to HSC emergence (Reese et al., 2004, Garriock et al., 2010, Garriock and Mikawa, 2011). In zebrafish and *Xenopus*, a single DA is formed at the midline. Medial convergence of angioblasts is regulated by *sonic-hedgehog (shh)* and *vascular endothelial growth factor (vegfa)* (Cleaver and Krieg, 1998, Gering and Patient, 2005, Wilkinson et al., 2009, Williams et al., 2010, Wilkinson et al., 2012).

Many of the signalling pathways required for endothelial development, such as *shh*, *vegfa* and *notch*, are also required for HSC specification indicating that their requirement in HSC specification might reflect their requirement in arterio-venous development. It is difficult to untangle these tightly interwoven developmental processes and the identification of signalling inputs that are required for one but not the other will help to shed light on the individual processes.

1.5.2.1 Establishing arterial identity

Mutants with a mis-specified arterial programme do not produce HSCs, suggesting that arterial over venous identity is a prerequisite for HSC specification (Adamo and García-Cardena, 2012). Initial evidence for genetic rather than physiological programming of arterial-venous identity came from the observation of markers restricted to arterial and venous endothelium, at E8.25, just prior to circulation (Wang et al., 1998). Lineage tracing revealed that endothelial cell clusters in the zebrafish lateral mesoderm are capable of contributing to the dorsal aorta or the posterior cardinal vein, but not both, supported by the observation of distinct groups of angioblasts migrating towards the midline, with those forming the DA arriving there first (Gering et al., 1998, Zhong et al., 2001, Williams et al., 2010). Additional experimental evidence reports the formation of an unspecified primitive midline vessel prior to arterio-venous segregation (Herbert et al., 2009).

Although the exact timing and location of arterio-venous specification remains debated, the genetic regulation of the event is less controversial: Sonic hedgehog (Shh) and Vegf signalling are fundamental for arterial specification upstream of Notch (Lawson et al., 2002) (Figure 1-3). Zebrafish embryos lacking the patterning signal Shh in the midline have an incorrectly programmed DA as indicated by loss of arterial genes, including those of the Notch pathway, and absence of circulation, with markers preferentially expressed in venous endothelium expressed at higher levels in the arteries (Lawson et al., 2002). *Vegfaa* expression in the somites is lost in these embryos and overexpression of *vegfaa* can rescue arterial development, placing Vegf signalling downstream of Shh in the genetic cascade governing arterial specification (Lawson et al., 2002). However, HH signalling can also act instructively on arterial induction by signalling through the

calcitonin receptor-like receptor (CRLR) in a Vegf-independent manner and mammalian studies found further Vegf-independent roles of HH signalling in lumenisation (Lawson et al., 2002, Vokes et al., 2004, Coultas et al., 2010, Wilkinson et al., 2012).

Notch signalling is required for arterial specification: arterial marker gene expression is lost in the absence of both Notch1 and Notch4, or Notch1 alone (Krebs et al., 2000), similar to compromised Notch activity by the loss of other Notch pathway components, CBF1/suppressor of hairless/Lag2 (Csl/Rbpjk), in mouse and zebrafish (Lawson et al., 2001, Krebs et al., 2004, Koo et al., 2005, Koo et al., 2007).

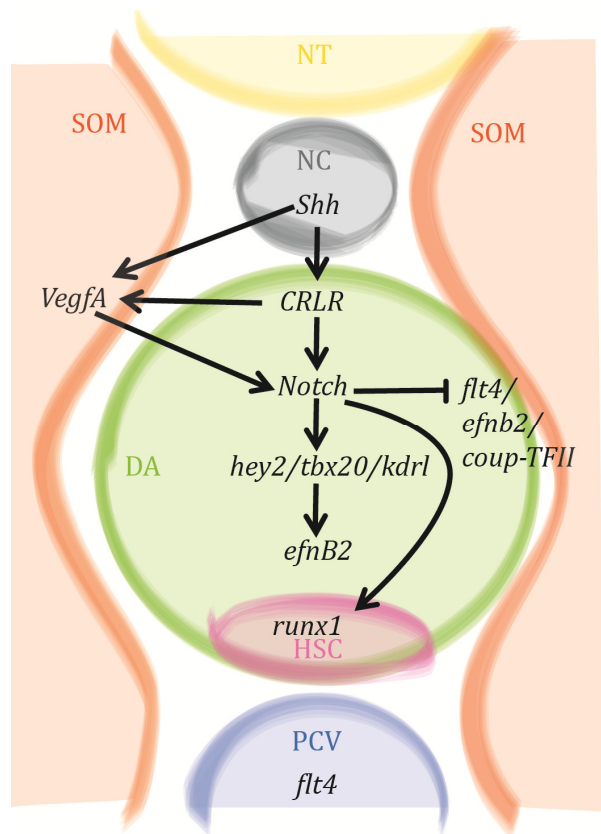


Figure 1-3. Schematic representation of the major pathways establishing arterial identity.

Shh signalling from the NC is required for *VegfA* and *CRLR* expression. These induce *notch* expression in the DA which suppresses venous markers *flt4* (*vegfr3*), *ephb4* and *coup-TFII* in the DA, allowing expression of *hey2* (*gridlock*), *tbx20* (*hrt*), *kdr1* (*flk1/vegfr2*) and *efnB2*. Key: somite (SOM), neural tube (NT), notochord (NC), dorsal aorta (DA), haematopoietic stem cell (HSC), posterior cardinal vein (PCV).

The zebrafish mutant, mindbomb (*mib*), a protein essential for Notch ligand maturation, further supports the importance of Notch in arterio-venous specification (Lawson et al., 2001). Mice with reduced activity or loss of the Notch ligand *DLL4*, have severe arterial defects in the mouse (Duarte et al., 2004, Krebs et al., 2004), and conditional overexpression of *Dll4* causes ectopic expression of arterial markers in the vein, accompanied by gross enlargement of the DA and premature fusion between arteries and veins, resulting in a severely reduced circulatory loop (Trindade et al., 2008). Similar results have been found in mice lacking the *ADAM metalloprotease* and γ -secretase, and some downstream-effectors such as *Hey1* and *Hey2* (also known as *Herp1*, *Herp2*, *Hesr2*, *Hrt2* and *gridlock*), supporting the requirement of a functional Notch signalling pathway for arterio-venous patterning (Herreman et al., 1999, Zhong et al., 2001, Hartmann et al., 2002, Fischer et al., 2004, Kokubo et al., 2005).

While loss of *hey2* (*gridlock*) in zebrafish leads to prominent vascular malformations and loss of *ephb2a* expression, ectopic expression of *hey2* in venous endothelium cannot induce arterial markers in this tissue, but instead leads to loss of venous identity, suggesting that the role of Notch in arteries is the suppression of venous fate (Zhong et al., 2000, Zhong et al., 2001, Rowlinson and Gering, 2010).

Notch regulates arterial specification through arterial but not venous activation of the ligand Ephrin B2 (*Efnb2*), which mediates arteriovenous segregation through interaction with its receptor *Ephb4*, expressed in venous endothelium (Wang et al., 1998, Grego-Bessa et al., 2007). Conversely, ectopic Notch signalling activation in veins precludes *ephb4* expression (Lawson et al., 2001).

It has been proposed that venous identity is the default state of endothelium, or that this was programmed by low levels or absence of HH signals, which set up arterial fate at

high concentrations (Thurston and Yancopoulos, 2001). It has since been shown that venous identity undergoes active programming, repressing expression of the arterial markers *Notch* and *Hey2* (You et al., 2005). Ablation of venous-expressed CoupTFII results in abnormal arterial identity and its overexpression results in arterio-venous fusions (Kawasaki et al., 1999, Huppert et al., 2000, You et al., 2005). MAPK, inhibiting P13K signalling downstream of Vegf, plays a role in the maintenance of venous identity in the zebrafish (Hong et al., 2006).

Dll1-null mutants show a requirement in arterial maintenance but not specification, consistent with *Dll1* expression in the arterial endothelium at E13-15 but not at E9.5-E10.5, when the arteries are specified (Beckers et al., 1999, Robert-Moreno et al., 2005, Sørensen et al., 2009), indicating input of multiple *notch* components for various developmental processes. This appears to be the case not only across developmental processes but also across different species: *Dll4* mouse mutants fail to initiate the arterial programme (Duarte et al., 2004, Krebs et al., 2004), while the absence of *dll4* in zebrafish merely causes later angiogenic defects in the intersegmental/intersomitic vessels (ISVs) in the presence of a fully functional axial vasculature (Leslie et al., 2007, Siekmann and Lawson, 2007, Hogan et al., 2009).

1.5.2.2 Polarising the dorsal aorta

While the mouse produces haematopoietic clusters in the dorsal and ventral aspects of the DA, haematopoietic stem cells are affiliated with the floor of the dorsal aorta in all vertebrate species studied to date (Taoudi, 2007, Yokomizo and Dzierzak, 2010). Different developmental processes are taking place at roughly the same time in the floor and the roof of the DA, with haematopoietic clusters forming in the floor and

intersegmental/somitic vessels forming from the roof, strongly indicating that this vessel is patterned in a dorso-ventral way.

The somites are a major source of angioblasts in chick and mouse, and play a major role in vascularisation (Noden, 1989, Pardanaud and Dieterlen-Lièvre, 1993, Wilting et al., 1994, Ambler et al., 2001, Ema et al., 2006). Vascularisation in the chick embryo appears to arise from two sources, with the somites contributing to the roof and the sides of the DA, and the floor originating from the splanchnopleural mesoderm (Pouget et al., 2006). While somite-derived angioblasts appear to be restricted to a vascular fate, the mesoderm-derived floor is also haemogenic, indicating a chimaeric and polarised nature of the DA (reviewed in Jaffredo et al, 2013). Further investigation employing chick/quail somitic grafts showed that over time the floor of the DA becomes replaced by somitic-derived angioblasts lacking haemogenic potential, providing an explanation for the transience of aortic haematopoiesis (Pouget et al., 2006). A recent study has shown that *runx1* expression in haemogenic endothelium and the formation of haematopoietic clusters are controlled by the sub-aortic mesenchyme, which itself is not a source of haematopoietic cells but induces *runx1* expression in the endothelium and collaborates with *Notch* signalling to control haematopoietic cell specification (Richard et al., 2013). In the zebrafish, a somitic contribution has also been observed, contributing to the floor of the DA. While these cells are not haemogenic themselves, they appear to provide an important signalling for the neighbouring haemogenic cells (Nguyen et al., 2014). Thus it appears that the somitic and mesenchymal contributions to the DA are conserved across vertebrate species, however their final location and role in haematopoiesis appears divergent and further studies are necessary to assess this.

This dorso-ventral homing of somite-derived angioblasts is mediated by a variety of signalling pathways such as VEGF, FGF and TGF β in the chick, and when their activity levels are changed, somite-derived angioblasts gain haemogenic potential and the ability to integrate into the floor of the DA (reviewed in Jaffredo et al, 2013). The signals that guide these migrations are not yet fully understood although BMP and Shh appear to be implicated in the mouse and zebrafish, albeit it remains to be shown that this polarisation involves non-endothelial cells (Gering and Patient, 2005, Durand et al., 2007, Wilkinson et al., 2009, Wilkinson et al., 2012).

The angiogenic processes of sprouting, elongation, division and anastomosis follow the vasculogenic development and generate more endothelial cells and the mature vasculature (Risau, 1997). This process is highly complex and regulated by many molecular cues, including Semophorins, Ephrins, Netrins, Slits, Sprouty, Angiopoietin/Tie and Tgf β signalling (reviewed in Patel-Hett and D'Amore, 2011). The molecular mechanisms controlling angiogenic events are highly conserved between fish and other vertebrates, and the *notch* pathway takes a prominent role in angiogenesis with involvement in various angiogenic processes ranging from developmental to tumor angiogenesis (Ellertsdóttir et al., 2010, Benedito and Hellström, 2013).

Tip cells are specialised endothelial cells that are primed to respond to angiogenic stimuli and migrate towards their source enabled by Vegf-mediated repression of Notch signalling. These cells trail endothelial stalk cells with them, which in turn have high levels of Notch, and consequently limited angiogenic behavior. This intricate interplay between Vegf and Notch signalling is required for maintenance of the integrity of the sprout. The Notch activity in this process is at least partially mediated through the Dll4 ligand (Siekman and Lawson, 2007, Hogan et al., 2009).

1.5.3 Haemogenic endothelium is specified from the ventral side of the artery and gives rise to HSCs

In all vertebrate species studied to date, a close relationship between the blood and endothelial lineages is observed: haematopoietic clusters appear in association with the dorsal aorta and there is considerable overlap of many markers in endothelial and haematopoietic cells. (Dieterlen-Lièvre and Martin, 1981, Garcia-Porrero et al., 1995, Garcia-Porrero et al., 1998, Godin et al., 1999, Ciau-Uitz et al., 2000, de Bruijn et al., 2000, Gering and Patient, 2005). More recently, it has been shown that HSC emergence is preceded by a loss of endothelial potential of cells still embedded in the endothelial layer, accompanied by the initiation of the haematopoietic programme (Swiers et al., 2013). These observations support the hypothesis that HSCs arise from an endothelial progenitor, and the term “haemogenic endothelium” designates cells with endothelial appearance and HSC capacity.

The first experimental evidence for an endothelial origin of HSCs came through endothelial lineage tracing experiments in the chick embryo (Jaffredo et al., 1998, Jaffredo et al., 2000). Avian aortic cells were labelled with an acetylated low density lipoprotein coupled with Dil (ac LDL-Dil), a dye which is specifically taken up by endothelial cells. From these experiments it became apparent that haematopoietic clusters in the DA contained this dye, as did cells in the ventrally adjacent mesenchyme, indicating that both cell populations had an endothelial origin (Jaffredo et al., 1998). The hypothesis that a subset of endothelial cells of the dorsal aorta egress into the mesenchyme and ingress into the lumen to form haematopoietic clusters was further supported by long-term retroviral labelling of endothelial cells (Jaffredo et al., 2000). While the labelled cells in the DA are haematopoietic clusters, those labelled in the

underlying mesenchyme are the precursors to the para-aortic foci, haematopoietic-like cells developing within the mesenchyme (Jaffredo et al., 2000). These early observations were supported by conditional labelling studies in the mouse, showing that HSCs have previously been expressing the endothelial gene *cdh5* (*VE-cadherin*) (Zovein et al., 2008, Chen et al., 2009a).

Arguably the strongest evidence for haemogenic endothelium comes through the *in vivo* direct imaging of the process of HSCs emerging from the ventral endothelium of the dorsal aorta in zebrafish and *ex vivo* in mice (Eilken et al., 2009, Lancrin et al., 2009, Bertrand et al., 2010a, Boisset et al., 2010, Kissa and Herbomel, 2010). The process of endothelial cells becoming haematopoietic in nature has been termed the “endothelial-to-haematopoietic transition” (EHT). It involves an endothelial cell bending out, rounding up and detaching from its endothelial context, forming a free-moving haematopoietic cell, leaving little doubt of an endothelial origin for HSCs (Kissa and Herbomel, 2010). However, this endothelial origin awaits formal proof, which is likely highly challenging as serial dilution studies estimate a single functional HSC present in the murine E11.5 DA (Kumaravelu et al., 2002, Medvinsky et al., 2011). Interestingly, even though all emergence from the endothelium observed to date comes from arterial endothelium, the direction of emergence differs between species: in the mouse, HSCs emerge from clusters in the lumen of the DA where they enter circulation (Figure 1-4-A), while HSCs in the chick emerge both dorsally into the lumen of the DA and ventrally into the underlying mesenchyme where they later form the para-aortic foci (Figure 1-4-B, reviewed in Jaffredo, 2005). In the zebrafish, HSCs emerge from the DA into the underlying mesenchyme to enter circulation through the vein (Figure 1-4-C).

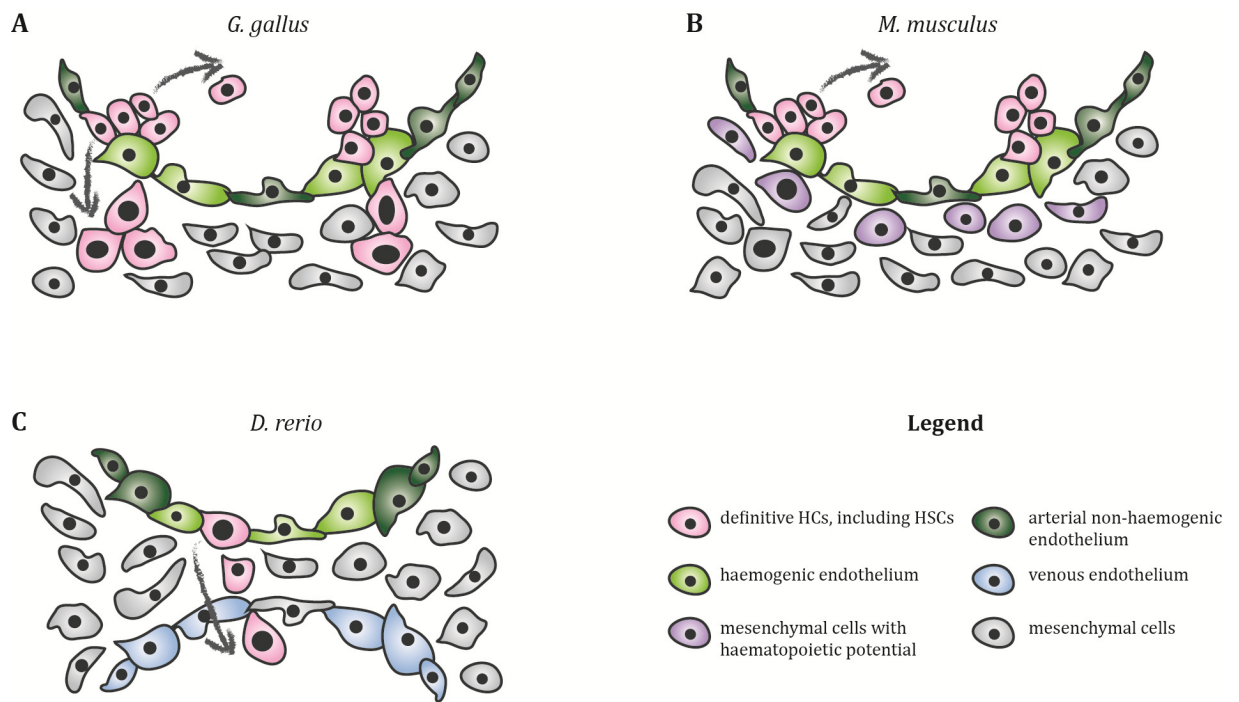


Figure 1-4. Haemogenic endothelium and HSC emergence across model species.

Schematic representation. **A** In chicken, haemogenic endothelium undergoes a phenotypic change, gradually losing endothelial and gaining haematopoietic character. Blood cells leave the endothelium in two ways: firstly into the lumen of the DA and thus into circulation, and secondly into the mesenchyme underlying the DA. Cells egressing into the mesenchyme later give rise to the para-aortic foci. **B** In the mouse, haematopoietic clusters release haematopoietic cells into the DA, while the contribution into the mesenchyme is unclear. The murine mesenchyme underlying the DA harbours mesenchymal cells with haematopoietic potential, where these derive from is still unclear. **C** In the zebrafish, no haematopoietic clusters are formed, but single cells undergo endothelial-to-haematopoietic transition from the haemogenic endothelium, egressing into the subjacent mesenchyme and entering the circulation through the posterior cardinal vein. **A** and **B** adapted from Jaffredo et al. 2005.

Despite the lack of any observation of HSC emergence from venous endothelium in wt embryos of any species, highly artificial experimental conditions have yielded HSC marker expression outside arterial endothelium (Urness et al., 2000, Sorensen et al., 2003, Burns et al., 2005, You et al., 2005, Ren et al., 2010). For example, mice with a defective *Alk1* (*Acvrl1*) or *Endoglin* (*CD150*) gene have been reported to show ectopic

haematopoiesis in vein-like vessels in the absence of expression of the arterial marker *Efnb2* (Urness et al., 2000, Sorensen et al., 2003, You et al., 2005).

Despite different ways of forming dorsal aortae across vertebrates, a lumenised aorta at the midline is a prerequisite for HSC emergence, and thus far, no unique cell surface markers have been found that allow the phenotypic identification and isolation of haemogenic endothelium. Haematopoietic progenitors have been extracted using a combination of different markers and stringencies, and this remains a source of inconsistency across studies. Although *runx1* is also expressed in other embryonic sites, an enhancer of *runx1* appears to be specific for sites of HSPC emergence and colonization, providing the single most appropriate means of HE isolation (Cai et al., 2000, Bee et al., 2010, Swiers et al., 2013). A recent study with this enhancer has further shown that the specification from the endothelial to the haematopoietic lineage occurs up to two days earlier in murine development than previously thought (Swiers et al., 2013).

It has been proposed that arterial development is likely a prerequisite for HSC gene expression, as *CouptFII* (*Nr2f2*) mouse mutants do not only ectopically express arterial markers in the vein, but also show ectopic haematopoietic cluster emergence from the veins, apparently through ectopic haemogenic endothelium (Xue et al., 1999). Although ectopic expression of the HSC marker *runx1* has been observed in venous endothelium in the absence of arterial gene expression, proof that these cells represent functional HSCs, and thus that HSCs can be specified in the absence of an artery is still outstanding (Xue et al., 1999, Burns et al., 2005).

Little is known about the genetic regulation of haemogenic endothelium, but it is an area of intense study and the mechanisms are starting to become elucidated. The

haematopoietic transcription factor Tal1 (Scl) is required for the generation of haemogenic endothelium (Porcher et al., 1996, Schlaeger et al., 2005, Lancrin et al., 2009, Van Handel et al., 2012). It is however not required once the haemogenic endothelium has formed, and acts thus upstream of *runx1* (Endoh et al., 2002, Chen et al., 2009a, Lancrin et al., 2009, Van Handel et al., 2012).

Another factor implicated in EHT is Sox17, which is expressed in the endothelium at the time of EHT, and which, when overexpressed, leads to an expansion of a cell population that has the capacity to down-regulate endothelial markers and generates haematopoietic cells, while endothelial-specific loss of Sox17 resulted in embryonic lethality (Kim et al., 2007, Clarke et al., 2013, Nakajima-Takagi et al., 2013). These studies suggest that Sox17 is suppressing the formation of haemogenic endothelium through a yet unknown mechanism, which stands in contrast to an earlier study that reported an inhibitory and apoptotic effect of increased Sox17 levels on haematopoietic progression in murine ES cell differentiation studies (Serrano et al., 2010).

The haematopoietic transcription factor *Runx1* is crucially involved and required for EHT: a subset of the arterial endothelium is highlighted by the expression of the transcription factor *Runx1*, which is part of the core-binding factor family (CBF). Here, *Runx1* expression precedes the formation of haematopoietic cells, and these cells have been interpreted as being haemogenic endothelium (reviewed in Swiers et al, 2010). Haemogenic endothelium does not require *Runx1* to form, but no haematopoietic cells emerge from *Runx1* negative haemogenic endothelium (North et al., 1999, Cai et al., 2000, Lacaud et al., 2002, Yokomizo et al., 2008, Kissa and Herbomel, 2010). Mice with an endothelial-specific ablation of *Runx1* have no HSCs, indicating that *runx1* is critically

required in this cell population (Chen et al., 2009a), and *runx1* deficient zebrafish initiate EHT but the cells die before completing the process (Kissa and Herbomel, 2010).

Runx1 appears to be responsible for the down-regulation of the endothelial programme in cells of the haemogenic endothelium prior to EHT (reviewed in Swiers et al, 2010). *Runx1* induces changes in the transcriptional profile of cells just before initiation of EHT: After initiation of *Runx1*, the binding profile of the key haematopoietic transcription factors Fli1, Tal1 (Scl) and C/EBP α changes to a profile much more similar to that of haematopoietic cells rather than haemogenic endothelium, indicating that *Runx1* plays a pivotal role in the re-organisation of the transcriptional profile within cells just prior to undergoing EHT (Lichtinger et al., 2012). This study provided an important insight into the role of *runx1* just prior to EHT while the mechanism remains unknown.

Another study indicates that initiation of *Runx1* expression in the haemogenic endothelium correlates with a down-regulation of *HoxA3* in the endothelium (Iacovino et al., 2011). Moreover, re-expression of *HoxA3* in nascent haematopoietic progenitors is able to re-activate and maintain endothelial marker expression, and Sox17 has been associated with this regulatory mechanism downstream of *HoxA3* (Iacovino et al., 2011, Clarke et al., 2013)

Lancrin and co-workers have shown in embryonic stem (ES-) cell derived cultures that while the *Runx1* targets *Cmyb* and *Pu.1* (*Sfpi1*) have no effect on the arterial programme in *Runx1* null haemogenic endothelium derived from ES-cells, *Gfi1*/*Gfi1b* down-regulated the expression of endothelial markers in these cells (Lancrin et al., 2012). These cells were further forming CD41 positive round cells reminiscent of haematopoietic progeny, but were unable to form haematopoietic colonies *in vitro*, unlike CD41 positive cells isolated from comparable wt ES cultures. In the absence of

these genes, cells were still expressing CD41, however they maintained endothelial marker expression and remained adherent (Lancrin et al., 2012). This study indicates that Gfi1 and Gfi1b are the Runx1 targets that are at least partially responsible for repression of endothelial markers at the onset of EHT (Lancrin et al., 2012).

HSC activity in the DA is transient and is lost after E.12. This loss of activity has been proposed to be facilitated through the replacement of the aortic floor by somite-derived angioblasts without haemogenic potential (Pouget et al., 2006). Following this initial peak of HSC activity in the AGM of the mouse, there is evidence that the placenta becomes a major haematopoietic tissue, although evidence of autonomous HSC generation from the placenta is still lacking (Gekas et al., 2005, Rhodes et al., 2008). After their initial formation, HSCs migrate to the bone marrow via the foetal liver.

1.6 The Notch pathway in haematopoiesis

Notch signalling is intricately interwoven with development, with multiple roles throughout development of different tissues, including the haemato-vascular lineages.

The *notch* pathway is a highly conserved cell-to-cell signalling pathway, based on the single transmembrane Notch receptors, which are activated by proteolytic cleavage induced upon interaction with a Notch ligand on a neighbouring cell (Figure 1-5). This cleavage releases the intracellular domain of the Notch protein (Notch intracellular domain, NICD) which trans-locates to the nucleus of the signal-receiving cell where it associates with the DNA binding protein CSL/Rbpj and its co-activator mastermind, enabling it to activate transcription of Notch targets such as *Hairy/Enhancer of Split* (*Hes*) (reviewed in Hori et al, 2013, Kopan and Ilagan, 2009 and Fortini, 2009).

While the Notch pathway and its mode of signalling is highly conserved across species, the number of receptors and ligands is variable: *Drosophila* has a single Notch receptor, while *C. elegans* has two redundant receptors and mammals have four Notch receptors (Notch1-4) and five canonical ligands (Jagged1-2, Delta1, 3, 4). *D. rerio* also has 4 Notch receptors (Notch1a, Notch1b, Notch2, Notch3), but four members of the Jagged family (Jag1a, Jag1b, Jag2a, Jag2b) and four members of the Delta family (Dla, Dlb, Dlc, Dld, Dll4). The evolutionary divergence across species in terms of numbers and types of receptors and ligands is likely due to gene/genome duplications, followed by subsequent loss or sub-functionalisation. Each of these components of the Notch signalling pathway has a distinct spatio-temporal expression pattern throughout development, giving a good indication of the potential role they play in different tissues during embryogenesis (reviewed in Gering and Patient, 2010).

In contrast to the important role of Notch signalling in endothelial development, early yolk sac haematopoiesis appears *Notch*-independent. *Notch1* and *RBPj* mouse mutants have normal numbers of primitive erythrocytes and their progenitors (Kumano et al., 2003, Robert-Moreno et al., 2005). The second primitive wave that generates primitive myeloid cells is also normal in these mutants (Kumano et al., 2003, Robert-Moreno et al., 2005). Primitive erythrocytes and erythro-myeloid progenitors are formed normally in *notch*-defective zebrafish mindbomb mutants (Burns et al., 2005, Bertrand et al., 2010a). These data indicate that the primitive waves are independent of Notch signalling, further supported by mouse chimaeras which demonstrated that *Notch1* mutant cells contribute to yolk sac and foetal liver haematopoiesis (Hadland et al., 2004).

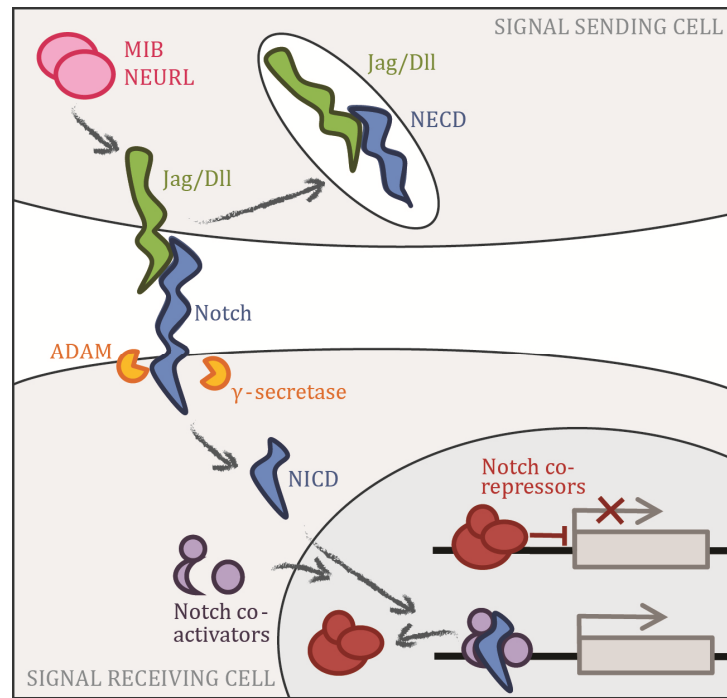


Figure 1-5. Schematic representation of Notch signalling between neighbouring cells.

Binding between Notch ligands and receptors initiated Notch signalling. A conformational change allows ADAM metalloprotease and γ -secretase to cleave the Notch receptor. Notch extracellular domain (NECD) is released from the transmembrane domain and endocytosed with the ligand by the signal sending cell. Notch intracellular domain (NICD) travels to the nucleus where it relieves target repression by association with Co-activators.

The role of Notch signalling in HSC specification has been difficult to address specifically, as this pathway is highly involved in the developmental processes immediately prior to HSC emergence, such as arterio-venous specification. Many Notch pathway mutants exhibit embryonic lethality, indicating the importance of the pathway but precluding its study in these models.

Initial evidence of a role for Notch signalling in definitive haematopoiesis was provided by the reduced ability of *Notch1*-null mouse mutants to generate repopulating long-term HSCs (LT-HSCs) in the AGM, and these cells were not capable of bone marrow colonisation (Kumano et al., 2003, Hadland et al., 2004). Unfortunately, detailed analysis

of the arterial programme was not carried out in these studies, leaving the possibility that the observed phenotype is secondary to a potential arterial deficiency. Haematopoietic specification in *mib*-deficient zebrafish embryos and *CSL*-deficient mouse P-Sp cultures can be rescued by *runx1* over-expression, indicating that these defects are specific to the haematopoietic lineage (Burns et al., 2005, Nakagawa et al., 2006).

Finally, evidence for a direct *notch* input into HSC specification was provided by *Jagged1* mouse mutants: despite embryonic lethality due to vascular remodelling defects and haemorrhaging of mice carrying a *Jag1* null mutation, these embryos show normal arterial specification, including the expression of *EphB2*, but an absence of expression of *Gata2* in the AGM, a gene required for definitive haematopoiesis in the mouse (Xue et al., 1999, Robert-Moreno et al., 2008). Despite apparently normal arterial development, *Jag1* mutants generate much less *ly6A*-GFP transgene expressing haemogenic endothelium and fewer HSCs (Robert-Moreno et al., 2008). Reduction of *Notch1* expression in the DA is concomitant with absence of HSC specification as a result of knockdown of *ETO2* activity in *Xenopus*, a process mediated via the long *VegfA* isoform (Leung et al., 2013). This requirement of *Notch1* in HSC emergence has been proposed to be through direct regulation of *Gata2* and *Hes1* in haematopoietic cells (Robert-Moreno et al., 2005, Robert-Moreno et al., 2008). These findings were supported by a recent study that has shown that manipulation of haematopoietic production in the aorta through the Notch pathway has no effect on arterial identity, as indicated by expansion of haematopoietic clusters within the dorsal aorta only (Richard et al., 2013). This study has further shown that Notch signalling decreases over time in HSCs and that this down-regulation is required for aortic haematopoiesis (Richard et al., 2013).

Further knowledge about the role of the *notch* pathway in haematopoiesis has been gained by the use of knockdown and conditional models: mice lacking the Notch1 transcriptional activation domain (TAD) show decreased Notch1 function and consequently survive longer during gestation than *Notch1* null mice, allowing the analysis of haematopoietic development (Gerhardt et al., 2014). Gerhardt and colleagues found that HSCs emerge and migrate successfully to the foetal liver in this model. However, by E14.5, these HSCs are decreased in number and are unable to compete with wt HSCs in bone marrow transplant experiments, similar to the conditional knockout of *Rbpj*, demonstrating important roles for Notch in foetal liver haematopoiesis (Gerhardt et al., 2014).

Similarly, *notch* overexpression studies have shown that *Notch* is sufficient to induce *runx1* in zebrafish, as indicated by *runx1* expression in venous cells that do not express *efnb2a* (Burns et al., 2005), although their functionality remains to be determined. Observations made in mouse showed that activation of *Notch1*, but not *Notch4*, in VE-cadherin positive cells resulted in absence of haematopoietic clusters and defects in foetal liver haematopoiesis (Tang et al., 2013).

Taken together, this evidence strongly supports an important role for Notch signalling in multiple aspects of HSC specification across models (Duncan et al., 2005, Gering and Patient, 2008, Maillard et al., 2008). The objective of this thesis is to further investigate the roles played by this pathway, employing the advantages of the zebrafish model.

1.7 The zebrafish as a model for HSC research

A dysfunctional cardio-vascular system in the developing mouse embryo generally leads to embryonic lethality. Despite being able to infer the type of cardio-vascular defect and possible underlying causes by evaluating the developmental timing of pre-natal lethality of the embryo, a detailed analysis of the molecular mechanisms is not possible in these animals due to their lethality (reviewed in Conway, 2003).

The use of cell lines to study haematopoiesis also has its limitations. At present, HSCs cannot be cultured; and while cell lines have the advantage of growing without contamination from other tissues, by definition this implies the lack of developmental signals from surrounding tissues. Considering the ontogeny of HSCs signalling inputs from surrounding cells are highly significant in establishing their fate.

The genetic regulatory mechanisms involved in haematopoietic development are highly conserved between zebrafish and other vertebrate models. Both HSC and endothelial specification are regulated by a common cascade of signalling pathways including HH, Vegf and Notch (Lawson et al., 2001, Lawson et al., 2002, Burns et al., 2005, Gering and Patient, 2005). Other conserved pathways specifically affect haematopoietic but not endothelial development, including BMP and Wnt signalling (Goessling et al., 2009, Wilkinson et al., 2009, Clements et al., 2011). These signals are upstream of the crucially required haematopoietic transcription factors *runx1* and *tal1 (scl)* (Kalev-Zylinska et al., 2002, Patterson et al., 2005, Kim et al., 2013).

Haematopoiesis occurs in analogous structures to those found in other vertebrates that provide equivalent haematopoietic niches (Figure 1-6 and reviewed in Paik and Zon, 2010 and Ciau-Uitz et al., 2014). Further, the transcriptional and functional profiles of

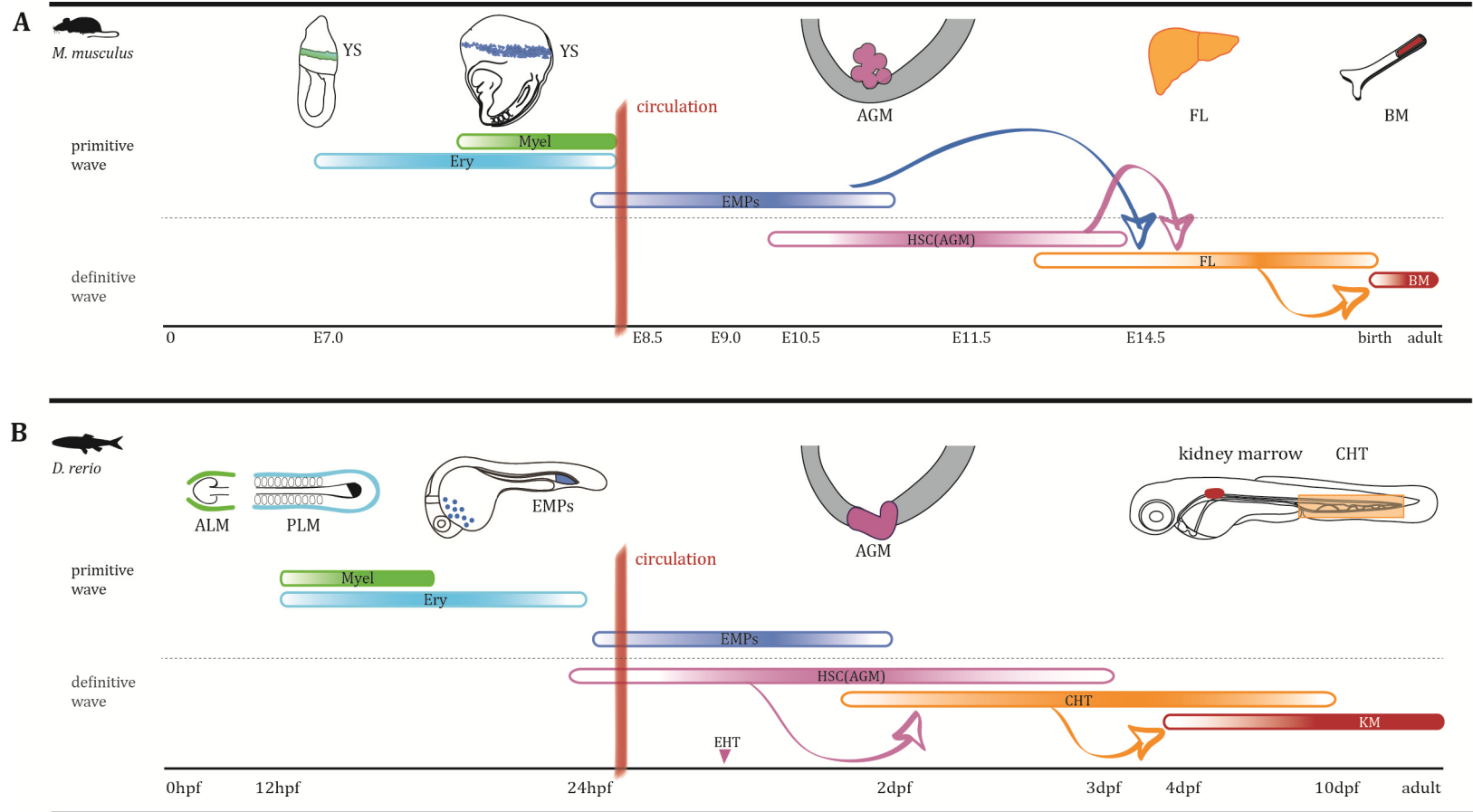


Figure 1-6. Comparative developmental haematopoiesis of mouse and zebrafish.

At least three distinctive waves of haematopoiesis are conserved between mouse (**A**) and zebrafish (**B**), the primitive and definitive waves. The first wave of primitive blood occurs in the yolk sac (YS) of the mouse (**A**) and the anterior (ALM) and posterior lateral mesoderm (PLM) in the fish (**B**), and produces primitive erythroid (light blue) and myeloid (green) cells. The second YS wave consists of a transient population of multipotent erythro-myeloid progenitors (EMPs, dark blue), and at least in the mouse, these cells seed the foetal liver where they give rise to erythroid and myeloid progeny (**A**). The emergence of HSCs from the floor of the DA through the process of endothelial-to-haematopoietic transition (EHT) marks the beginning of the definitive wave in both species. HSCs enter circulation in both organisms and seed the major site of embryonic haematopoiesis, the foetal liver (FL) and the equivalent tissue in zebrafish (caudal haematopoietic tissue, CHT) (orange). HSCs eventually seed the bone marrow in the mouse and the kidney in the zebrafish, which form the sites of adult haematopoiesis (purple). Bars represent developmental timing of haematopoietic activity with the colour-gradient indicating activity peaks. Arrows represent migration and seeding of haematopoietic sites. Embryonic staging is represented as the x axis, with the staging being represented as days postcoitum (E) for mouse and hours/days post fertilisation for zebrafish. Adapted from Cia-Uitz et al., 2014 .

the individual cellular lineages that make up the haematopoietic system are also conserved between zebrafish and other vertebrates: zebrafish erythrocytes remain nucleated but express globin genes as found in mammalian erythrocytes and are likely to fulfil similar functions (Ganis et al., 2012); CD41-expressing platelets (thrombocytes) are also present in zebrafish and have blood-clotting properties like their mammalian counterparts (Jagadeeswaran et al., 1999, Lin et al., 2005). The zebrafish innate immune system is comprised of granulocytes, macrophages and neutrophils, exhibiting anti-inflammatory and phagocytic activity, indicating a functionally conserved myeloid lineage that allows exploitation as a model for infectious disease (Herbomel et al., 1999, Bennett et al., 2001, Lieschke et al., 2001, Davis et al., 2002). The adaptive immune system in the zebrafish equally comprises all lineages represented in other vertebrates: T- and B- lymphocytes, Natural Killer (NK) cells and dendritic cells have been reported

in the zebrafish although the site of maturation for all but T-lymphocytes remains controversial (Willett et al., 1997, Willett et al., 1999, Yoder et al., 2001, Danilova and Steiner, 2002, Yoder et al., 2004, Lugo-Villarino et al., 2010, Page et al., 2013).

The zebrafish is an attractive developmental model, due to its rapid *ex utero* development, and genetic tractability (reviewed in Carroll and North, 2014). Its visual clarity allows real time *in vivo* observation of developmental processes, and morpholino-mediated knockdown approaches allow the rapid and highly specific functional assessment of candidate genes. New technologies enabling targeted mutagenesis and chemical screening are emerging and promise a widened repertoire for functional *in vivo* assessment.

1.8 Zebrafish haematopoiesis

The zebrafish embryo starts undergoing patterning processes crucial for HSC generation as early as 5 hours post fertilization (hpf), just prior to gastrulation (reviewed in Paik and Zon, 2010). Induced by Nodal and further specified by BMP, the ventral-most mesendoderm takes on haemato-vascular fates while the dorsal mesendoderm develops myeloid fate (Kimmel et al., 1990, Warga and Nüsslein-Volhard, 1999, Lieschke et al., 2002). Specification of the lateral mesoderm into vascular, haematopoietic and pronephric duct (PND) progenitors proceeds during early somitogenesis.

Bilateral cell populations of the ALM and PLM co-express blood and endothelial genes and give rise to both blood and endothelial lineages. These cells are considered to be haemangioblasts and begin co-expressing the transcription factors *tal1 (scl)*, *lmo2*, *gata2*, *fli1* and *etsrp (etv2)* from 10.5 hpf (3 ss), indicative of their haematopoietic and

endothelial potential (Stainier et al., 1995, Choi et al., 1998, Vogeli et al., 2006). Haemangioblasts located in the ALM begin expressing *spi1b (pu1)* or *kdrl (flk1/vegfr2)* from 10ss (15 hpf), indicating their myeloid or endothelial potential. Those haemangioblasts in the PLM give rise to *gata1a*-expressing erythroid and *spi1b (pu1)*-expressing myeloid progenitors as well as endothelial cells (Lieschke et al., 2002). These progenitors start migrating towards the midline shortly afterwards (12ss, 15.5 hpf), starting with the anterior-most population (Brown et al., 2000, Patterson et al., 2005). *Kdrl* positive angioblasts reach the midline first, at 15 ss (16 hpf), where they eventually form the dorsal aorta, closely followed by the primitive erythroid progenitors and angioblasts that will form the posterior cardinal vein, reaching the midline by 18 ss (17 hpf) (Gering and Patient, 2005). Primitive erythroid progenitor cells form the intermediate cell mass (ICM), an arrangement that is functionally analogous to the yolk sac blood islands in the mouse (Detrich et al., 1995). At this time, neutrophils and thrombocytes are also produced, their cellular origins and lineage relationships to the primitive erythrocytes and macrophages however are still unresolved (Le Guyader et al., 2008, Warga et al., 2009).

Angioblasts derived from the most posterior PLM migrate to the midline to form the posterior blood island (PBI), where the transient erythro-myeloid progenitors (EMPs) begin appearing independently of HSCs at around 24 hpf (Bertrand et al., 2007). These cells are analogous to the transient yolk sac-derived erythro-myeloid progenitors found in the mouse and represent a transient population with some, albeit limited, multi-lineage potential (Bertrand et al., 2007, Bertrand et al., 2010b). The PBI develops into the caudal haematopoietic tissue (CHT) around 48 hpf, comprising the tissue equivalent to the murine foetal liver, and thus it is difficult to assess the extent of migration and

seeding of the CHT by EMPs (Murayama et al., 2006, Jin et al., 2007). While these EMPs can give rise to erythroid and myeloid progeny *in vitro*, there is no evidence yet for their *in vivo* contribution to either kidney or thymus, supporting their classification as haematopoietic progenitors rather than HSCs (Bertrand et al., 2007).

At 22 hpf the DA begins to lumenise and polarises from 23 hpf, resulting in cells in the roof of the vessel being functionally and transcriptionally distinct from those in the floor, further indicated by the onset of *runx1* expression in the ventral DA and the initiation of angiogenic sprouting from the roof (Isogai et al., 2003, Wilkinson et al., 2009). The posterior cardinal vein (PCV) lumenises at 25 hpf, just prior to the onset of circulation at 26 hpf, when the first slowly moving primitive erythroid progenitors can be seen circulating through the major axial vessels. Figure 1-7 shows marker genes expressed in these vessels used for assessment of their respective programming in Chapters 4 to 7. Some of the primitive myeloid cells also enter circulation while others migrate through the tissues and differentiate in primitive macrophages and later neutrophils (Herbomel et al., 1999, Herbomel et al., 2001, Lieschke et al., 2002, Le Guyader et al., 2008). At the same time, the first angiogenic events are taking place: endothelial tip cells sprout from the roof of the DA and migrate dorsally along the somite boundaries, trailing stalk cells behind them to form the primary intersomitic vessels (ISVs). The tip cells reach their most dorsal position at 32 hpf where they branch and connect with their anterior and posterior neighbours to form the dorsal longitudinal anastomotic vessel (DLAV) (Isogai et al., 2003). From 32 hpf, the second wave of angiogenesis initiates from the roof of the axial vein, generating sprouts that either connect to the primary ISVs or contribute to the lymphatic vasculature (Isogai et al., 2003, Yaniv et al., 2006, Geudens et al., 2010).

The definitive wave of haematopoiesis initiates with the expression of *runx1* in cells of the ventral dorsal aorta from 23 hpf, which is dependent on Notch and Shh signalling (Figure 1-7)(Kalev-Zylinska et al., 2002, Burns et al., 2005, Gering and Patient, 2005, Zhang and Rodaway, 2007, Wilkinson et al., 2009). At least some of these *runx1* positive cells undergo endothelial-to-haematopoietic transition (EHT) from 30 hpf onwards (arrowhead in Figure 1-7), a process that initiates but is not completed in the absence of *runx1* (Bertrand et al., 2010a, Kissa and Herbomel, 2010). The maintenance of the definitive haematopoietic programme is reliant on circulation exerting haemo-dynamic forces, as is the maturation of the DA (Adamo et al., 2009, North et al., 2009, Wang et al., 2011).

Lineage tracing in the zebrafish has shown that HSCs migrate to secondary transient haematopoietic niches before colonization of the adult site of haematopoiesis, the kidney marrow. After EHT, HSCs enter circulation through the vein and subsequently colonise the caudal haematopoietic tissue (CHT, Figure 1-7), the functional analogue of the murine foetal liver, where they expand and undergo limited lineage commitment (Murayama et al., 2006, Bertrand et al., 2008, Kissa et al., 2008). The homing process to the CHT is mediated by a fine balance of Cxcr4 and Cxcl12 (Sdf1) signalling, which is in turn controlled by *cmyb* (Zhang et al., 2011).

The first HSCs in the CHT can be identified by their expression of HSC-associated markers such as *runx1*, *ikzf1* (*ikaros*), *cmyb* and *itga2b* (*CD41*) by 48 hpf (Figure 1-7). As lineage commitment begins when these cells arrive in the CHT, none of these markers are specific to the HSCs, but are also expressed in the myeloid / erythroid fated lineages. The transgenic line Tg(*itga2b:gfp*), however can be used to identify HSCs by the

intensity of transgene expression, with low levels of gfp present in HSCs and high levels in HSC-derived thrombocytes (Lin et al., 2005, Bertrand et al., 2008).

In the CHT, differentiation into erythroid and myeloid but not lymphoid progenitors takes place (Murayama et al., 2006, Monteiro et al., 2011). By 4 dpf, definitive

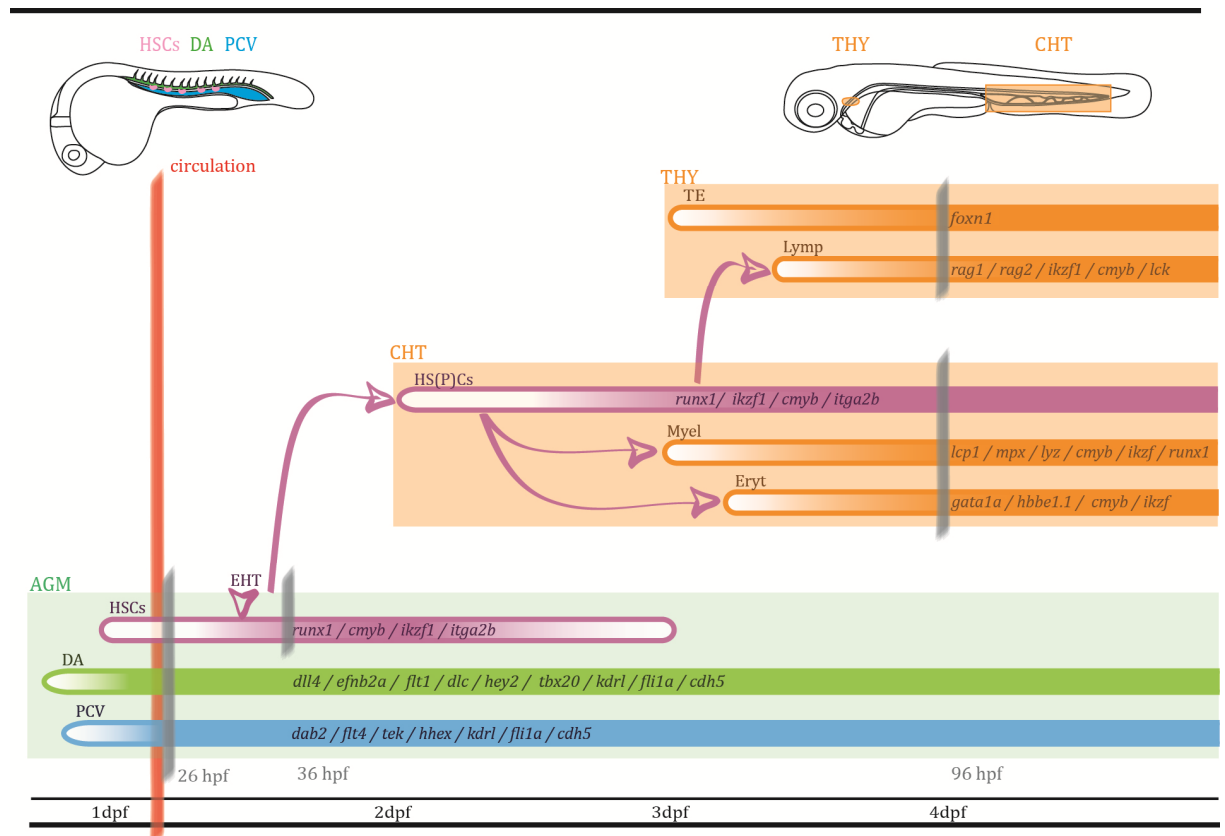


Figure 1-7. Tissues and markers of zebrafish definitive haematopoiesis.

Schematic representation of the tissues and markers of definitive haematopoiesis in the zebrafish assessed in this thesis. Tissues are indicated by boxes, cell types within these tissues by horizontal coloured bars. Markers used to assess the programming of the respective cell types are indicated. Developmental time is indicated on the x axis. Grey vertical bars indicate time points assessed, intersecting with the tissues assessed at the given time point. Arrows indicate cell migrations. Key: thymus (THY), thymic epithelium (TE), lymphocytes (Lymph), haematopoietic stem (progenitor) cells (HS(P)Cs), myeloid cells (Myel), erythroid cells (Eryt), aorta-gonad-mesonephros equivalent (AGM), haematopoietic stem cells (HSCs), dorsal aorta (DA), posterior cardinal vein (PCV), endothelial-to-haematopoietic transition (EHT).

erythrocytes are present in the CHT and express markers such as *gata1a* and *hbbe1.1* (*βe1 globin*) (Figure 1-7), which are no longer expressed by the still circulating primitive erythrocytes. From 5 dpf, these definitive erythrocytes will enter the circulation and slowly replace primitive cells; however, when this process is complete is still unclear (Bertrand and Traver, 2009). From 3 dpf, the first definitive myeloid cells can be detected employing markers such as *lyz* (*lysozyme c*), *mpx* (*mpo*) and *lcp1* (*l plastin*) (Figure 1-7), however, at this time primitive myeloid cells are also still present in the embryo. This is no longer the case at 4 dpf, when definitive myeloid cells have replaced the vast majority of primitive cells expressing these markers in the embryo (Bennett et al., 2001, Berman et al., 2005). *Mpx* (*mpo*) expression marks functional definitive granulocytes, and together with the absence of *spi1b* (*pu1*) expression, indicates the transition from early myeloid precursor cells to more mature myeloid-restricted cells (Bennett et al., 2001, Lieschke et al., 2001, Berman et al., 2005). *Lcp1* (*l plastin*) and *lyz* (*lysozyme c*) mark amoeboid-like monocytes and macrophages that phagocytose erythroblasts and bacteria, similar to mammalian macrophages (Herbomel et al., 1999, Bennett et al., 2001, Liu and Wen, 2002). Definitive thrombocytes are observed in the CHT from 3 dpf (Lin et al., 2005).

From the CHT, *ikzf1* (*ikaros*) positive HSCs and progenitors seed the thymus from 3.5 dpf onwards, where they begin to mature into T-lymphocytes from 4 dpf (Willett et al., 1999, Murayama et al., 2006, Kissa et al., 2008). The kidney marrow is colonised by haematopoietic stem (progenitor) cells (HS(P)Cs) from 5 dpf onwards, where they remain throughout the lifetime of the fish (Murayama et al., 2006, Kissa et al., 2008). There is evidence suggesting that a small but significant population of HSCs can seed the kidney marrow without going through the CHT (Bertrand et al., 2008).

1.9 Overview and objectives

The difficulties in the generation of functional HSCs *de novo* from ES and iPS cells, or through reprogramming, reflects the holes in our understanding of the processes that govern HSC specification (Ledran et al., 2008, Szabo et al., 2010, Choi et al., 2012, Pereira et al., 2013). This suggests that crucial developmental inputs are not represented in these systems, highlighting the importance of investigating the genetic, cellular, and environmental factors governing these processes.

The characterisation of the genetic regulatory networks required for HSC specification in the embryo is of crucial importance to further our understanding of HSC biology, and enable *in vitro* generation of functional HSCs for clinical treatment of disorders of the blood system, such as leukaemia. The Notch pathway is critical in establishing many embryonic tissues and is known to play a vital role in HSC specification (Gering and Patient, 2010, Bigas and Espinosa, 2012). However, it has been difficult to assess the role of this pathway in HSC specification, due to its requirement in arterial and vascular specification. As the first haematopoietic stem cells arise in the embryo proper from aortic haemogenic endothelium, its appropriate specification is essential for HSC emergence. Therefore, it is not possible to investigate the role of the Notch pathway in HSC specification in mouse mutation models that show a prior requirement of Notch in arterial specification, often resulting in embryonic lethality (Hrabě de Angelis et al., 1997). While progress has been made in the investigation of the role of Notch receptors and the Jagged family of ligands in haematopoiesis, the Delta family of Notch ligands has not been investigated in this context, due to major arterial defects in the mouse models used (Duarte et al., 2004, Krebs et al., 2004).

The main objective of this thesis is to further the understanding of the role of the Notch pathway in HSC specification, with specific focus on its ligands, by identifying and studying the most promising of these. Through functional assessment and identification of target genes, the elucidation of the genetic regulatory network downstream of Notch signalling is the main objective in this thesis.

Chapter 2

Materials and Methods

2.1 Genes and their nomenclature

Gene names and symbols used in this thesis are current as of 2014, and were obtained from either ENSEMBL (Flicek et al., 2014) or zfin (Bradford et al., 2011) databases. The zebrafish community is aiming for all zebrafish genes to reflect their mammalian orthologues whenever possible and thus many genes have recently changed names. For convenience and reference all current gene names used in this thesis have been listed in Table 2-2 with their unique and consistent ENSEMBL IDs and their previous names. Where new nomenclature may be misleading in the main text, previous gene names have been added in parentheses.

In this thesis, all zebrafish genes and their products are formatted according to the conventions agreed on by the Zebrafish Nomenclature Committee (based on the genetic nomenclature guide (Mullins, 1995, Bae et al., 2000)). In short, the same name and abbreviation as the mammalian orthologue is used for established zebrafish orthologous genes and transcripts, with all letters lower case and italicised. Protein symbols are non-italic, with the first letter in upper case.

Similarly, nomenclature follows the respective genetic nomenclature guides for genes and proteins of other species (Crittenden et al., 1995, McAlpine, 1995, Eppig, 2006, Bowes et al., 2008). An example of comparative nomenclature, is given in Table 2-1.

Table 2-1. Comparative nomenclature across model organisms.

species	gene notation (example)	protein notation (example)	convention / source
<i>Homo sapiens</i>	<i>DLL4</i>	DLL4	(McAlpine, 1995)
<i>Mus musculus</i>	<i>Dll4</i>	DLL4	(Eppig, 2006)
<i>Gallus gallus</i>	<i>DLL4</i>	DLL4	(Crittenden et al., 1995)
<i>Xenopus spp.</i>	<i>dll4</i>	Dll4	(Bowes et al., 2008)
<i>Danio rerio</i>	<i>dll4</i>	Dll4	(Mullins, 1995)

Table 2-2. Zebrafish genes referred to in this thesis and their symbols, IDs and previous names.

All gene symbols, names and previous names are with reference to zfin (Bradford et al., 2011) and gene IDs are from the ENSEMBL database (Flicek et al., 2014). All information in this table is recent as of 2014. Genes screened for their expression patterns in Chapter 4 are not included in this list, for ENSEMBL numbers of these genes refer to Appendix Table 8-4 (p. 252).

gene symbol	ENSEMBL ID	gene name	previous names
<i>actb1</i> (<i>βactin1</i>)	ENSDARG00000037746	<i>actin, beta 1</i>	<i>actine, B-ACTZF, bact, bactin1, wu:fd18f05</i>
<i>actb2</i> (<i>βactin2</i>)	ENSDARG00000037870	<i>actin, beta 2</i>	<i>bact2, bactin2, fa56g06, fb38a10, wu:fa56g06, wu:fb38a10</i>
<i>alas2</i>	ENSDARG00000038643	<i>aminolevulinate, delta-, synthetase 2</i>	<i>alas-e, sau, cb1063, sauternes, si:dkey-24f17. 4, si:dkey-24f17none</i>
<i>b2m</i>	ENSDARG00000053136	<i>beta-2-microglobulin</i>	<i>β2mic, bwm, wu:fa94c05, wu:fb10a09</i>
<i>baxa</i>	ENSDARG00000020623	<i>bcl2-associated X protein, a</i>	<i>bax, zBax1, fj16e01, wu:fc50b10, wu:fj16e01</i>
<i>ccnd1</i> (<i>cyclinD1</i>)	ENSDARG00000035750	<i>cyclin D1</i>	<i>cb161, cycd1, etID37810. 7, fb52e01, fc45c08, fc83a12, id:ibd1198, wu:fb52e01, wu:fc45c08, wu:fc83a12</i>
<i>cdh5</i> (<i>VE-cadherin</i>)	ENSDARG00000075549	<i>cadherin 5</i>	<i>cadherin 5, type 2, VE-cadherin (vascular epithelium), fc88a07, vascular endothelial cadherin, vecdn, wu:fc88a07</i>
<i>cdkn1a (p21)</i>	ENSDARG00000076554	<i>Cyclin-dependent kinase inhibitor 1A</i>	<i>cip1, p21, waf1, si:ch1073-48m5. 2</i>
<i>cebpz</i>	ENSDARG00000045857	<i>CCAAT/enhancer binding protein (C/EBP), zeta</i>	<i>wu:fi26d06, zgc:112104</i>
<i>cldn5b</i>	ENSDARG00000014059	<i>claudin 5b</i>	<i>zgc:103419</i>
<i>cmyb</i>	ENSDARG00000053666	<i>transcription factor cmyb</i>	<i>c-myb</i>
<i>col2a1a</i>	ENSDARG00000069093	<i>collagen type II, alpha-1a</i>	<i>col2a1, coll2a1, fc10c01, fb38c06, wu:fb38c06, wu:fc10c01</i>
<i>cxcl12a</i> (<i>sdf1a</i>)	ENSDARG00000037116	<i>chemokine (C-X-C motif) ligand 12a (stromal cell-derived factor 1)</i>	<i>cxcl12, SDF-1, sdf-1a, sdf1a, unm t30516, unm_t30516, wu:fa55e10, wu:fc16h12, wu:fj84c02</i>
<i>cxcr4a</i>	ENSDARG00000057633	<i>chemokine (C-X-C motif) receptor 4a</i>	<i>cb824</i>
<i>dab2</i>	ENSDARG00000031761	<i>disabled homolog 2 (Drosophila)</i>	<i>sb:cb778</i>
<i>dla</i>	ENSDARG00000010791	<i>deltaA</i>	<i>cb335, delta A, fa04c10, wu:fa04c10</i>
<i>dlb</i>	ENSDARG00000004232	<i>deltaB</i>	<i>deltab, etID309872. 5, sc:d0400</i>
<i>dlc</i>	ENSDARG00000002336	<i>deltaC</i>	<i>bea, beamter</i>
<i>dld</i>	ENSDARG00000020219	<i>deltaD</i>	<i>aei, after eight, sc:d0402</i>
<i>dll4</i>	ENSDARG00000070425	<i>delta-like 4 (Drosophila)</i>	<i>si:ch211-244p18. 5, zgc:136596</i>
<i>eef1a1l1</i> (<i>ef1a</i>)	ENSDARG00000020850	<i>eukaryotic translation elongation factor 1 alpha 1, like 1</i>	<i>ef1a, ef1 alpha, ef1a, EFL1-alpha, chunp6927, ik:tdsubc_2a3, ik:tdsubc_2b3, tdsbuc_2a3, wu:fa91c07, wu:fa94b03, wu:fi13b09, xx:tdsubc_2a3, xx:tdsubc_2b3</i>
<i>efnb2a</i>	ENSDARG00000020164	<i>ephrin B2a</i>	<i>efnb2, ephrin-B2a, id:ibd5072</i>
<i>ephb4a</i>	ENSDARG00000040346	<i>eph receptor B4a</i>	<i>cb646, ephB4, ephb4a, rtk5</i>

gene symbol	ENSEMBL ID	gene name	previous names
<i>etv2 (etsrp)</i>	ENSDARG00000053868	<i>ets</i> variant 2	<i>er71</i> (1), <i>ets1b</i> , <i>etsrp</i> (2), <i>ETS1</i> -related protein (1), <i>v-ets</i> erythroblastosis virus E26 oncogene homolog 1b, <i>wu:fc49b07</i> , <i>zgc:153819</i>
<i>figf (vegfd)</i>	ENSDARG00000056624	<i>c-fos</i> induced growth factor	<i>vegfd</i> , <i>vegfd</i> , <i>vegfd</i>
<i>fli1a</i>	ENSDARG00000054632	<i>friend leukemia integration 1a</i>	<i>fli</i> , <i>fli-1</i> , <i>fli1</i> , <i>cb855</i> , <i>wu:fc45b11</i>
<i>flt1 (vegfr1)</i>	ENSDARG00000019371	<i>fms</i> -related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	<i>vegfr1</i> , <i>VEGFR1</i>
<i>flt4 (vegfr3)</i>	ENSDARG00000015717	<i>fms</i> -related tyrosine kinase 4	<i>vegfr3</i> , <i>cb1059</i> , <i>expando</i> , <i>VEGFR3</i>
<i>fn1a</i>	ENSDARG00000019815	<i>fibronectin 1a</i>	<i>Fn</i> , <i>fn1</i> , <i>FN1</i> , <i>fn1l</i> , <i>FN2</i> , <i>nat</i> , <i>fb80d10</i> , <i>natter</i> , <i>wu:fb80d10</i>
<i>foxn1</i>	ENSDARG00000011879	<i>forkhead box N1</i>	<i>whnb</i> , <i>nude</i>
<i>foxc1b</i>	ENSDARG00000055398	<i>forkhead box C1b</i>	<i>id:ibd5079</i>
<i>foxd1</i>	ENSDARG00000029179	<i>forkhead box D1</i>	<i>foxd1. 1</i> , <i>foxd1l</i> , <i>zgc:76875</i>
<i>foxo4</i>	ENSDARG00000055792	<i>forkhead box O4</i>	n/a
<i>g6pd</i>	ENSDARG00000071065	<i>glucose-6-phosphate dehydrogenase</i>	<i>fj78b06</i> , <i>si:dkey-90a13. 8</i> , <i>wu:fj78b06</i>
<i>gapdh</i>	ENSDARG00000043457	<i>glyceraldehyde-3-phosphate dehydrogenase</i>	<i>gapd</i> , <i>bb02e05</i> , <i>cb609</i> , <i>mg:bb02e05</i> , <i>wu:fb33a10</i> , <i>wu:ft80f05</i>
<i>gata1a</i>	ENSDARG00000013477	<i>GATA</i> binding protein 1a	<i>gata1</i> , <i>gta1</i> , <i>vlt</i> , <i>zg1</i> , <i>etID309774. 11</i> , <i>vlad tepes</i>
<i>gfi1aa</i>	ENSDARG00000020746	<i>growth factor independent 1aa</i>	<i>gfi1. 1</i> , <i>gfi1a</i> , <i>zgc:113277</i>
<i>gfi1ab</i>	ENSDARG00000044457	<i>growth factor independent 1ab</i>	<i>gfi1</i> , <i>gfi1. 2</i> , <i>gfi1b</i>
<i>gnb2l1</i>	ENSDARG00000041619	<i>guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1</i>	<i>rack1</i> , <i>wu:fb80d08</i> , <i>wu:fk65d12</i>
<i>hbbe1.1 (βe1 globin)</i>	ENSDARG00000089963	<i>hemoglobin beta embryonic-1. 1</i>	<i>βe1 globin</i> , <i>hbbe1</i> , <i>beta e1 globin</i> , <i>betae1globin</i> , <i>si:by119c3. 2</i> , <i>zgc:109871</i>
<i>her8a</i>	ENSDARG00000016363	<i>hairy-related 8a</i>	<i>her8b</i> , <i>wu:fb75f05</i> , <i>wu:fc06c04</i> , <i>zgc:85712</i>
<i>hes6</i>	ENSDARG00000019335	<i>hairy and enhancer of split 6 (Drosophila)</i>	<i>her13. 2</i>
<i>hey2 (gridlock)</i>	ENSDARG00000013441	<i>hairy/enhancer-of-split related with YRPW motif 2</i>	<i>gridlock</i> , <i>grl</i> , <i>herp2</i> , <i>zgc:136746</i>
<i>hhex</i>		<i>hematopoietically expressed homeobox</i>	<i>hex</i>
<i>hif1ab</i>	ENSDARG00000034293	<i>hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) b</i>	<i>hif1a</i> , <i>HIF1B</i> , <i>wu:fb63a10</i> , <i>zgc:55405</i>
<i>hppt1</i>	ENSDARG00000008884	<i>hypoxanthine phosphoribosyltransferase 1</i>	<i>hppt</i> , <i>id:ibd1344</i> , <i>id:ibd5108</i> , <i>wu:fc10g09</i> , <i>zgc:56221</i> , <i>zgc:86608</i>
<i>ikzf1 (ikaros)</i>	ENSDARG00000013539	<i>IKAROS</i> family zinc finger 1 (<i>Ikaros</i>)	<i>ikaros</i> , <i>znfn1a1</i>
<i>itga2b</i>	ENSDARG00000018687	<i>integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41B)</i>	<i>GPIIb</i> , <i>CD41</i> , <i>si:dkey-27b3. 6</i>
<i>jag1a</i>	ENSDARG00000030289	<i>jagged 1a</i>	<i>jag1</i> , <i>jagged1a</i> , <i>cb243</i> , <i>sb:cb243</i>
<i>jag1b</i>	ENSDARG00000013168	<i>jagged 1b</i>	<i>jag3</i> , <i>jagged1b</i> , <i>kmi</i> , <i>puc</i> , <i>unp1069</i>

gene symbol	ENSEMBL ID	gene name	previous names
<i>jag2a</i>	ENSDARG00000014246	<i>jagged 2a</i>	<i>zmp:0000000546</i>
<i>jag2b</i>	ENSDARG000000021389	<i>jagged 2b</i>	<i>jag2, jagged2, serB, serrateB, unp1068, zgc:152855</i>
<i>kdr1</i> (<i>flk1/vegfr2</i>)	ENSDARG000000015815	<i>kinase insert domain receptor like</i>	<i>kdr, kdra, foetal liver kinase, fk52c05, flk, flk-1, flk1, VEGFR-2, vegfr2, vegfr4, vegr2, wu:fk52c05</i>
<i>lck</i>	ENSDARG000000059282	<i>lymphocyte-specific protein tyrosine kinase</i>	<i>zgc:136695</i>
<i>lcp1</i> (<i>l plastin</i>)	ENSDARG000000088098	<i>lymphocyte cytosolic plastin 1</i>	<i>cb245, fj24b12, l plastin, L-plastin, leucocyte-specific plastin, wu:fj24b12</i>
<i>lyz</i> (<i>lysozyme c</i>)	ENSDARG000000057789	<i>lysozyme</i>	<i>lys C, lysozyme C, zgc:136734</i>
<i>mpx (mpo)</i>	ENSDARG000000019521	<i>myeloid-specific peroxidase</i>	<i>mpo, drf, fj80f04, myeloperoxidase, wu:fj80f04</i>
<i>myod1</i>	ENSDARG000000030110	<i>myogenic differentiation 1</i>	<i>myod, MyoD, Myod1, etID309723. 25, wu:fb57a01, zgc:136744</i>
<i>nfatc1</i>	ENSDARG000000036168	<i>nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1</i>	<i>si:ch211-198c22. 2</i>
<i>nkx2. 5</i>	ENSDARG000000018004	<i>NK2 transcription factor related 5</i>	<i>nk2. 5, nkx2-5, tinman homeobox homolog, zgc:111912</i>
<i>notch1a</i>	ENSDARG000000044841	<i>notch homolog 1a</i>	<i>n1a, notch, deadly seven, des, wu:fc01e02, wu:fc37a05</i>
<i>notch1b</i>	ENSDARG000000052094	<i>notch homolog 1b</i>	<i>etID309871. 5, wu:fc55e11</i>
<i>notch2</i>	ENSDARG000000043130	<i>notch homolog 2</i>	<i>notch6, sb:cb884</i>
<i>notch3</i>	ENSDARG000000052139	<i>notch homolog 3</i>	<i>notch5, fa14b08, wu:fa14b08</i>
<i>pax2a</i>	ENSDARG000000028148	<i>paired box gene 2a</i>	<i>Pax-2, pax-b, Pax2. 1, pax2. 1, pax2a1, pax[zf-b], paxb, cb378, no isthmus, noi</i>
<i>pelo</i>	ENSDARG000000055477	<i>wu:fa12c12, wu:fb97f07, zgc:56550</i>	<i>pelota homolog (Drosophila)</i>
<i>polr2d</i>	ENSDARG000000076509	<i>polymerase (RNA) II (DNA directed) polypeptide D</i>	<i>rnap, wu:fa95b11, zgc:110435</i>
<i>ppiab</i>	ENSDARG000000042247	<i>peptidylprolyl isomerase Ab (cyclophilin A)</i>	<i>ppial, cyclophilin, wu:fj18g05, zgc:73102, zgc:86688</i>
<i>rag1</i>	ENSDARG000000052122	<i>recombination activating gene 1</i>	<i>etID309719. 25, recombinase</i>
<i>rag2</i>	ENSDARG000000052121	<i>recombination activating gene 2</i>	<i>zgc:136743</i>
<i>rpl13a</i>	ENSDARG000000044093	<i>ribosomal protein L13a</i>	<i>fd05d08, hm:zehp0384, wu:fa95e06, wu:fb08g07, wu:fd05d08</i>
<i>rplp0</i>	ENSDARG000000051783	<i>ribosomal protein, large, P0</i>	<i>arbp, arp, fa56b12, wu:fa56b12, wu:fb04a12, wu:fb15a08, wu:fk48c12</i>
<i>rspo</i>	cb1071 (1), fj81e05, wu:fj81e05, zgc:92153	<i>r-spondin homolog (Xenopus laevis)</i>	<i>cb1071, fj81e05, wu:fj81e05, zgc:92153</i>
<i>runx1</i>	ENSDARG000000025921	<i>runt-related transcription factor 1</i>	<i>aml1, runx1a, runxa</i>
<i>sdha</i>	ENSDARG000000016721	<i>succinate dehydrogenase complex, subunit A, flavoprotein (Fp)</i>	<i>im:7141001, zgc:56051</i>
<i>shha</i>	ENSDARG000000068567	<i>sonic hedgehog a</i>	<i>shh, syu, vhh1, fc83d08, sonic you, sonic-you, vertebrate hedgehog homolog, wu:fc83d08</i>
<i>spi1b (pu1)</i>	ENSDARG000000000767	<i>spleen focus forming virus (SFFV) proviral integration oncogene spi1b</i>	<i>pu. 1, pu1, spi1, cb1086, si:by184l24. 2</i>

gene symbol	ENSEMBL ID	gene name	previous names
<i>ssh1a</i>	ENSDARG000000056780	<i>slingshot homolog 1a (Drosophila)</i>	<i>ssh1</i> , <i>si:ch211-108c6. 1</i>
<i>tal1 (scl)</i>	ENSDARG000000019930	<i>T-cell acute lymphocytic leukemia 1</i>	<i>scl</i> , <i>scl/tal1</i> , <i>tal-1</i> , <i>zgc:85605</i>
<i>tbp</i>	ENSDARG000000014994	<i>TATA box binding protein</i>	<i>fd20h06</i> , <i>si:zc101n13. 2</i> , <i>wu:fd20h06</i>
<i>tbx20 (hrt)</i>	ENSDARG000000005150	<i>T-box 20</i>	<i>cb826</i> , <i>ZF-TBX20</i> , <i>hrt</i> , <i>hrT</i>
<i>tek (tie2)</i>	ENSDARG000000028663	<i>TEK tyrosine kinase, endothelial</i>	<i>tie-2</i> , <i>tie2</i>
<i>tie1</i>	ENSDARG000000004105	<i>endothelium-specific receptor tyrosine kinase 1</i>	<i>tie-1</i> , <i>sb:eu718</i>
<i>tp53</i>	ENSDARG000000035559	<i>tumor protein p53</i>	<i>brp53</i> , <i>drp53</i> , <i>p53</i> , <i>etID22686. 5</i> , <i>fb40d06</i> , <i>wu:fb40d06</i> , <i>zgc:111919</i>
<i>tuba1b</i>	ENSDARG000000045367	<i>tubulin, alpha 1b</i>	<i>cb944</i> , <i>tuba1</i> , <i>fb22g06</i> , <i>wu:fb22g06</i>
<i>ubc</i>	ENSDARG000000067755	<i>ubiquitin C</i>	<i>im:7042025</i> , <i>wu:fb37h06</i> , <i>zgc:153686</i>
<i>urod</i>	ENSDARG000000006818	<i>uroporphyrinogen decarboxylase</i>	<i>wu:fc43e09</i> , <i>yqe</i> , <i>yquem</i>
<i>vegfaa</i>	ENSDARG000000045971	<i>vascular endothelial growth factor Aa</i>	<i>vegf</i> , <i>vegfa</i> , <i>etID309976. 16</i> , <i>wu:fj82c06</i>
<i>vegfc</i>	ENSDARG000000069640	<i>vascular endothelial growth factor c</i>	n/a
<i>zfat</i>	ENSDARG000000077987	<i>zinc finger and AT hook domain containing</i>	n/a

2.2 Materials

2.2.1 Solutions

All solutions prepared for this thesis were made up in MilliQ water and autoclaved as appropriate. Their composition and storage conditions as used in this thesis are detailed in Table 2-3 below.

Table 2-3. Solutions.

solution	composition	storage conditions
Agarose gel	1% Agarose, 0. 5x TEA	RT
alkaline lysis reagent	25 mM NaOH, 0. 2 mM disodium EDTA, pH 12. 0	RT
AP Buffer	Alkaline phosphatase buffer: 100 mM Tris-HCl pH 9. 5, 100 mM NaCl, 50 mM MgCl ₂ , 0. 1% Tween	prepared fresh
Bleaching solution	10% hydrogen peroxide, 5% formamide in 0. 5% SSC, 0. 1% Tween	prepared fresh

solution	composition	storage conditions
Chloroform	24x CHCl ₃ , 1x 3-Methyl-1-butanol ((CH ₃) ₂ CHCH ₂ CH ₂ OH)	RT
Collagenase/ Trypsin	trypsin, 25% 10x HBSS with 2. 5% Collagenase	prepared fresh
Developing solution	50% BM Purple (Roche), 50% AP Buffer	prepared fresh, RT
DMSO	Dimethyl sulfoxide (Sigma-Aldrich): (CH ₃) ₂ SO	RT
E3 medium (60x)	1. 75% NaCl, 0. 08% KCl, 0. 3% CaCl ₂ •2H ₂ O, 0. 5% MgCl ₂ •6H ₂ O, pH 7. 2 + methylene blue	autoclave, 4°C
EDTA	0. 5M EDTA, pH8	RT
0.1M glycine	0. 1 M glycine, pH 2. 2, 0. 1% Tween	RT
Hanks solution	1x HBSS, 0. 01M HEPES pH8. 0, 25% BSA	prepared fresh
Hanks + Hoechst	Hanks solution + 1µg/ µl Hoechst	prepared fresh
Hoechst	Hoechst 33258, Pentahydrate (bis-Benzimide) (Life Technologies)	4°C
Hybe ^{-/-}	50% Formamide, 5x SSC, 9. 2 mM Citric Acid, 0. 1% Tween	4°C
Hybe ^{+/+}	50% Formamide, 5x SSC, 9. 2 mM Citric Acid, 0. 1% Tween, 0. 05% tRNA, 0. 005% Heparin	4°C
Hybe ^{+/+} /PBStw	50% PBStw, 50% Hybe ^{+/+}	4°C
LB (Agar)	1% Bactotryptone, 0. 5% Bactoyeast, 1% NaCl, pH 7. 0 (1. 5% Bactoagar)	4°C
LB + Ampicillin	0. 1% Ampicillin in LB	4°C
gel loading dye	2. 5% Ficoll-400, 11mM EDTA, 3. 3mM Tris-HCl, 0. 017% SDS, 0. 015% bromophenol blue, pH 8. 0	RT
MAB Block	0. 1 M maleic Acid, 0. 15 M NaCl, pH 7. 5, 2% Boehringer's Blocking Reagent	-20°C
MABtw	0. 1 M maleic Acid, 0. 15 M NaCl, 0. 1% Tween, pH 7. 5	autoclave, 4°C
methylene blue	C ₁₆ H ₁₈ N ₃ SCl in solution	RT
MS222	Tricaine mesylate: 0. 4% Ethyl-3-amino benzoate methanesulfonate salt, pH 7. 0	4°C
NH ₄ Acetate	Ammonium acetate: NH ₄ C ₂ H ₃ O ₂	RT
neutralising reagent	40 mM Tris-HCl, pH 5. 0	RT
PBS	Phosphate Buffered Saline: 0. 8% NaCl, 0. 15% Na ₂ HPO ₄ , 0. 025% KH ₂ PO ₄ , 0. 02% KCl, pH 7. 2	RT
PBStw	PBS tween: 0. 8% NaCl, 0. 15% Na ₂ HPO ₄ , 0. 025% KH ₂ PO ₄ , 0. 02% KCl, pH 7. 2 + 0. 1% tween	RT
PFA	Paraformaldehyde: 4% PFA, 96% PBStw	RT
pronase (10x)	10 mg/ml Protease from <i>Streptomyces griseus</i> (Sigma-Aldrich) in PBS	-20°C
pronase (1x)	10% pronase (10x) in 90% PBStw	prepared fresh
proteinase K	20 mg/ml proteinase K (Sigma-Aldrich) in dH ₂ O	-20°C
ringer's solution	6. 5 g NaCl, 0. 42 g KCl, 0. 25 g CaCl ₂ and 1 M NaHCO ₃ in 1 l dH ₂ O	autoclave, RT
20x SSC	saline-sodium citrate buffer (20x): 3 M NaCl, 0. 3 M Na ₃ C ₆ H ₅ O ₇ , pH 7. 0	autoclave, RT
2x SSC	10% 20x SSC, 0. 1% Tween	RT

solution	composition	storage conditions
0. 2x SSC	1% 20x SSC, 0. 1% Tween	RT
TE	10 mM Tris, 0. 1 mM EDTA, pH 7. 6	autoclave, RT
TAE	40 mM Tris, 20 mM CH ₃ COOH, 1 mM EDTA.	RT
TRIS	tris(hydroxymethyl)aminomethane: 0. 1 M Tris-HCl, pH 9. 5	autoclave, RT
Trypsin	0. 05% Trypsin/0. 53mM EDTA in 1xHBSS, With Sodium Bicarbonate, without Calcium and Magnesium (Mediatech)	-20°C

2.2.2 Morpholinos

All MOs used in this thesis were ordered from Gene Tools, LLC (Philomath, USA). They were dissolved to make a 50 μ M stock solution, stored at -20°C. Where not previously published, MOs were designed using Gene Tools' Oligo Design Service. All MOs were aligned against the zebrafish genome to assess predicted off-target effects. Only MOs binding specifically to the target site were used in this thesis unless otherwise indicated. All MOs have a sequence length of 25 bp; an off-target effect was considered to be a match to another site with more than 16 bp identity. Morpholino information is given below.

Table 2-4. Morpholinos used for knockdown analyses.

For ENSEMBL IDs and full names of genes, please refer to Table 2-2 (p. 47).

target gene	MO	MO target	MO sequence (5' - 3')	source
<i>dll4</i>	MO1	Exon 2 / Intron 2	GTTCGAGCTTACCGGCCACCCAAAG	5ng (Siekmann and Lawson, 2007)
	MO4	Exon 4/ Intron 4	TGATCTCTGATTGCTTACGTTCTTC	6ng (Hogan et al., 2009)
<i>runx1</i>	MO1	Exon 2 / Intron 2	AGCGCTCTTACCGTATTTGGCGTCC	4ng (Gering and Patient, 2005)
	MO2	Intron 5/ Exon 6	GTGTGAATGTGTAAGTACAGTGT	5ng (Peterkin and Pinheiro, unpublished)
<i>cldn5b</i>	MO	5' UTR	CCAAACTCTAAATCTGTCAGAAGCA	10ng this thesis
<i>foxc1b</i>	MO1	ATG	GCATCGTACCCCTTTCTTCGGTACA	8ng (De Val et al., 2008)
	MO2	UTR	AAGTGAAATGAAGACTATGCAGACG	(Topczewska et al., 2001a)
<i>foxd1</i>	MO	ATG	TCATGGCTCTCTCGGGTTTCCCGAT	2.5ng (Balikova et al., 2010)
<i>hif1ab</i>	MO	Intron 1 / Exon 2	ACCCTACAAAGAAAGAAGGAGAGC	0.5ng (Mendelsohn et al., 2008)
<i>jag1a</i>	MO	Exon1 / Intron 1	AAGCCAAACCCGCACATACCCGCAT	(Yamamoto et al., 2010)
<i>jag1b</i>	MO	Exon1 / Intron 1	AAATCAAGACTCACCGTCGTCCGCA	(Yeo and Chitnis, 2007)

target gene	MO	MO target	MO sequence (5' - 3')		source
<i>zfat</i>	MO1	Exon 1 / Intron 1	CTCTCTTTATAGCGGTTTACCTGAA	20ng	this thesis
	MO2	Exon 4 / Intron 4	CATGTGTATCTCACCAGGCATGGCC	30ng	this thesis
<i>hes6</i>	MO	ATG	TGCAGTTCAGGACGCTTGAATGGG	3ng	(Kawamura et al., 2005b)
<i>her8a</i>	MO	Intron 2 / Exon 3	AGATTGCTGTGGGAATTCAAATTAT	2.5ng	(Chung et al., 2011a)

2.2.3 Chemical inhibitors

Chemical inhibitors used in this thesis, their targets, solubility, and concentration are detailed below.

Table 2-5. Chemical inhibitors used in this thesis.

inhibitor	name	primary targets	solubility	conc.	source
<i>SU5416</i>	3-[(2,4-Dimethylpyrrol-5-yl)methylidene]-indolin-2-one	VEGF-R (KDR/Flk-1) and PDGF-R tyrosine kinases	DMSO	1μM	calbiochem (Merck/Millipore)

2.2.4 Primers

All primers designed for this thesis were designed using the Primer3 web service (Koressaar and Remm, 2007, Untergasser et al., 2012) and ordered from Sigma-Aldrich (RP purified). All primers were re-suspended to a stock solution of 100 μM (stored at -20°C) and used at 10 pmol per reaction.

Primers for quantitative real-time PCR

Primer pairs for qPCR designed in this thesis were designed to amplify a product as close to 100 bp as possible. The following parameters were applied to all designed qPCR primers: melting temperature (T_m) of 62°C, GC content between 40% and 60% and a primer length of 19-29 bp. If qPCR primers for a given gene have been previously

published, these were used. Melting curves were tested for all qPCR primers used in this thesis.

Table 2-6. QPCR primer pairs used in this thesis.

For ENSEMBL IDs and full names of genes, please refer to Table 2-2 (p. 47).

gene	forward primer sequence (5' → 3')	reverse primer sequence (5' → 3')	source
<i>cldn5b</i>	ACCGTCACCGGGACTCTTT	AGATGTTTTGCGCCACCAC	this thesis
<i>dll4</i>	CTTCACCGGACCCCTCTGT	TGGAAGCGGTCTTGAGTTTCTC	(Siekman and Lawson, 2007)
<i>foxc1b</i>	GCCTTATATCCCCGAGACC	TGCGTGCGTGAGAGTACAT	this thesis
<i>foxd1</i>	GACCGACATTGACGTGGTTG	TTGAGCAGGATCTCGTCGTG	this thesis
<i>g6pd</i>	GTCCCGAAAGGCTCCACTC	CCTCCGCTTTCCTCTC	(McCurley and Callard, 2008)
<i>gapdh</i>	GGTCATTGATGGTCATGCAATC	CACCTGCATCACCCACTTA	(Simões et al., 2011)
<i>her8a</i>	AAGTCGCGAAACCATTGAT	TGGTCCAAATTGTAGGCATCC	this thesis
<i>hes6</i>	GGATGCGCAGGTAAATATGG	CACACTGTCAGCCTCCTTCG	this thesis
<i>hif1ab</i>	ACGTGGAAGGTTCTTCACTGC	AAGGTAAGTGACAGGGGGCTCT	this thesis
<i>tp53</i>	TTAAGTGATGTGGTGCCTGCCT	AGTTCTTTCCCTGTTTGGGCT	(Peterkin and Pinheiro, unpublished)
<i>zfat</i>	CCTGGTGCCTCGAAGATTGT	TTTAATCGCTGCCTCCTGAGA	this thesis

Primer sets for testing splice morpholino efficiency

Primers designed in this thesis for testing splice MO efficiency were designed in sets, with each primer having a melting temperature (T_m) of 60°C, 40% to 60% GC content and a primer length of 20 bp (+/- 2bp).

Table 2-7. Primer sets for testing MO and mutant efficiency.

gene	MO/mutant	forward primer sequence (5' → 3')	reverse primer sequence (5' → 3')
<i>cmyb</i>	<i>cmyb</i> ^{hkz3}	CAACAGAAAACGAGCTGAAGG	CTCTGGTAAAGTGGAGCTGGA
<i>dll4</i>	<i>dll4</i> MO1	TGCAGGACTTCTTCCGAGT	GAGTGCCAGGCCTCAATAAT
	<i>dll4</i> MO4	GCCTGGCACTCACCTTACTC	CTCACTGCATCCCTCCAGA
<i>zfat</i>	<i>zfat</i> MO1	TGGTTCGTCAGTCTTCATGTG	ACATGCGATTGCACCTTCTTG
	<i>zfat</i> MO2	TGGTTCGTCAGTCTTCATGTG	ATGGCGCACATAGATGAA

Primerpairs used to create WISH probes

For all novel wholemount *in situ* hybridisation (WISH) probes created, primer pairs were designed against the ensembl cDNA sequence (Flicek et al., 2014) using Primer3

(Koressaar and Remm, 2007, Untergasser et al., 2012). For genes with several protein coding splice isoforms, a probe was designed to bind to all isoforms, where possible. WISH probes were designed to be 1 kb or shorter in length, as dictated by gene size. All primers were designed with the following parameters: melting temperature (T_m) of 60°C, GC content between 40% and 60% and a primer length of 20 bp (+/- 2bp).

Details of primers designed for the expression screen detailed in Chapter 4 are listed together with all other details of WISH probe generation (p.56).

Table 2-8. Primer pairs for general WISH probes of interest.

For ENSEMBL IDs and full names of genes, please refer to Table 2-2 (p. 47).

gene	forward primer sequence (5' → 3')	reverse primer sequence (5' → 3')
<i>dla</i>	TGTCCCTTTCACAGTCTCC	TTGGCTGGCAGAATAAAACC
<i>her8a</i>	TCCGGAGAAGAATTCAACG	CTCCACATGGAAGGATCGAC
<i>hes6</i>	TCACGACGAGGATTATTACGG	GCCAAGTGCCAGAGTTGTTAG
<i>jag1a</i>	AGGCAAGAACTGCTCTCAGC	GTCTTCAGCCCACTTTGCTC
<i>jag2a</i>	AGGACCAATGCGACACCTAC	CTCCATATGAGCACGGGTTT

2.2.5 Plasmids

Antisense probe constructs for WISH

Table 2-9. General haematopoiesis-related antisense probes used in this thesis and their sources.

For ENSEMBL IDs and full names of genes, please refer to Table 2-2 (p. 47).

gene / probe	plasmid	restriction enzyme	polymerase	source
<i>alas2</i>	pBK-CMV	BamHI	T7	RZPD clone ID: LLKMp964E092
<i>cdh5 (VE-cadherin)</i>		EcoRI	T7	IMAGE:6793191
<i>cmyb</i>	pBS	EcoRI	T7	(Thompson et al., 1998)
<i>col2a1a</i>	pCMV-SPORT1	SalI / SmaI	T7	Accession ID AAH93433
<i>dab2</i>	PBK-CMV	SalI	T7	(Song et al., 2004)
<i>dlb</i>	pBSSK(-)	EcoRI	T7	(Haddon et al., 1998)
<i>dlc</i>	pBSSK	XbaI	T7	(Appel and Eisen, 1998)
<i>dld</i>		XbaI	T7	(Lawson et al., 2001)
<i>dll4</i>		SpeI	T7	(Martin Gering, <i>unpublished</i>)
<i>efnb2a</i>	pBS	XhoI	T3	(Lawson et al., 2001)
<i>ephb4</i>	pBS RTK	XbaI	T7	(Lawson et al., 2001)

gene / probe	plasmid	restriction enzyme	polymerase	source
<i>fli1a</i>	pBS	<i>XhoI</i>	T3	(Brown et al., 2000)
<i>flt1 (vegfr1)</i>	pBS	<i>EcoRI</i>	T7	(Fouquet et al., 1997)
<i>flt4 (vegfr3)</i>	pGEMT Easy	<i>NcoI</i>	SP6	(Bussmann et al., 2007)
<i>foxn1</i>	pBS	<i>EcoRI</i>	T7	(Thompson et al., 1998)
<i>gata1</i>		<i>SalI</i>	T7	(Thompson et al., 1998)
<i>gfi1aa</i>		<i>XbaI</i>	T7	(Detrich et al., 1995)
<i>hbbe1.1 (βe1 globin)</i>	pExpress1	<i>EcoRI</i>	SP6	IMAGE:7049024
<i>her8a</i>	pGEM-T easy	<i>ApaI</i>	Sp6	this thesis
<i>hes6</i>	pGEM-T easy	<i>ApaI</i>	Sp6	this thesis
<i>hey2 (gridlock)</i>	pBS SK(-)	<i>XhoI</i>	T3	(Quinkertz and Campos-Ortega, 1999)
<i>hhex</i>	pGEM-T easy	<i>SalI</i>	T7	(Martin Gering, <i>unpublished</i>)
<i>ikzf</i>	pSPORT1	<i>NotI</i>	T7	(Liao et al., 2000)
<i>jag1b</i>	pCRII TOPO	<i>ApaI</i>	SP6	(Zecchin et al., 2005)
<i>jag2</i>	pCRII TOPO	<i>EcoRv</i>	Sp6	(Zecchin et al., 2005)
<i>kdr1 (flk1/vegfr2)</i>	pCRII TOPO	<i>SpeI</i>	T7	(Zecchin et al., 2005)
<i>lck</i>	pCMV-Sport6. 1	<i>KpnI</i>	T7	IMAGE:6795451
<i>lcp1 (l plastin)</i>	pBSKII	<i>NotI</i>	T7	(Herbomel et al., 1999)
<i>lyz (lysozyme c)</i>	pBluescript II KS(+)	<i>BamHI</i>	T3	(Liu and Wen, 2002)
<i>mpx (mpo)</i>	pBK-CMV	<i>EcoRI</i>	T7	RZPD clone ID: CHB0p575111 20Q3 fj79a06.y1
<i>myod1</i>	pBS II SK	<i>BamHI</i>	T7	(Eric Weinberg and Geoff Doerre, <i>unpublished</i>)
<i>notch1a</i>	pSport1	<i>HindIII</i>	T7	IMAGE:3725324
<i>notch1b</i>	pGEM-T easy	<i>NcoI</i>	SP6	(Lynn Poulton, <i>unpublished</i>)
<i>notch2</i>		<i>BamHI</i>	T3	(Chen et al., 2009b)
<i>notch3</i>	pGEM-T	<i>PstI</i>	T7	(Lawson et al., 2001)
<i>rag1</i>	pBS-SK-	<i>HindIII</i>	T7	(Wilkinson et al., 2009)
<i>rag2</i>	pBS-SK-	<i>NdeI</i>	T3	(Willett et al., 1997)
<i>runx1</i>	pBluescript SK(+)	<i>HindIII</i>	T7	(Kalev-Zylinska et al., 2002)
<i>spi1b (pu1)</i>	Bluescript II	<i>EcoRI</i>	T7	(Lieschke et al., 2002)
<i>tal1 (scl)</i>	pZE62	<i>EcoRI</i>	T7	(Gering et al., 1998)
<i>tbx20 (hrt)</i>		<i>BamHI</i>	T7	(Griffin et al., 2000)
<i>tek</i>	Bluescript SK	<i>XhoI</i>	T3	(Lyons et al., 1998)
<i>tie1</i>	Bluescript SK-	<i>EcoRI</i>	T7	(Lyons et al., 1998)
<i>uroD</i>	pSPORT	<i>EcoRI</i>	SP6	RZPD clone ID: DKFZp717E081
<i>vegfaa</i>	pBSK-	<i>XbaI</i>	T7	(Gering and Patient, 2005)

Table 2-10. Primers, plasmids, restriction enzymes and polymerases used for each gene included in the expression screen conducted in Chapter 4.

Overview of the cloning details of WISH probes created with primer pairs, restriction enzymes (RE) and polymerases (POL) used. All PCR products were cloned into pGEMT-easy plasmids. For full gene names and the respective RNA-seq values, refer to Appendix Table 8-4 (p. 252))

gene/probe	class	ENSEMBL ID	forward primer	reverse primer	RE	POL
<i>aar2</i>	UG(20)	ENSDARG00000018658	AGACATGGACCCTGAAGTGG	CGCGTTGAGAATGTAAGTGC	<i>SpeI</i>	T7
<i>abhd17aa</i>	UG(29)	ENSDARG000000091433	CCTGCCACAATCAGGAAGAT	GAAGGCGTCAAAGCAGTAGG	<i>SpeI</i>	T7
<i>adarb2</i>	OTHER	ENSDARG000000071823	AGTGGATGGCAGGATATTCG	GCCACTGAGTAATGGCTGGT	<i>SpeI</i>	T7
<i>angpt1</i>	SIG	ENSDARG000000030364	GTGGAGACCCAGGTTTGGAA	TACACACCGTTCAGGTTGGA	<i>SpeI</i>	T7
<i>apex2</i>	TF	ENSDARG00000008472	TGAGAAAACAGCGATTGTCG	GATTCAGGTTTCCCGGATT	<i>SpeI</i>	T7
<i>atf4b1</i>	TF	ENSDARG000000039515	CTGGACCAGGATGAGGAGAG	GTTTGCTCTTGCGCTGTTT	<i>ApaI</i>	Sp6
<i>atf6b</i>	TF	ENSDARG000000069883	TCACACACACACCACAGTGC	TTCCCGTCAGACTTCCTGTT	<i>ApaI</i>	Sp6
<i>atpif1b</i>	UG(17)	ENSDARG000000044092	GGCTAGGTTTCTGAACAGCAAT A	GTTTCTTGATTTTGCTCTCGTG	<i>AatII</i>	Sp6
<i>bmp1a</i>	SIG	ENSDARG000000028071	TGGGTGATCACAGCAGAGAA	TCACGTCCACATTTCTTCA	n/a	n/a
<i>btbd10b</i>	TF	ENSDARG000000020252	AGCTCAGAGGGCACCTACAA	TTGGGAAGAATGTCACGCTC	<i>ApaI</i>	Sp6
<i>btg2</i>	SIG	ENSDARG000000020298	AGTTTGTGTTGCCGGCTGT	AGGAGGTTTTTGGGTGGACT	<i>SpeI</i>	T7
<i>BX548000. 3</i>	UG(30)	ENSDARG000000088772	ATCAGCACCACTTGGCTACC	TGCGGTCCACTAAGTTGAAA	<i>SpeI</i>	T7
<i>BX936343. 1</i>	UG(18)	ENSDARG000000054645	TCTGGATGCCAGGGAACATAC	GTTCTTCCACCAACGCAAT	<i>SpeI</i>	T7
<i>BX957362. 1</i>	UG(32)	ENSDARG000000052074	GCCTGTTTGGAGAACAGCTC	GCCGAAGGAAAATGAATGAA	<i>ApaI</i>	Sp6
<i>C5H8orf4</i> (<i>zgc:110340</i>)	UG(28)	ENSDARG000000079497	TCCGTCAGCTCTCCACTTTT	GGCATAGGGACAAGGCATAA	<i>SpeI</i>	T7
<i>C9H3orf70</i> (<i>zgc:162707</i>)	UG(25)	ENSDARG000000061664	TACAGTGGAGCGCAGAAGG	GTTGCAGTATTTGGGCGATT	<i>AatII</i>	Sp6
<i>CABZ0106923</i> <i>9. 1</i>	UG(19)	ENSDARG000000090031	ATGGAGATGGACGGTTTCG	CGGTTGTCGTGTGTGTGTG	<i>AatII</i>	Sp6
<i>ccdc85ca</i>	TF	ENSDARG000000005232	GAGGACAACCAGGAGCTCAG	AAGGGCCTTTGAAGTGGTTT	<i>ApaI</i>	Sp6
<i>cct4</i>	SIG	ENSDARG000000013475	CGGGATCAAAAACAAAGGAA	TGCCGATAGCTTTCAGATG	<i>NcoI</i>	Sp6
<i>chchd4</i>	TF	ENSDARG000000040707	GTCTGAAAGCGCGCTCTACT	CTTGCCGAGTTAACCCTCACT	<i>SpeI</i>	T7
<i>cldn10b</i>	UG(26)	ENSDARG000000005377	GAGGAACATGCGGAGAGAGA	TTATACGTAGCGGTTTTGTGCG	<i>SpeI</i>	T7
<i>cldn5b</i>	SIG	ENSDARG000000014059	CGCCAAAAGAGAGAAATCG	CTGCACGTTTACAGGCAGAA	<i>PstI</i>	T7
<i>crebzf</i>	TF	ENSDARG000000089787	GTGAACAACACACGACACC	CATGGTCTTTGTCCACATGC	<i>NcoI</i>	Sp6
<i>crygn2</i>	OTHER	ENSDARG000000030411	GCTGTCTGGAGGTGTATGGAG	GACTGGATGTGGGATTCTG	<i>ApaI</i>	Sp6
<i>CU457819. 3</i>	UG(33)	ENSDARG000000090426	GCGACCTCATCGTCAAGACT	TGCTGGTTTCTTGCCTTCT	<i>SpeI</i>	T7
<i>CU633479. 6</i>	UG(16)	ENSDARG000000092357	CGTGTGACTTTCCAGCTCAA	CAGGGAGAAATGAGCCTCTG	<i>AatII</i>	Sp6
<i>dhhs3b</i>	SIG	ENSDARG000000044803	CTGCGTGGTGTCTTACCTA	CCAGTGCACCTCTGTGGTAGC	<i>SpeI</i>	T7
<i>dhx15</i>	SIG	ENSDARG000000015392	CCGGCTCTACACTGAGAAGG	CGTTCCAGTTGCCTCTTAGC	<i>ApaI</i>	Sp6
<i>elf4a3</i>	SIG	ENSDARG000000014472	CAGCCACCTTCTGTGTCTCA	GATCAGATCAGCCACGTTCA	<i>ApaI</i>	Sp6
<i>fhad1</i>	UG(03)	ENSDARG000000056723	AAAGGCCTGGTTCAAATGAG	TTCTCTGGGACTCATCGTC	<i>SpeI</i>	T7
<i>fip1l1</i>	SIG	ENSDARG000000070431	GAAGAGGCCAACTGACAGC	GGACCAGGAGGAAAGAAAGG	<i>ApaI</i>	Sp6
<i>foxc1b</i>	TF	ENSDARG000000055398	CCAAGGACATGGTGAAACCT	GAGTCGGTTCTGTGCAAGT	<i>SpeI</i>	T7
<i>foxd1</i>	TF	ENSDARG000000029179	AGACCACATTTGACGTGGTT	CGCTAGTGCCACTGACAATC	<i>ApaI</i>	Sp6
<i>foxd1 (2)</i>	UG(43)	ENSDARG000000079699	TTAGAAGCCGTCTCGCATCT	TAAATCCACGTGGCTGTGAG	<i>SpeI</i>	T7
<i>FP015790. 3</i>	UG(08)	ENSDARG000000089541	CCCGTTTATGGAAGTGAAGG	GAACATCTGAGCGACGCTTT	<i>ApaI</i>	Sp6
<i>grb2</i>	SIG	ENSDARG000000038059	GGCCATTGCCAAGTATGATT	TCGGAGAAAGATCTGCTGGT	<i>SpeI</i>	T7

gene/probe	class	ENSEMBL ID	forward primer	reverse primer	RE	POL
<i>h2afy-13</i>	UG(38)	ENSDARG00000087235	CAGGGCTTCAGTTTCCAGTC	TGGGTAGTACACCACCCTGAG	<i>SpeI</i>	T7
<i>h2afy-8</i>	UG(46)	ENSDARG00000070280	GACCAAGATGAGCGGAAGAG	CTGAGGCTGATTAGTTTAATGC AA	<i>SpeI</i>	T7
<i>her8a</i>	OTHER	ENSDARG00000016363	TCCGGAGAAGAATTTCAACG	CTCCACATGGAAGGATCGAC	<i>ApaI</i>	Sp6
<i>hes6</i>	OTHER	ENSDARG00000019335	TCACGACGAGGATTATTACGG	GCCAAGTGCCAGAGTTGTTAG	<i>ApaI</i>	Sp6
<i>hif1ab</i>	TF	ENSDARG00000034293	TGGTTTTGTGTGGTTGAGA	TGTGCTGCTGGTAGATGAGG	<i>ApaI</i>	Sp6
<i>hist1h4a</i>	UG(31)	ENSDARG00000091446	GGTCGAGGCCAAAGGAGGTA	AACCTCCAAAGCCGTACAGA	<i>SpeI</i>	T7
<i>hlcs</i>	SIG	ENSDARG00000039934	GCAGCAGTTCCAGAAGATCC	TGGAGGATGTGACGTCAGTG	<i>ApaI</i>	Sp6
<i>hm:zewp0073</i>	UG(21)	ENSDARG00000076126	GACGAGGCTCAGAGCAAGAG	GAGGGACCTGCCTCATCATA	<i>SpeI</i>	T7
<i>hnrnpd</i>	SIG	ENSDARG00000059246	AAGACCCGACGATGAAGATG	CCACCGTAGCCTTGATTGTT	<i>SpeI</i>	T7
<i>jam3b</i>	SIG	ENSDARG00000061794	CTAAACCGTGGTCAACGAG	AAATCCCCTCATCCTCTGT	<i>ApaI</i>	Sp6
<i>lig4</i>	TF	ENSDARG00000060620	TTGGAAGATTCGTTGCAGTG	TGGGTCATGAAGTTGTCTGC	<i>SpeI</i>	T7
<i>lrig3</i>	TF	ENSDARG00000079363	AGAAACGGCATCAGCAGACT	GTCAGTTCCGCTGTCTCTCC	<i>SpeI</i>	T7
<i>lrp6</i>	SIG	ENSDARG00000076053	ACCCCTGCACGTCTGATAAC	AGCTGTAGGAGCGGTGTGTT	<i>SpeI</i>	T7
<i>lrrc8c</i>	TF	ENSDARG00000059718	AGGTTTCCACAATGCCTGTC	TCTTGAATACAGGGGATCG	<i>SpeI</i>	T7
<i>mafbb</i>	TF	ENSDARG00000070542	GTCTTCTGGACCTGGCAAAA	GGTTTTTACAAGAGGGTCCA	<i>ApaI</i>	Sp6
<i>maff</i>	TF	ENSDARG00000028957	CAACAGGTTTCAGGACACA	CTTTCACGTCTGTGTTGGA	<i>ApaI</i>	Sp6
<i>mplkip</i>	UG(23)	ENSDARG00000078986	GAGACCACAGTTTCGCCATC	TCGCTTTGTTTGGAGGTTTT	<i>AatII</i>	Sp6
<i>mta3</i>	TF	ENSDARG00000054903	AGGCTGATGTCCCTGAAATG	TTTGGGCTACCACTGTTC	<i>ApaI</i>	Sp6
<i>muc5ac</i>	UG(24)	ENSDARG00000070331	TGGAAGGTGGATGTGTGA	GCAGGTTGATCCACAGTTTT	<i>SpeI</i>	T7
<i>nkx3.2</i>	TF	ENSDARG00000037639	AGAGGAGTCTCGCCATCTGA	CCTTCACCGCCACTTTCTT	<i>SpeI</i>	T7
<i>nr1d2b</i>	TF	ENSDARG00000009594	GAGATGTGGCGTCAGGATTT	CTTTGCAAATTCACCACCT	<i>SpeI</i>	T7
<i>nsmaf</i>	SIG	ENSDARG00000051920	CGATGATGCTGTGAAGCCTA	AGGCTCAGGCATGTCTCTGT	<i>ApaI</i>	Sp6
<i>pde8a</i>	SIG	ENSDARG00000088905	ATAGAGGCGCCCATCACTAA	CCAGTGGTTTGTGATGCTG	<i>SpeI</i>	T7
<i>pl10</i>	SIG	ENSDARG00000005774	AGAACAGCGCACAGGAAAAT	GCTCATACGCCAGACTCTCC	<i>SpeI</i>	T7
<i>plcd4b</i>	SIG	ENSDARG00000040684	TGGAGGACCAGCTAAAAGGA	TAGGCAGCTGTTGTCCACTG	<i>ApaI</i>	Sp6
<i>plod3</i>	SIG	ENSDARG00000076317	GTGATCACCGCTGTACAGA	GCCTGGTCATGTTTGAGGTT	<i>SpeI</i>	T7
<i>ppp1r3cb</i>	SIG	ENSDARG00000014554	ATGAATTGCACCAGGGTTCT	TCACCAATAGGGGGTGTGT	<i>SpeI</i>	T7
<i>prrc2b</i>	TF	ENSDARG00000079639	ACCCGATCCAAAGAGTTCTT	TCCACTTCCTCATGGAGACC	<i>AatII</i>	Sp6
<i>psmd5</i>	SIG	ENSDARG00000059046	CAGAAGATCCGATCAGGAG	TAATGCCTTGTGTGCCAGA	<i>SpeI</i>	T7
<i>pth1b</i>	SIG	ENSDARG00000091961	TTGTGCTGTGGAGTCTTGC	CCTTCCCCCTTCTTAAACG	<i>ApaI</i>	Sp6
<i>ralgps1</i>	TF	ENSDARG00000089443	GAGCGCATCTGTTCCAAGA	GTTGACTGACCGCGTACAT	<i>ApaI</i>	Sp6
<i>rassf8</i>	UG(01)	ENSDARG00000090577	CAGCGTGCAAGGATTTTACA	AGGAGCTTTTGGTTGGTTT	<i>AatII</i>	Sp6
<i>rgs6</i>	SIG	ENSDARG00000015627	CTCCAAGATGAGGCTGAAGG	CTGAATTCCGACTCCAGGAA	<i>ApaI</i>	Sp6
<i>rnps1</i>	SIG	ENSDARG00000015853	GAACGAGGGAAGGAGAAAAC	CCGAGACCTTCTTCTCATGC	<i>SpeI</i>	T7
<i>sema4ba</i>	SIG	ENSDARG00000074414	CGCAATGAAGTGGGAGAAAT	TGTGCCAATGAAAAGCACAT	<i>ApaI</i>	Sp6
<i>sf3b2</i>	SIG	ENSDARG00000018049	GGTATCGAGAAACCCCATTT	GTCCAACCTCAGCTCCTCTG	<i>ApaI</i>	Sp6
<i>si:ch211-117m20.5</i>	UG(12)	ENSDARG00000091996	GCCACTCCCTGAATTTCTCA	GGTTTCATTTGCAACCATGA	<i>SpeI</i>	T7
<i>si:ch211-125e6.8</i>	UG(15)	ENSDARG00000094518	GTTGAAAAAGACCAGGCTGAA	GCATCTTCCAAAGTTAGACCAC A	<i>SpeI</i>	T7
<i>si:ch211-278f9.2</i>	UG(14)	ENSDARG00000093624	TTTCCTCGATTTCTGGATACG	CTTGGGGTTTCACTTGTGCT	<i>SpeI</i>	T7
<i>si:dkey-15h8.1</i>	UG(06)	ENSDARG00000095009	CTCTGCCGTCATCAGTGAAA	TGGTTTGACCATGTGTCTT	<i>SpeI</i>	T7
<i>si:dkey-15i15.2</i>	UG(02)	ENSDARG00000095084	TAGCCACATGATGGAGACA	ATTGCCGCTAGTGAAGAGGA	<i>AatII</i>	Sp6
<i>si:dkey-6f10.4</i>	UG(04)	ENSDARG00000092390	TGGTGTGCCAAAACCTCAA	GTTCAACAGAGTGCCAGCAA	<i>SpeI</i>	T7

gene/probe	class	ENSEMBL ID	forward primer	reverse primer	RE	POL
<i>si:dkeyp-13d11.2</i>	UG(10)	ENSDARG00000077519	TGGTGTGGCCAAAACCTCAA	GTTCAACAGAGTGCCAGCAA	<i>SpeI</i>	T7
<i>si:dkeyp-51b9.3</i>	UG(05)	ENSDARG00000092379	GTGATGTCAGAGGGCTGGTT	AAATCATAAGCACGCGATCC	<i>SpeI</i>	T7
<i>slc27a2</i>	UG(22)	ENSDARG00000067520	TGAGGAGTCTGAGCAGAGCA	GAGGACGTCCGAGACTTCAG	<i>SpeI</i>	T7
<i>smarcb1b</i>	SIG	ENSDARG00000011594	AGGCGAAGAGGAACAGTCAA	CATCTCCTGTGTGCGAATG	<i>SpeI</i>	T7
<i>socs9</i>	SIG	ENSDARG00000074493	TGGACGAGGGAAGAAGAAGA	CCCCAGTAGCACGACAGATT	<i>ApaI</i>	Sp6
<i>sp9</i>	TF	ENSDARG00000010462	TTGCAAAGTTCGTTGCACTC	ACTCCAGACCCGTTTCCTTT	<i>ApaI</i>	Sp6
<i>srr</i>	UG(07)	ENSDARG00000096045	AGGCCTGTGAGACTGTGAGG	TCCACTGAGAATCACCACCA	<i>SpeI</i>	T7
<i>srsf2a</i>	SIG	ENSDARG00000010906	GTCGAGGGCATGACATCTTT	TCTGGAGACTTGGGTCTGCT	<i>ApaI</i>	Sp6
<i>stmn1b</i>	SIG	ENSDARG00000033655	GCGTCCTCTGGAGCTGATA	CGCCTCCCTCTTTTGTTCCT	<i>ApaI</i>	Sp6
<i>tfdp1b</i>	TF	ENSDARG00000016304	CCAGAGTCCTGGAAGAGCTG	CAGCTGGATGATGGTGTGTTG	<i>SpeI</i>	T7
<i>tjp2b</i>	SIG	ENSDARG00000023443	ACGGTTTGGGAGCAGTACAC	ATATCGCCCTCTGGAGATT	<i>PstI</i>	T7
<i>tnni1d</i>	UG(13)	ENSDARG00000016728	AGGCCTGGAATCCAAAAGT	TCACCAACCTCACTGGTCAA	<i>AatII</i>	Sp6
<i>yipf1</i>	TF	ENSDARG00000014474	AAGCACAACAGTGACCATCG	GAGTAGGACACCAGGCTTGC	<i>ApaI</i>	Sp6
<i>zc3h18</i>	TF	ENSDARG00000062506	CTGACAATGAGCTGCTGGAG	AGGCAGAATGGGTTTCCTTT	<i>SpeI</i>	T7
<i>zfand3</i>	TF	ENSDARG00000052833	CAGCAAGCCGCTAGTATTC	ACCAGAGCAGTTCTCCGAAA	<i>ApaI</i>	Sp6
<i>zfai</i>	TF	ENSDARG00000077987	GCGAGTGACACAAGAAGATCA	GCTGTCGTTTCTGCATCTGT	<i>ApaI</i>	Sp6
<i>zfp36</i>	TF	ENSDARG00000044395	TTCAGCCGACACAGTAGTGG	TCTCCATGGCAGATGTTGAT	<i>ApaI</i>	Sp6
<i>zgc:110425</i>	UG(41)	ENSDARG00000058479	GCAGAGACGAAGACGAAACC	CTGCTGTTGTTGACATGCT	<i>ApaI</i>	Sp6
<i>zgc:162816</i>	UG(27)	ENSDARG00000070416	GAAAAGTCTGGAGCGTCTGG	CATTTGGCTCAGATCAGCA	<i>PstI</i>	T7
<i>zgc:163061</i>	UG(40)	ENSDARG00000075538	AAGCTCAAGATCCGCTCAGA	CAGAAATGAGTGGGTGGCTCT	<i>AatII</i>	Sp6
<i>zgc:165409</i>	UG(36)	ENSDARG00000091385	CTGCATATGTTGAGCGGGTA	TTTGGAGCTACCAAGGGAGA	<i>ApaI</i>	Sp6
<i>zgc:195633</i>	UG(37)	ENSDARG00000088291	GGTAAACCGGAGGAAAAGC	TTATTTCTTGGCGGCCTTC	<i>ApaI</i>	Sp6
<i>znf503</i>	TF	ENSDARG00000018492	CCTACGGGAAGAGACAGAGC	TTGGATCCTGAGGTGGTCTC	<i>AatII</i>	Sp6

Plasmids for injection

Plasmids for either DNA or RNA injection, with the appropriate restriction sites and polymerases used, are listed in Table 2-11.

Table 2-11. Plasmids for injection.

name	plasmid	inject.	restr. site	polym.	amount injected	source
<i>pCS2-FA-transposase</i>	pCS2	RNA	NotI	Sp6	30 pg	(Wilkinson, <i>unpublished</i>)
<i>runx1_cDNA</i>		DNA	n/a	n/a	50 pg	(Pinheiro, <i>unpublished</i>)

2.3 Zebrafish husbandry and manipulation of embryos

2.3.1 Zebrafish maintenance

Danio rerio (zebrafish) were housed in flowing system water at a temperature of 28.5°C, 450-550 µS conductivity range, a pH of 7.1-7.2 and a light/dark cycle of 14h of light and 10h of darkness. A mixed diet of high protein pellets, flake food and enriched brine shrimp was fed three times a day (BMS Staff, University of Oxford). All zebrafish work was approved by the Research Ethics Committee of the University of Oxford.

2.3.2 Fin-clipping zebrafish

Adult fish were anaesthetised in 50% 1.5 g/l MS222 pH 7, 50% system water for the minimum period required, transferred to a petri-dish, and a small section of the caudal fin was removed with a sterile scalpel before returning the fish to a tank. The dissected tissue was collected in a PCR strip (Starlab) for extraction of genomic DNA.

Fish were kept in isolation after the procedure until results were obtained. All fin-clipping was carried out in accordance to ASPA regulations and fish were monitored for adverse effects during and after the procedure.

2.3.3 Breeding, embryo collection and maintenance

Boxes filled with marbles for obtaining embryos were placed in fish tanks at the end of the previous light cycle, approximately 15 hrs before collection. Alternatively, breeding pairs were set up as and when necessary (eg. crossing different lines).

Fertilised eggs from natural spawning were washed and collected in system water with methylene blue to inhibit mold growth. This was replaced later the same day by E3 Medium + methylene blue when any dead or unfertilised embryos and any debris were removed from the dishes.

Embryos were generally grown in incubators at 28.5°C. For collection of embryos at 36 hpf, the temperature was raised to 32.5°C. For other stages, the temperature was adjusted as appropriate within the range of 26.5°C-32.5°C. To estimate the time (T_{dev}) to reach the desired developmental stage (DS, in hpf) at the required temperature (t), the following equation was used: $T_{dev} = DS / ((0.055 * t) - 0.57)$ (Kimmel et al., 1995). Embryos were staged according to morphology under a dissection microscope (Kimmel et al., 1995).

2.3.4 Micro-injections of zebrafish embryos

1-2 cell stage embryos were aligned against a glass slide placed in a petri dish for microinjections of morpholinos. Morpholinos, RNA or DNA were diluted to working concentration in dH₂O. MOs were co-injected with 10% phenol red (Sigma-Aldrich) to aid visualisation of injections into the yolk. DNA and RNA-injections were conducted into the cell.

2.3.5 Dechoriation and fixation of zebrafish embryos

Embryos younger than 20 hpf were fixed in their chorions for a minimum of 3 hpf at RT in 4% paraformaldehyde, before being washed in PBStw to be dechorionated manually. Embryos fixed or imaged between 20 hpf and hatching were dechorionated before

fixing, to prevent curvature of the anterior-posterior axis. Manual dechoriation was achieved using INOX number 5 forceps (FST); for enzymatic dechoriation, a 10 min treatment with pronase followed by a series of washes in PBStw was used. Embryos collected after 4 dpf were allowed to hatch naturally before fixation.

All fixed embryos collected for whole mount *in situ* hybridisation (WISH) were washed 3x in PBStw before being dehydrated through a series of 25%, 50% and 75% MeOH washes to 100% MeOH and stored at -20°C and processed for WISH as described on p. 72.

2.3.6 Dissection of trunks and tails

Embryos were dechorionated manually and placed in Sterilin™ Petri dishes (Thermo Scientific) lined with 1% Agarose and filled with E3 + MS222. Using INOX number 5 forceps (FST) the trunk and tail region was separated from the head and yolk region under a dissecting microscope.

2.3.7 Zebrafish lines

Most experiments conducted in this thesis employed wt^{kcl} zebrafish; all other zebrafish lines used in this thesis are outlined in Table 2-12.

Table 2-12. Fish lines used in this thesis.

Table 2-12-A. Transgenic fish lines used in this thesis.		
line name	transgene expression	source
Tg(<i>itga2b</i> :gfp)	thrombocytes (gfp ^{high}) and HSCs (gfp ^{low})	(Lin et al., 2005)
Tg(<i>kdr1</i> :gfp)	endothelial cells	(Jin et al., 2005)
Tg(<i>dll4</i> :gfp)	arterial/endothelial cells	(Sacilotto et al., 2013)

Table 2-12-B. Mutant fish lines used in this thesis.			
line name	mutation type	allele	source
<i>cmyb</i> ^{hkz3}	point mutation	hkz3	(Zhang et al., 2011)

2.3.8 Isolation of single cells and fluorescence-activated cell sorting

Embryos were kept in E3 without methylene blue to reduce auto-fluorescence. Up to 70 embryos per experimental condition were dechorionated and transferred to Ringer's solution + MS222 to dissect trunk/tail tissues (p. 50). Dissected tissues were transferred into SafeSeal low-binding 1.7 ml tubes (Soerenson) pre-chilled on ice, fresh Ringer's solution was added. All following steps were carried out in a tissue culture hood. Samples were briefly spun at 4000 rpm, rinsed in Ringer's solution, rinsed in 500 µl 32°C Trypsin (Mediatech); 3-5 µl of 32°C collagenase/trypsin solution was added per embryo or 300 µl, whichever was larger, and samples were incubated in a shaking thermomixer (Eppendorff) at 32°C. Tails were homogenised with a p1000 pipette every 5 min, until all tissue was dissolved; the reaction was stopped by adding the dissociated cells to 5 ml Hanks solution and mixed carefully with a 5-ml glass pipette (Pyrex) before cells were spun at 500 rcf for 10min at RT. The supernatant was removed and samples were re-suspended in 5 ml of Hanks+ Hoechst, passed through a 40-µm cell strainer (Falcon) and spun for 10 min at 750 rcf at RT. The supernatant was removed, leaving 300-500 µl to re-suspend the cells which were transferred to a 5 ml FACS tube (Falcon) and kept in the dark on ice for FACS sorting.

Cells were immediately sorted at room temperature on a MoFlo cell sorter (Kevin Clark, WIMM FACS facility). Gfp auto-fluorescent cells were excluded from the analysis by

excluding the overlap of the gfp^{positive}/hoechst^{positive} samples with the gfp^{negative}/hoechst^{positive} control. The same was applied to exclude hoechst auto-fluorescent cells by excluding the overlap of the gfp^{positive}/hoechst^{positive} samples with the gfp^{positive}/hoechst^{negative} control. Live cells were Hoechst-negative cells gated inside the side-scatter, forward-scatter double positive cloud. From this fraction, GFP positive cells were collected in Hanks solution.

Collected cells were spun immediately after sorting for 4 min at 2000g and 4°C, and washed in 500 µl ice cold autoclaved 1x PBS. 100 µl lysis buffer (RNAqueous microkit, Ambion (Life Technologies / Invitrogen)) was added on ice for 15min, vortexed every 2 min, before samples were snap-frozen on dry ice and stored at -80°C. RNA was extracted from lysed cells using the RNAqueous microkit (Ambion, Life Technologies / Invitrogen) according to the manufacturer's instructions.

2.4 Extraction, preparation and manipulation of DNA

2.4.1 Transformation of chemically competent *Escherichia coli*

To transform cloned pGEMT plasmids, DH5α competent cells (NEB) were defrosted on ice, 4 µl of the ligation reaction was added and incubated for 15-30 min on ice, followed by a 45 sec heat shock at 37°C. Cells were then incubated on ice for 2 min before 500 µl LB without antibiotic was added close to a flame and grown for 1hr at 37°C before being streaked on ampicillin plates, next to a flame. Volume of transformed cells streaked on plates varied according to efficiency of the transformation reaction but would usually

range between 50 µl and 250 µl. Plates were incubated at 37°C oN (p. 65); colonies containing transformed plasmids were verified by Colony PCR (p. 68).

2.4.2 Growing bacterial cultures for DNA harvests

Bacteria were cultured at 37°C for 9–18 h either in shaking liquid LB culture or streaked on solid LB agar culture. Cultures were supplemented with 100 µg/ml of ampicillin or kanamycin and stored at 4°C, as required.

2.4.3 Isolation and extraction of plasmid DNA

After identification of successfully transformed bacterial colonies, these were grown in 12 ml or 250 ml LB Agar + Ampicillin O/N, as necessary.

Bacterial cells from liquid culture were pelleted and plasmid DNA isolated by alkaline lysis on an immobilised column. For 12 ml preps, plasmid DNA was extracted using the QuickLyse Miniprep kit (Quiagen), according to the manufacturer's instructions. Plasmid DNA from 12 ml preps was eluted in 50 µl dH₂O. For 250 ml preps, plasmid DNA was extracted using the GenElute™ HP Plasmid Maxiprep Kit (Sigma-Aldrich) according to the provided protocol. Plasmid DNA from 250 ml preps was eluted in 3 ml dH₂O. Plasmid concentration of all extractions was determined using the ND-1000 (p. 67) and plasmids were sequenced (p. 69).

2.4.4 Genomic DNA extraction from zebrafish embryos

Genomic DNA was extracted from 22-27 hpf wt fish using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) as advised by the provider. 100 embryos were lysed for 2.5 hrs at 55°C in 180 µl Lysis solution T with 400 µg ProteinaseK. DNA was eluted with 200 µl elution buffer. DNA concentration was measured and DNA quality was assessed using the ND-1000 (p. 67).

2.4.5 Genomic DNA extraction from fin-clips

Genomic DNA was extracted as described before (Truett et al., 2000). In short, collected tissue samples were immersed in 75 µl alkaline lysis reagent and incubated at 95°C for 1 hr. Samples were cooled to 4°C before adding 75 µl neutralising reagent. Samples were well mixed by pipetting before using 5 µl per PCR reaction. PCR products were cleaned up with the Quiaquick PCR purification kit (Quiagen), according to the manufacturer's protocol, and sequenced.

2.4.6 Agarose gel electrophoresis

Nucleic acid or DNA ladders (NEB, 1 kB or 100 bp) were mixed 1:5 with loading dye, to be run on a 1% agarose gel made up with 0.5% TAE and 0.01% GelRed™ (VWR). Typically, the gel was run at 135V in 0.5% TAE for the required amount of time and visualised with trans-UV light, then photographed at 300 dpi.

2.4.7 Determining nucleic acid concentration

A260 was used to determine nucleic acid concentration with an NanoDrop (ND)-1000.

An extinction coefficient of 50 was used for double-stranded DNA, 40 for RNA.

2.4.8 Restriction enzyme digest

Restriction enzyme digests were performed to linearise plasmids before probe synthesis. Before linearisation for RNA-probe synthesis, restriction enzymes used were chosen based on absence of restriction sites within the insert, using the NEBcutter V2.0 analysis tool (Vincze et al., 2003). Digests were usually conducted in 40 µl to 100 µl with 10 µg plasmid and 1 µl restriction enzyme (NEB) with the appropriate restriction buffers (NEB) and BSA (NEB) in a water bath or hot block at 37°C (unless otherwise specified) for 1-14 h. Cut and uncut fragments were analysed by Agarose gel electrophoresis (p. 66) to assess digest efficiency.

2.4.9 Polymerase chain reaction (PCR)

PCR was conducted using JumpStart™ REDTaq® ReadyMix™ (Sigma-Aldrich) reaction mix with 10 µM of each primer per reaction, in a total volume of 20 µl. Primers were designed with a length of 20 bp, a GC content between 40% and 60%, and a melting temperature of 60°C, wherever possible and unless otherwise stated. Detailed information on primers and sequences used is provided in Section 2.2.4.

The standard programme used has an initial denaturation step at 95°C for 4 min. The following PCR cycles have a denaturation step at 95°C for 15 sec, followed by an annealing step at 55°C (= T_m -5°C) for 45 sec. Extension at 72°C was 1 min per kb,

depending on the length of the PCR product. The number of PCR cycles depended on the concentration of the PCR product and the length, followed by a 7 min final extension step. PCR was carried out on a thermocycler with a heated lid (DNA Engine).

After the reaction, 2 µl of the PCR product was visualised by agarose gel electrophoresis (p. 66) to assess the efficacy of the reaction. If necessary, the PCR product was purified using the Quiaquick PCR purification kit (Quiagen), according to the manufacturer's protocol.

Colony PCR

To screen bacterial colonies after cloning of PCR products into pGEMT vector (p. 68), colony PCR was performed after transformation (p. 64). Colonies were picked from the plate with a sterile pipette tip and re-suspended in 30 µl dH₂O. 5 µl of this solution was added to a standard PCR reaction with Sp6 and T7 primers, designed to amplify part of the pGEMT vector including the cloned insert. 2 µl of the PCR reaction was visualised by agarose gel electrophoresis (p. 66) to assess individual colonies.

2.4.10 Cloning of PCR products

PCR products were cloned into expression vectors using the pGEM®-T Vector System (Promega), according to the manufacturer's suggestions. In short, up to 5.5 µl PCR product was incubated with 1 µl T4 ligase and 1 µl pGEMT vector in 2x ligation Buffer in a total volume of 15 µl for 1 hr at RT or O/N at 4°C.

2.4.11 DNA sequencing

All DNA sequencing was carried out using the Sanger DNA Sequencing facility at the WIMM. For all sequencing reactions, primers were used at a concentration of 3.2 pmol/ μ l.

For sequencing PCR products, PCR was performed at the standard conditions (if not stated otherwise), agarose gel electrophoresis and the PCR products were purified and concentration was assessed using the ND-1000 (p. 67). The amount of PCR product submitted to sequencing depended on product size as follows: 100-800 bp of product required 20 ng of DNA per reaction and 800-1500 bp required 30 ng.

The concentration of plasmids submitted for sequencing was determined using the ND-1000 (p. 67). Plasmids sequenced for this thesis never exceeded 5 kb; all plasmids were submitted with a minimum of 300 ng DNA per reaction.

2.5 Extraction, preparation and manipulation of RNA

2.5.1 RNA isolation from tissue samples

For RNA-extraction, 20-200 embryos or trunk/tail samples were usually collected at the appropriate developmental stage. For trunk/tail samples, embryos would be manually de-chorionated and then dissected in E3 + MS222 (p. 50); samples were then collected on ice. For whole embryo preparations, neither of these steps was conducted. E3 (+MS333) was replaced by 200 μ l TRI Reagent® RNA Isolation Reagent (Sigma-Aldrich) in all tissue samples, snap-frozen on dry ice and stored at -80°C O/N or longer, if

necessary. For further tissue processing, samples were thawed on ice and homogenised with a pestle (Sigma-Aldrich, autoclaved).

800 µl Tri-Reagent (Sigma-Aldrich) was added to the sample to avoid saturation. Samples were vortexed and spun at 12000 g/rcf for 10 min at 4°C; the supernatant was then transferred to a fresh RNase-free tube, 200 µl chloroform was added, and then the samples were vortexed again for 30 sec. Samples were allowed to stand for 5 min at RT, then spun at 12000 g/rcf for 10 min at 4°C. The aqueous phase was transferred to a fresh RNase-free tube, and 500 µl isopropanol was added and samples mixed. Samples were allowed to stand for 5 min at RT and then spun at 12000 g/rcf for 10 min at 4°C. The supernatant was removed and the pellet washed in 1 ml 75% EtOH, spun at 7500 g/rcf for 5 min at 4°C before the EtOH was removed and the pellet air-dried. RNA pellets were re-dissolved in 87.5 µl RNase-free H₂O (RNeasy Micro Kit (Quiagen)) and RNA concentration was determined on the ND-1000 (p.66).

DNaseI digestion of RNA

DNaseI treatment was carried out to remove any residual DNA in the RNA samples according to the protocol for the RNeasy Micro Kit (Quiagen). 10 µl RDD buffer (provided) and 2.5 µl DNaseI (provided) were added to the samples at room temperature for 15 min .

Samples were then cleaned up according to the protocol for the RNeasy Micro Kit (Quiagen): 350 µl Buffer RLT (provided) was added to the samples and mixed well. Then 250 µl of 96-100% Ethanol was added and the samples were centrifuged in the provided spin column for 15 sec at ≥8000 xg. Wash steps were carried out as follows: 700 µl of Buffer RW1 (provided), 500 µl of Buffer RPE (provided) both at ≥8000 xg for 15 sec; 500

µl of 80% EtOH for 2 min at ≥ 8000 xg. Columns were dried by spinning at full speed for 5 min before eluting with 20 µl RNase-free H₂O (provided) into an RNase-free 1.5 ml eppendorff tube by centrifuging for 1 min at full speed.

2.5.2 Reverse transcription of RNA

For cDNA synthesis, 2 µl Random primers (Thermo Scientific) and 2 µl of 10mM dNTP Mix (Thermo Scientific) were added to 1.5 µg RNA, and made up to 14 µl total volume with nuclease-free dH₂O (Ambion) and heated at 65°C for 5 min, then briefly chilled on ice for 3 min. Samples were briefly spun, before 4 µl 5x First Strand Buffer (Thermo Scientific), 2 µl 0.1M DTT (Thermo Scientific) and 0.5 µl RNaseOUT (Thermo Scientific) were added, mixed gently and incubated at RT for 2 min. 0.5 µl Superscript III enzyme (Thermo Scientific) was added to each sample, mixed, let stand at RT for 10 min before incubation at 42°C for a minimum of 50 min. Enzyme activity was then heat-inactivated at 70°C for 15 min.

2.5.3 *In vitro* synthesis of capped mRNA for injection into embryos

Plasmids used to synthesise mRNA for injection are listed in Table 2-11 (p. 59). After linearisation (p. 67), 1 µg of plasmid was transcribed *in vitro* using the mMessage mMachine Kit (Life Technologies) according to the manufacturer's instructions. In brief, the transcription reaction was assembled in a total volume of 20 µl, including Nuclease-free water, 2x NTP/CAP, 10x Reaction buffer and Enzyme mix (all provided). Reactions were incubated at 37°C for 2 hrs before TURBO DNase (kit) was added for 15 min to remove template DNA. The reaction was stopped and precipitated with LiCl

Precipitation Solution (kit) at -20°C for a minimum of 30 min before centrifugation for 15 min at 4°C. Obtained pellets were washed once in 70% EtOH before re-suspension in dH₂O. RNA concentration was determined using the Nanodrop before aliquotting and storage at -80°C.

2.5.4 *In vitro* synthesis of antisense RNA-probes

For RNA-probe synthesis 1 µg linearised plasmid (p. 67) was incubated for 2-18 hrs at 37°C with 10x concentrated NTP Labeling Mixture, 10x concentrated Transcription Buffer, 10U RNase inhibitor (RNaseOut, Invitrogen) and 20U SP6, T7 or T3 RNA Polymerase (Roche). Template DNA was digested with DNaseI (Roche) for 15 min at 37°C. The reaction was stopped with 1 µl EDTA, the volume was then made up to 100 µl with dH₂O. Probes were precipitated with 1/3 volume of 10M NH₄Acetate and 2.5 volumes of 100% EtOH on ice for a minimum of 10 min. Probes were then pelleted by centrifuging for 10 min at 4°C, washed with 70% EtOH and air-dried. Probes were re-suspended in 100 µl dH₂O and 5 µl of the boiled probe was run on a 1% agarose gel to analyse quality and purity. Typically, probes were diluted 1:200 in Hybe^{+/+} for use in WISH (p. 72).

2.5.5 Whole mount *in situ* hybridisation

Whole mount *in situ* hybridisation (WISH) was carried out as described before (Jowett and Yan, 1996). Embryos stored in MeOH (p. 61) were re-hydrated through a series of 5 min washes of 75%, 50% and 25% MeOH, followed by 3x 5 min washes in PBStw. Embryos older than 26 hpf were bleached for 5-30 min in Bleaching Solution on a light

box or until all pigment had been bleached. Bleached embryos were then washed 3-5 times for 5 min in PBStw. Embryos between 24 and 48 hpf were treated with 0.05% proteinaseK in PBStw for 3 to 20 min, according to developmental stage. Embryos between 49 hpf and 4 dpf were treated with 0.1% proteinaseK in PBStw for 15 to 35 min. ProteinaseK activity was stopped by 2x 5 min washes in 0.1M glycine, followed by a 20 min fixation in PFA. Embryos were washed 3-5x in PBStw for 5 min, followed by a wash in Hybe^{+/+}/PBStw for 5 min. Embryos were pre-hybridised in Hybe^{+/+} for 1-3 hrs at 65°C before hybridisation with probes in Hybe^{+/+} (usually at a dilution of 1:200) at 65°C O/N.

Probes were replaced with pre-heated Hybe^{-/-} and re-used multiple times. The following steps of the protocol were performed in a BioLane™ HTI-16V (intavis AG). Embryos were washed through a series of 10 min washes in 75%, 50% and 25% Hybe^{-/-} in 2x SSC, followed by a wash in 2x SSC, all at 65°C. 4 washes in pre-heated 0.2x SSC were followed by a series of 25%, 50%, 75% and 100% MABtw in 0.2x SSC before the blocking step in MABBlock for 1 hr. Anti-Digoxigenin (Roche) antibody was diluted in MABBlock and incubated O/N at 4°C. Embryos were then washed 8x at RT in MABtw.

Embryos were washed 3x for 5 min in AP buffer before addition of developing solution. Staining was developed in the dark at RT or 4°C, as appropriate. To stop the staining process, embryos were washed in PBStw and fixed in PFA for at least 20 min. For storage, embryos were washed 3x in PBS for 5 min, transferred to 80% glycerol and stored in an air- and light-tight environment at RT. Pictures were taken on a dissecting microscope in 100% glycerol.

2.5.6 Quantitative polymerase chain reaction (qPCR)

Quantitative real-time PCR (q-RT-PCR) was carried out using SYBR green on an Applied Biosystems 7500 real-time cycler, according to the manufacturer's instructions. Reactions were adjusted to 20 µl total volume and cycled 40 times before melting curve analysis. Only primers and reactions with a single-peaked melting curve were used in this thesis; thresholds were automatically determined by the 7500 fast software (Applied Biosystems). Data were analysed using the $\Delta\Delta CT$ method as described previously (Dussault and Pouliot, 2006). The mean value of the gene of interest (GOI) was calculated by determining the arithmetic mean of the 3 replicates. Where applicable, a combined value for several housekeeping genes (HK) was determined as the geometric mean of the individual arithmetic means of the housekeeping genes. The $\Delta\Delta CT$ values between control and sample was determined using the following equation:

$$\Delta\Delta CT = (Ct_{GOIcontrol} - Ct_{HKcontrol}) - (Ct_{GOItest} - Ct_{HKtest})$$

Fold change of genes of interest were approximated by: $\text{Fold change} = 2^{-\Delta\Delta CT}$

Results from biological replicates were combined and error bars are representative of the standard error about the mean:

$$SEM = Stdev * \sqrt{n}$$

Significance was assessed using a two-tailed Student's t-test.

2.5.7 RNA sample preparation for RNA sequencing and quality/quantity determination

10 µl of RNase-free H₂O were added to each sample after elution, adding up to 28 µl of total RNA sample assuming that 2 µl of the sample were lost during the elution process. 2 µl was taken out as a control to be retained at -80°C in case confirmation of results was

needed. A further 1 µl was diluted with 9 µl RNase-free H₂O for quality and quantity control using the 2100 bioanalyzer Pico kit (Agilent technologies).

RNA-seq and differential expression analysis was carried out by the Wellcome Trust Centre for Human Genetics (University of Oxford).

2.5.8 Nanostring

70ng of RNA were used for the nanostring experiment. The hybridisation reaction was set up according to the manufacturer's instructions, with a final solution of 10 µl Reporter CodeSet, 10 µl hybridization buffer, a total volume of 5 µl of sample RNA, and 5 µl Capture ProbeSet (Nanostring technologies). Samples were hybridised in a thermocycler with heated lid (DNA engine) at 65°C over night, before transfer to the ncounter prep station (nanostring technologies).

2.6 Microscopy, imaging and statistical analysis

2.6.1 Still digital photography

Phenotypes were scored by assessing the expression pattern in wt embryos. The number of embryos exhibiting a phenotype was counted and representative pictures were captured with a MicroPublisher 5.0 rtv camera attached to a Nikon SMZ-U 1500 dissecting microscope. Images were captured with the Q-Capture Pro 7™ software. White balance and exposure were set automatically for control samples and carried over for test samples. Embryos were illuminated from the top and bottom on a glass

depression slide, or from the top on a white depression slide, as appropriate. Pictures represent the majority of the phenotype across a minimum of 3 biological replicates, unless otherwise indicated. A detailed breakdown of numbers of embryos showing an increase, decrease or no effect in/on marker gene expression for each figure is given in Appendix Table 8-1 (p.235)

2.6.2 Wide-field fluorescence imaging

Live embryos were imaged in E3 medium + MS222 or mounted in 3% methylcellulose. Embryos were assessed and imaged on a SteREO Lumar V12 microscope (Zeiss). Exposure was manually fixed for each experiment. Image acquisition and analysis was conducted with the Axio Vision software (Zeiss).

2.6.3 Image analysis and representation

Acquired images were adjusted and assembled for figures with Adobe Photoshop CS5 or CS6. Images were adjusted for levels, curves and colour, where appropriate. Adjustments were always conducted on control images; images of test embryos were adjusted identically to control embryos. All images are represented from a lateral view, with anterior to the left and dorsal to the top, unless otherwise stated.

2.7 Bioinformatic tools

2.7.1 Genomic, transcript, and protein sequence information

All sequence information (genomic, transcript, and protein) for zebrafish, mouse and human referred to in this thesis have been obtained from the Ensembl database (Flicek et al., 2014). Reference genomes are Zv. 9 (*D. rerio*), GRCh37 (*H. sapiens*) and GRCm38 (*M. musculus*).

2.7.2 Analysis of homology, identity, and synteny

All multiple sequence protein alignment has been carried out using ClustalW2 or ClustalOmega (both EMBL-EBI) as previously described (Sievers et al., 2011). Synteny of genomic loci was evaluated using the Genomicus browser, and to obtain gene trees (Muffato et al., 2010, Louis et al., 2013).

2.7.3 Predictions of conserved protein domains

Conserved protein domains were predicted and obtained from the Conserved Domain Database (CDD; NCBI) (Marchler-Bauer et al., 2011).

Chapter 3

The role of the Notch ligand *dll4* in definitive haematopoiesis

3.1 Introduction

The highly conserved Notch signalling pathway plays multiple important roles during vertebrate development (reviewed in Andersson et al., 2011) and haematopoiesis (reviewed in Bigas and Espinosa, 2010 and Gering and Patient, 2010). The involvement of Notch receptors in arterial development and in the emergence of haematopoietic stem cells (HSCs) is well established; however, less is known about the Notch ligands required (Lawson et al., 2001, Burns et al., 2005, Gering and Patient, 2005, Robert-Moreno et al., 2008, Clements et al., 2011, Bigas and Espinosa, 2012). The aim of this chapter is to identify the most promising ligand for a regulatory role in HSC emergence, if any, and subsequent functional analysis thereof.

Each component of the Notch signalling pathway has a distinct spatio-temporal expression pattern throughout embryonic development (Gering and Patient, 2010), and expression activity is highly indicative of functional roles. Therefore, the expression patterns of all known Notch ligands in the zebrafish have here been investigated at two pivotal timepoints in HSC development, 26 hpf and 36 hpf. The Notch ligand *Dll4* is expressed in the arterial and neural tissues across species before and around the time of HSC emergence (Kumano et al., 2003, Benedito and Duarte, 2005, Leslie et al., 2007, Siekmann and Lawson, 2007, Robert-Moreno et al., 2008). While mice carrying *Dll4*^{-/-} null mutations exhibit severe arterial abnormalities resulting in a non-functional vasculature, knockdown in the zebrafish has previously been described to cause only a late angiogenic and lymphatic phenotype (Krebs et al., 2000, Duarte et al., 2004, Leslie et al., 2007, Siekmann and Lawson, 2007, Hogan et al., 2009, Geudens et al., 2010). Developmental gene expression profiles in the DA are largely normal in the absence of *dll4* in the zebrafish and no arterial phenotype has been observed prior to 28-32 hpf

(Hogan et al., 2009). The absence of a comparably severe arterial phenotype in *dll4* zebrafish morphants offers the possibility of assessing the role of this Notch ligand in HSC development without a prior effect on the DA as in the mouse (Robert-Moreno et al., 2008). As no assessment of the haematopoietic programme has been conducted in the absence of *dll4* in the zebrafish, and has only focussed on HSC derivatives in the mouse and human (Koch et al., 2008, Poirault-Chassac et al., 2010, Billiard et al., 2011), the main objective in this chapter has been to assess the role of *dll4* in HSC development.

Here I show the earliest yet known requirement for *dll4* in zebrafish development, namely the specification of HSCs from 24 hpf onwards, and confirm that this phenotype is not due to abnormal arterial development. All markers for HSCs and their derivatives assessed here are down-regulated if not absent in *dll4* morphants and *runx1* is sufficient to rescue the down-regulation of *cmyb* at 36 hpf in *dll4* morphant embryos.

3.2 Expression pattern of Notch ligands around the time of haematopoietic stem cell emergence in the zebrafish embryo

In order to investigate which Notch ligands are good candidate genes for a role in HSC emergence, their expression was assessed at 26 hpf, when expression of *runx1* has just been initiated in the dorsal aorta (DA) (Kalev-Zylinska et al., 2002). These cells are believed to contain those that will ultimately become HSCs (Burns et al., 2005, Gering and Patient, 2005). Expression was also assessed at 36 hpf, when *runx1* positive cells first start to undergo the endothelial-to-haematopoietic transition (EHT) and express further HSC markers such as *cmyb* and *ikzf1* (*ikaros*) (Gering and Patient, 2005, Kissa and Herbomel, 2010, Soza-Ried et al., 2010). As definitive haematopoiesis is restricted to

the posterior tissues (reviewed in Ciau-Uitz et al., 2014), the expression analysis of Notch ligands has here focussed on their expression in this part of the embryo.

There are 4 members of the *jagged* family of Notch ligands in the zebrafish, and 5 of the *delta* family (Bradford et al., 2011). Both vertebrate *jagged* genes are duplicated in the zebrafish (Table 3-1) and are expressed broadly. *Jag1a* appears to be expressed broadly at low levels across the tissues of the trunk/tail region, including the central nervous system (CNS, Figure 3-1-A). This low-level broad expression persists at 36 hpf, with higher expression levels observed in the CNS, gut and lateral line (Figure 3-1-B).

Similarly *jag1b* expression is broad at low levels throughout the tissues of the trunk and tail. However, higher levels are observed in the somites and the CNS at 26 hpf (Figure 3-1-C). Expression in these tissues is reduced to basal levels at 36 hpf, while higher expression is present in the notochord (NC) and pronephric duct (Figure 3-1-D).

Table 3-1. Notch receptor and ligand orthologues.

Orthologue assignment was carried out using the Ensembl orthologue annotation function (Flicek et al., 2014).

	D. rerio	H. sapiens	M. musculus	G. gallus	X. tropicalis
notch receptors	<i>notch1a, notch1b</i>	<i>NOTCH1</i>	<i>Notch1</i>	<i>NOTCH1</i>	<i>notch1</i>
	<i>notch2</i>	<i>NOTCH2</i>	<i>Notch2</i>	<i>NOTCH2</i>	<i>notch2</i>
	<i>notch3</i>	<i>NOTCH3</i>	<i>Notch3</i>	-	<i>notch3</i>
	-	<i>NOTCH4</i>	<i>Notch4</i>	-	<i>notch4</i>
jagged ligands	<i>jag1a, jag1b</i>	<i>JAG1</i>	<i>Jag1</i>	<i>JAG1</i>	<i>jag1</i>
	<i>jag2a, jag2b</i>	<i>JAG2</i>	<i>Jag2</i>	<i>JAG2</i>	<i>jag2</i>
delta ligands	<i>dla, dlb, dlc, dld</i>	<i>DLL1</i>	<i>Dll1</i>	<i>DLL1</i>	<i>dll1</i>
	-	<i>DLL3</i>	<i>Dll3</i>	-	-
	<i>dll4</i>	<i>DLL4</i>	<i>Dll4</i>	<i>DLL4</i>	<i>dll4</i>

The *JAG2* orthologue has also undergone a gene duplication in the zebrafish, resulting in *jag2a* and *jag2b* (Table 3-1). *Jag2a* has only been recently annotated, and expression analysis conducted here reveals that it is expressed broadly at low levels throughout the embryo and at higher levels in the brain tissues, the pronephric duct and the ectoderm at 26 hpf (Figure 3-1-E). Ectodermal expression follows a gradient of high expression in the posterior to low expression in the anterior, persisting at 36 hpf, however with a generally less extreme gradient at lower levels of expression (Figure 3-1-F). Pronephric expression is not visible at 36 hpf, while expression appears in the lateral line and is maintained in the brain.

The other *Jag2* paralogue, *jag2b*, appears to be expressed in most tissues at low levels at 26 hpf, with higher expression in the brain and NT, the somites, the pronephric duct and the ectoderm. Similar to *jag2a*, *jag2b* is expressed in the ectoderm and the somites along an anterior-posterior gradient with higher expression levels towards the posterior (Figure 3-1-G). At 36 hpf, this expression is maintained with additional expression in the NC (Figure 3-1-H). Again, similarly to *jag2a* expression, the gradient of expression appears less extreme at this later timepoint.

There are 5 members of the *delta* family of Notch ligands in the zebrafish: *dla*, *dlb*, *dlc*, *dld* and *dll4*. The first four are paralogues of *DLL1*, while *dll4* is the single orthologue of *DLL4* (Table 3-1). At 26 and 36 hpf, *dla* appears to be exclusively expressed at high levels in cells of the neural tissues (Figure 3-2-A&B), as is *dlb* (Figure 3-2-C&D). *Dlc* is also expressed in neural tissues at both timepoints. Expression is also observed in the dorsal aorta and intersomitic vessels (ISVs) at 26 hpf (Figure 3-2-E) until 30 hpf (Burns et al., 2005, Patterson et al., 2007, Qian et al., 2007), but not at 36 hpf (Figure 3-2-F). At 26 hpf,

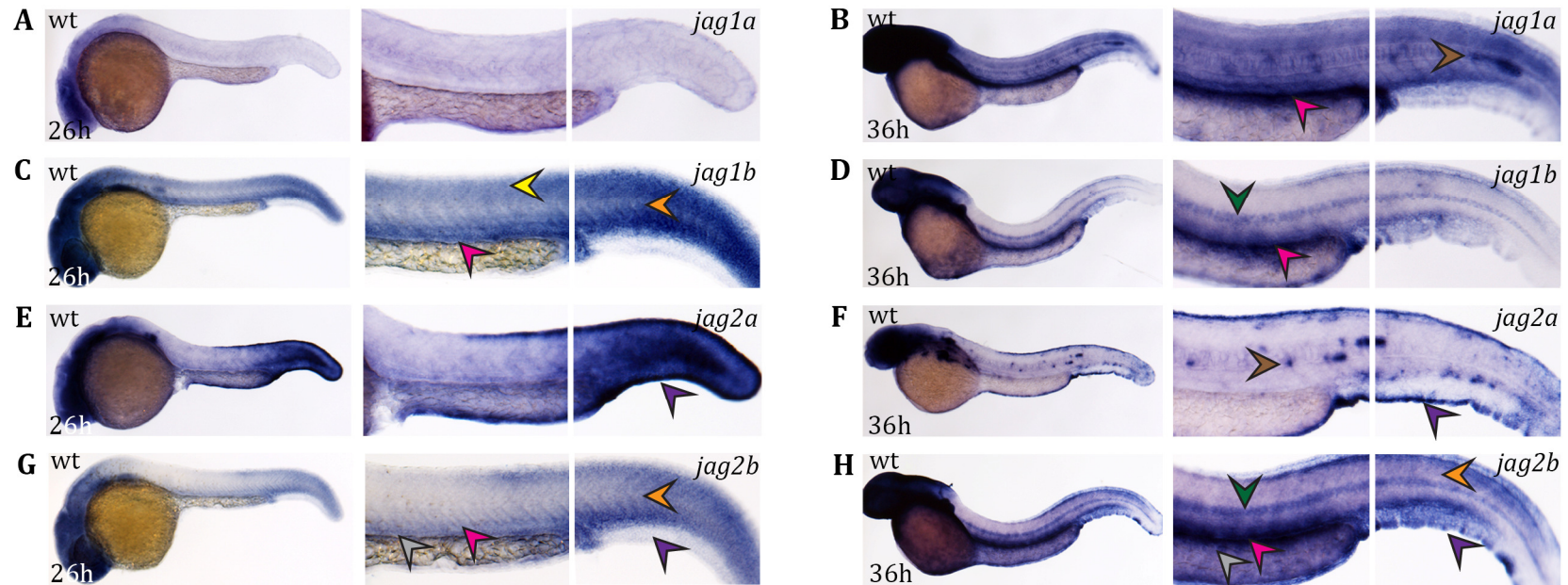


Figure 3-1. Expression of the *jagged* family of Notch ligands in the zebrafish embryo around the early stages of HSC development.

jag1a is expressed in the brain and broadly at low levels at 26 hpf (A), at 36 hpf expression is broad and at higher levels in the CNS, lateral line and gut (B). *jag1b* is expressed in the CNS, the somites, the pronephric duct and the otic vesicle at 26 hpf (C), at 36 hpf, expression is broad with higher levels in the NC and pronephric duct (D). *jag2a* is expressed broadly at low levels and at higher levels in the brain, pronephric duct and ectoderm at 26 hpf (E); expression is maintained in the ectoderm at 36 hpf and the lateral line also expresses *jag2a* (F). *jag2b* (*jag2*) is expressed in the CNS, epidermis, pronephric duct and the somites at 26 hpf (G), additional expression in the NC is observed at 36 hpf (H). Arrows indicate posterior tissues: CNS (NT, yellow), somites (orange), NC (dark green), gut (pink), lateral line (brown), pronephric duct (grey) and ectoderm (purple).

dlc expression is visible in the somites and persists until 36 hpf, in contrast to expression in the NC which is detectable at 36 hpf, but not before (Figure 3-2-F).

The single *DLL4* orthologue in *Danio rerio* (Table 3-1, p. 81) is expressed in the DA and the ISVs but not the posterior cardinal vein (PCV) at 26 hpf (Figure 3-2-I), but not at 36 hpf (Figure 3-2-J). In addition, *dll4* is also expressed in a subset of ventrolateral neurons in the NT and parts of the brain at both timepoints (Figure 3-2-I&J), while expression in the NC is detected at 36 hpf only. (Figure 3-2-J).

I have shown here that, of all 9 zebrafish Notch ligands, only *dlc* and *dll4* are expressed at high levels in the DA around the time of HSC specification. Expression in the DA indicates that these genes are active in the appropriate tissue for a role in HSC development, and both have indeed been implicated in vertebrate HSC development before (Robert-Moreno et al., 2008, Clements et al., 2011). Since *dlc* has been shown to be required for HSC development through its somitic activity very early in zebrafish development (Clements et al., 2011), and *dlc* is one of four paralogues of the mammalian *Dll1*, this gene was not considered as interesting for further analysis as *dll4*. *Dll4* has been implicated in haematopoiesis in the mouse, through the loss of *runx1* expression at E9.5 (Robert-Moreno et al., 2008), however its role in HSC specification has not been studied in depth, due to its preceding requirement in arterial specification in the mouse (Krebs et al., 2000, Duarte et al., 2004), a phenotype that is not observed in the zebrafish (Leslie et al., 2007, Hogan et al., 2009).

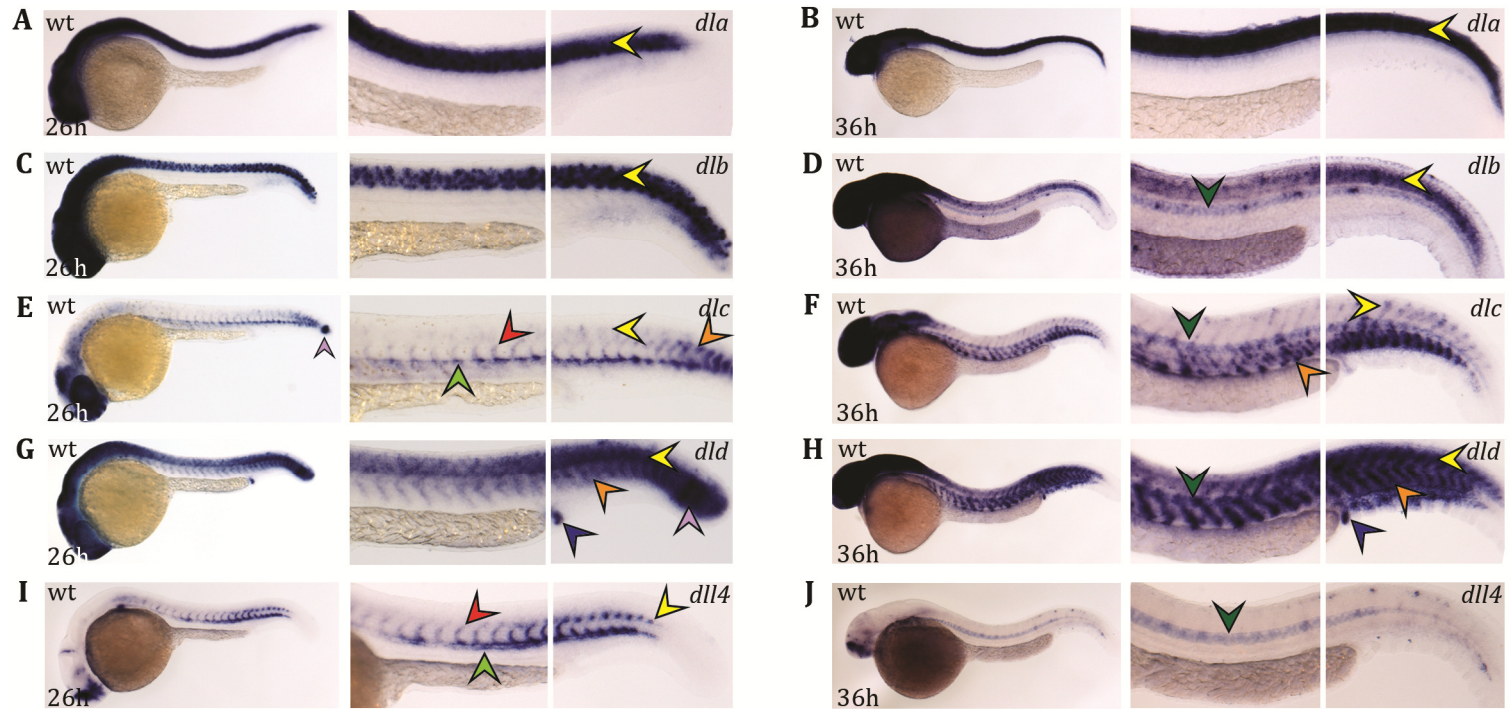


Figure 3-2. Expression of the *delta* family of Notch ligands in the zebrafish embryo around the early stages of HSC development.

dla (A,B) and *dlb* (C,D) are expressed in the CNS at 26 and 36 hpf, *dlb* is also expressed in the NC at 36 hpf (D). *dlc* is expressed in the DA and ISVs, somites, the NT and the eyes at 26 hpf (E). At 36 hpf, expression in the somites and NT is increased, expression in the NC is gained and expression in the DA and tailbud is lost (F). *dld* is expressed in the CNS, the anus, the somites and the tailbud at 26 hpf (G), its somitic expression increases by 36 hpf and expression in the NC is gained, tailbud expression is lost (H). *dll4* is expressed in parts of the brain and NT, the DA and ISVs at 26 hpf (I); at 36 hpf expression in DA and ISVs is not detectable by WISH and the NC expresses *dll4* (J). Arrows indicate posterior tissues: DA (light green), ISVs (red), CNS (NT, yellow), somites (orange), NC (dark green), tail bud (lilac), gut (pink) and anus (dark blue).

Published data agrees that arterial *dll4* expression fades between 30 and 48 hpf in the zebrafish (Siekman and Lawson, 2007, Wang et al., 2011); however the exact timepoint is less clear. Therefore, the expression pattern of *dll4* has here been investigated in more detail: at 10ss, no expression of *dll4* can be discerned by WISH (Figure 3-3-A); however, when using RT-PCR on whole-embryo RNA extractions, *dll4* expression is detected (Figure 3-3-F), suggesting low-level expression below the detection threshold of WISH, consistent with previous reports (Leslie et al., 2007). At 20ss, *dll4* is expressed in the newly formed DA and neural cells (green and yellow arrows, Figure 3-3-B). Arterial expression of *dll4* is expanded into the forming ISVs at 24 hpf (red arrow, Figure 3-3-C). All arterial cells express *dll4* at this stage (Leslie et al., 2007). *Dll4* expression in the DA at 26 hpf is indistinguishable from that at 24 hpf, and it is thus considered to be expressed by all arterial cells of the trunk/tail at this stage (Figure 3-2-I). By 27 hpf in contrast, expression is fading in the anterior parts of the DA and ISVs (Figure 3-3-C) and is not detectable by WISH in the vasculature at 34 hpf and 36 hpf, while persisting in the brain (Figure 3-3-E and Figure 3-2-J, p. 85). PCR on whole-embryo RNA extractions, still show *dll4* RNA at these developmental stages, as well as at 2, 3 and 4 dpf (Figure 3-3-F), likely reflecting expression of *dll4* in the brain and potentially at low levels in other tissues.

In conclusion, it has here been confirmed that *dll4* is expressed in the arterial cells of the trunk/tail region in the zebrafish embryo between 20ss and 26 hpf, as previously suggested (Leslie et al., 2007). *Dll4* expression fades at 27 hpf in an anterior-to-posterior fashion and is not detectable in the arterial trunk vasculature by WISH at 34 hpf, consistent with findings by Siekman et al (2007). This suggests that a role for *dll4* in HSC

development, if any, is likely to be before 27 hpf, while *dll4* is still expressed in the region of the DA where HSCs emerge.

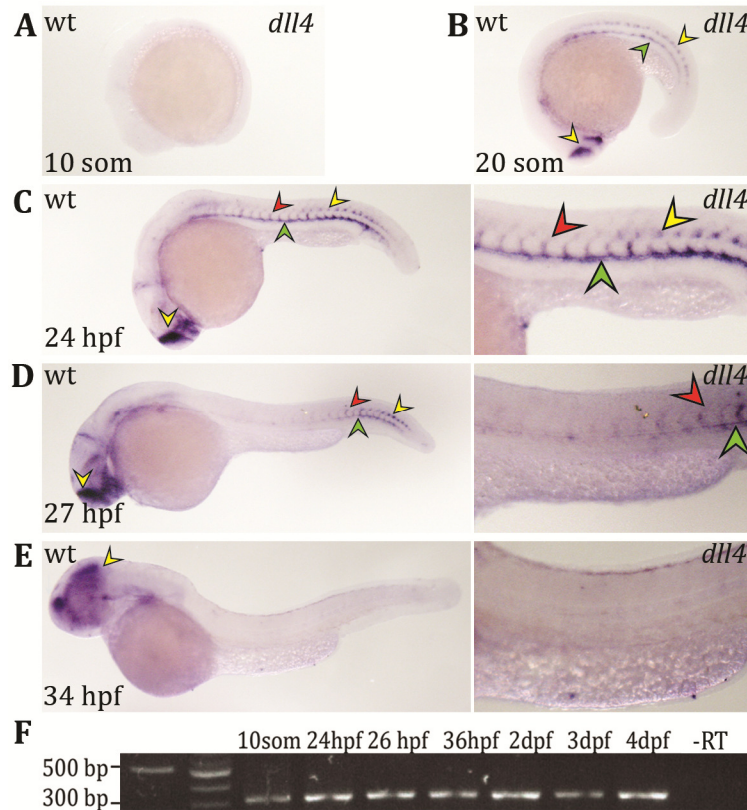


Figure 3-3. *Dll4* expression in the developing zebrafish embryo.

A-E Timecourse of *dll4* expression by WISH: at 10ss, no specific expression of *dll4* can be visualised using WISH (**A**). First specific expression of *dll4* is observed at 20ss in the DA precursors, neural cells and the telencephalon (**B**). At 24 hpf, expression of *dll4* is uniform in the DA along the AP axis and expression extends into the sprouting ISVs (**C**). By 27 hpf expression in the DA, ISVs and neural tissues is confined to the most posterior part of the tail (**D**). All localised expression in the trunk and tail is absent by 36 hpf while expression in the telencephalon persists (**E**). Arrows indicate DA (green), ISVs (red) and neural tissues (yellow). **F**. *Dll4* expression timecourse analysis by RT-PCR, indicating that *dll4* is expressed from 10ss until at least 4 dpf.

3.3 Choosing and titrating *dll4* morpholinos

In the absence of zebrafish *dll4* mutants at the time of experimentation, a MO strategy was chosen. Morpholinos that have previously been shown to be specific to *dll4* and not to cause non-specific off-target effects were available (Siekmann and Lawson, 2007, Hogan et al., 2009). Six MOs have been published for *dll4* (Leslie et al., 2007, Siekmann and Lawson, 2007, Hogan et al., 2009, Geudens et al., 2010, Biyashev et al., 2012), and here MO1 (Siekmann and Lawson, 2007) and MO4 (Hogan et al., 2009) have been used for phenotypic analysis, based on their published high phenotypic penetrance and thorough characterization. Both morpholinos are splice inhibitors, with MO1 binding between the N-terminus of the Notch ligand domain and the Delta-Serrate-Lag2 (DSL) domain (Appendix Figure 8-2-B, p. 245), and was shown to result in a non-functional *dll4* protein (Siekmann and Lawson, 2007). *Dll4* MO4 binds downstream of this domain (Appendix Figure 8-2-B, p. 245), and has been also shown to result in a non-functional protein product (Hogan et al., 2009). In order to confirm that these morpholinos work, RT-PCR analysis of cDNA synthesized from wt and *dll4* morphants has been conducted here. Levels of the housekeeping gene *eef1a1l1* (*ef1 α*) are comparable between wt and morphant embryos. Two bands are amplified in wt samples with a primer pair designed to amplify the region between exon 1 and 3, the smaller being representative of *dll4* mRNA and the larger of yet unspliced *dll4* pre-mRNA. In *dll4*MO1 samples, only the larger band is observed and its levels are increased (Figure 3-4-B). These results show that *dll4*MO1 targets *dll4* and that at least part of intron 1 is retained in these morphants (Figure 3-4-D), confirming previous reports (Siekmann and Lawson, 2007).

Similar results are obtained for *dll4*MO4: a primer pair with an exonic forward and an intronic reverse primer amplify a more intense band in *dll4*MO4 samples as compared

to wt, while semi-quantitative PCR for *eef1a111* (*ef1α*) reveals slightly lower levels of RNA present in dll4MO4 samples as compared to wt (Figure 3-4-C). This indicates that intron 4 is at least partially retained in embryos injected with dll4MO4 (Figure 3-4-D), consistent with previous reports (Hogan et al., 2009).

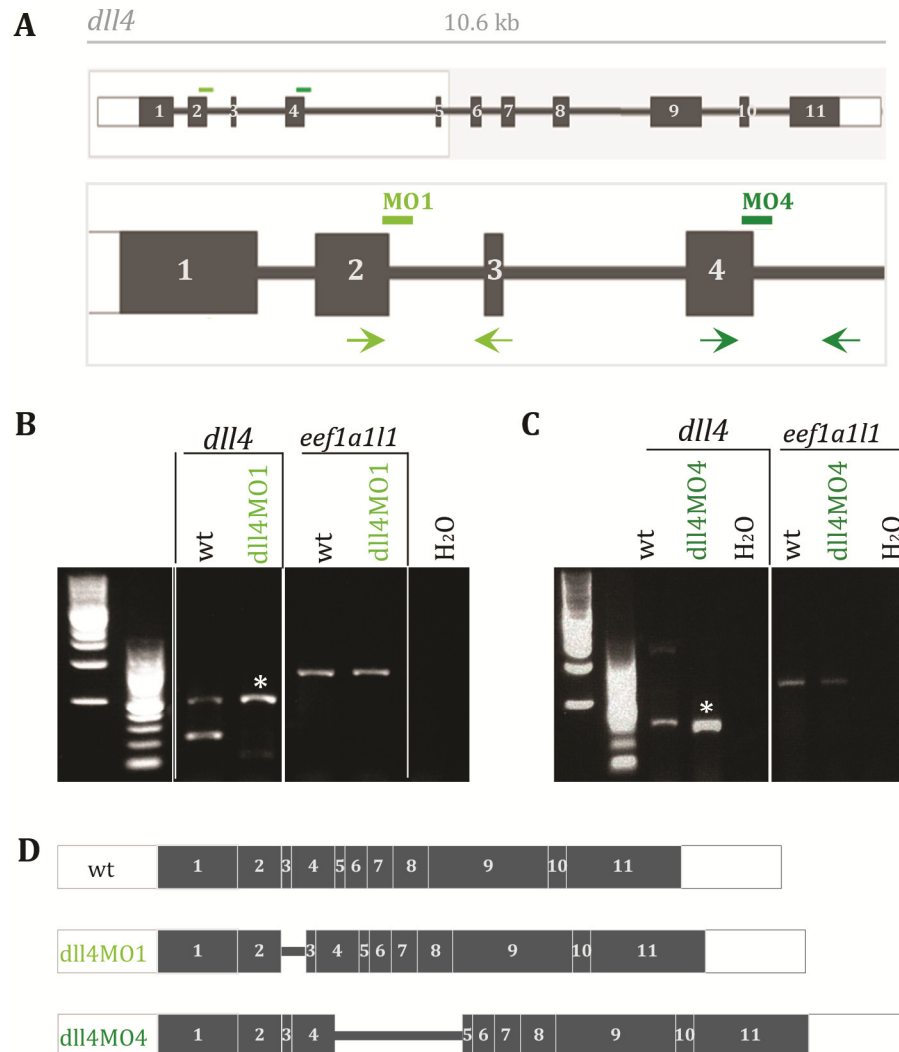


Figure 3-4. *Dll4* morpholino design and testing of specificity.

A Schematic representation of the exon-intron structure of the *dll4* gene with MO binding sites and primer pair design (arrows). **B-C** Comparative RT-PCR analysis of splicing in wt embryos and siblings injected with 5ng *dll4*MO1 (**B**) and 6ng *dll4*MO4 (**C**), respectively. Asterisks indicate enhanced levels in morphant cDNA. **D** Schematic representation of the effect of *dll4*MO1 and *dll4*MO4 on *dll4* mRNA; both MOs retain at least part of intron 2 and 4, respectively.

The detailed assessment of the expression pattern of *dll4* and the reproduction of morpholino specificity for *dll4*MO1 and *dll4*MO4 at the same concentrations as previously published (Siekman and Lawson, 2007, Hogan et al., 2009), provided the baseline for a detailed functional analysis of *dll4* in HSC development.

For the novel characterization of the HSC phenotype, both MOs have been simultaneously injected for all experiments and their phenotypes compared, to ensure specificity for loss of *dll4* function, according to the standard procedures proposed previously (Pickart et al., 2006).

3.4 *Dll4* is required for definitive haematopoiesis in the zebrafish embryo

The HSC programme has not previously been assessed in the absence of *dll4* in the zebrafish. When assessing the expression of *runx1* from when it first appears in the DA in the zebrafish embryo around 24 hpf, a near loss of *runx1* positive cells is observed (Figure 3-5-A). This could either be due to an absence of HSCs or a delay in the onset of the HSC programme. In order to address this question, a timecourse of *runx1* expression was conducted every 2 hrs (Figure 3-5), up to 32 hpf, by which time the HSC programme is established in the zebrafish and the very first EHT events are observed in wt embryos (Kissa and Herbomel, 2010). Near absence of *runx1* positive cells is still observed at 26 hpf (Figure 3-5-B). At 28 hpf *runx1* positive cells have increased in numbers in the wt and morphants, albeit at lower numbers (Figure 3-5-C). *Runx1* cells continue increasing at 30 hpf and 32 hpf, with many fewer cells visible in the *dll4* morphants (Figure 3-5-D&E). The timecourse revealed a consistent reduction in *runx1* positive cells in

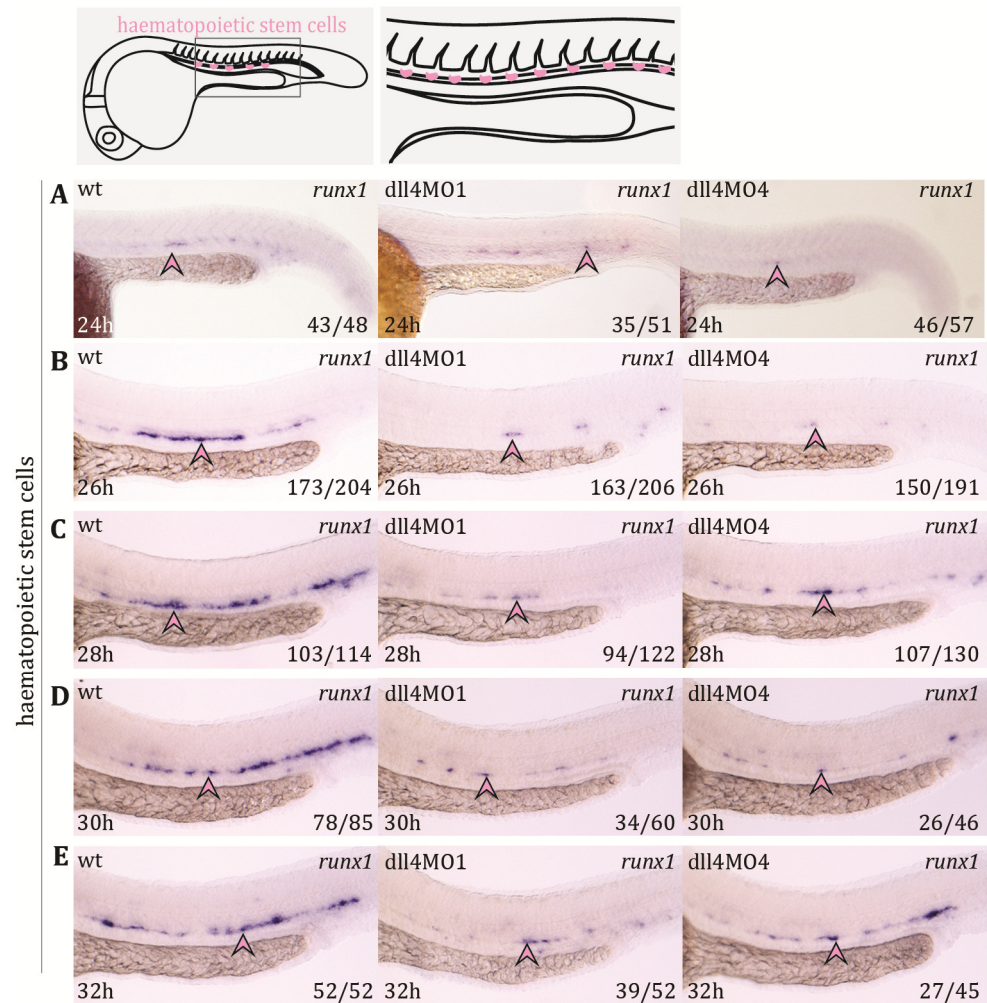


Figure 3-5. *Runx1* positive cells are reduced in the DA in *dll4* morphants.

Timecourse of loss of *runx1* expression in *dll4* morphants. Numbers of *runx1* positive cells in the DA are reduced between 24 hpf (A) and 32 hpf (D) in both *dll4*MO1 and MO4. Arrows indicate HSCs (pink). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

the DA of *dll4* morphant fish (Figure 3-5), arguing against merely a delayed onset of definitive haematopoiesis in the absence of *dll4*.

Another way to address the question of whether the reduction in *runx1* is merely a temporal phenomenon or represents a true, permanent effect on the definitive haematopoietic programme, is the assessment of additional HSC marker genes. This

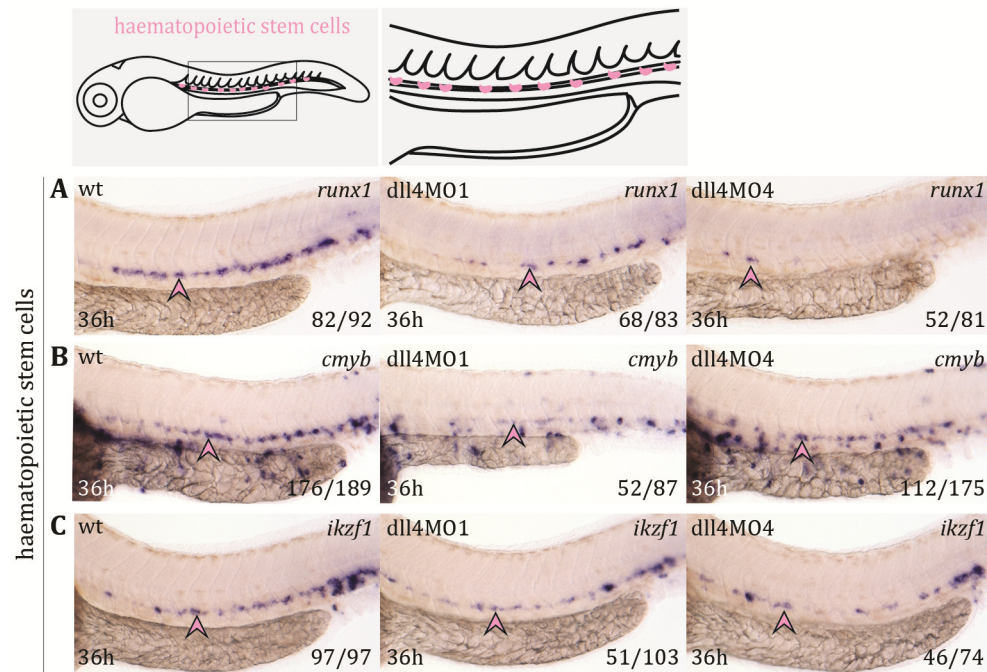


Figure 3-6. The loss of *runx1* in *dll4* morphants represents a loss of HSCs.

The number of *runx1* positive cells is reduced at 36 hpf in both *dll4* morphants (A). Expression of *cmyb* is also markedly reduced in the DA as compared to in the wt siblings (B), as are the numbers of *ikzf1* (*ikaros*) positive cells. Arrows indicate HSCs (pink). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

further allows to distinguish between an effect on *runx1* gene expression and an effect on the HSC programme. At the early timepoints of HSC emergence, the only reliable marker for assessment is *runx1* (Gering and Patient, 2005), as other HSC markers such as *ikzf1* (*ikaros*) and *cmyb* are also expressed in primitive blood at these timepoints (Thompson et al., 1998, Patterson et al., 2005), which overlap in location with that of definitive haematopoiesis in the zebrafish (Davidson and Zon, 2004). Therefore, assessment of additional markers was only possible at later stages, i.e. 36 hpf, when expression of *ikzf1* and *cmyb* is restricted to the definitive lineage (Gering and Patient, 2005). *Runx1* expression is still severely down-regulated in both *dll4* morphants at 36 hpf (Figure 3-6-A). Numbers of *cmyb* expressing cells are also reduced (Figure 3-6-B), as

is expression of *ikzf1* in the DA of *dll4* morphants at this timepoint (Figure 3-6-C), consistent with the notion that *dll4* is required for definitive haematopoiesis, all HSC markers assessed at 36 hpf are reduced in *dll4* morphants.

All HSC gene expression assessed here is down-regulated in *dll4* morphants, proposing a role for *dll4* in emergence of HSCs, upstream of *runx1*. If *dll4* is only upstream of *runx1*, it would be expected that overexpression of *runx1* in *dll4* morphants could rescue the HSC phenotype. To test this hypothesis, embryos were injected with *dll4*MO4 alone or with a full length *runx1* cDNA construct driven by *eef1a1l1* (*ef1α*) and flanked by *tol2* sites together with *tol2* mRNA (constructs kindly provided by Dr. Philip Pinheiro). Injection of the *runx1* construct + *tol2* mRNA alone and uninjected wt embryos acted as controls (Figure 3-7-A). In this experiment, *cmyb* is severely down-regulated in *dll4* morphants as compared to uninjected wt siblings (Figure 3-7-B), consistent with data shown above. The *runx1* overexpression construct rescues *cmyb* staining in the DA induced by *dll4*MO4 to wt levels (Figure 3-7-B), showing that *runx1* is downstream of *dll4* and that the effect of *dll4* on *runx1* expression is sufficient to explain the reduction of *cmyb* in the *dll4* morphants.

Next, I tested whether *runx1* can also regulate *dll4*. In order to test whether there is a feedback loop, *dll4* expression was assessed in dissected trunk/tail tissues of embryos injected with *dll4*MO4 or *runx1*MOe1 (Gering and Patient, 2005) by qPCR to confirm the observed absence of an effect as indicated by WISH analysis (data not shown). qPCR values were normalized to *g6pd*, wt values were set to 1. Log2 fold changes were calculated, resulting in wt values and values for genes not differentially expressed, represented at a value of 0. In the absence of known *runx1* targets at this early developmental stage (26 hpf), a positive control for *runx1*MOe1 could unfortunately not

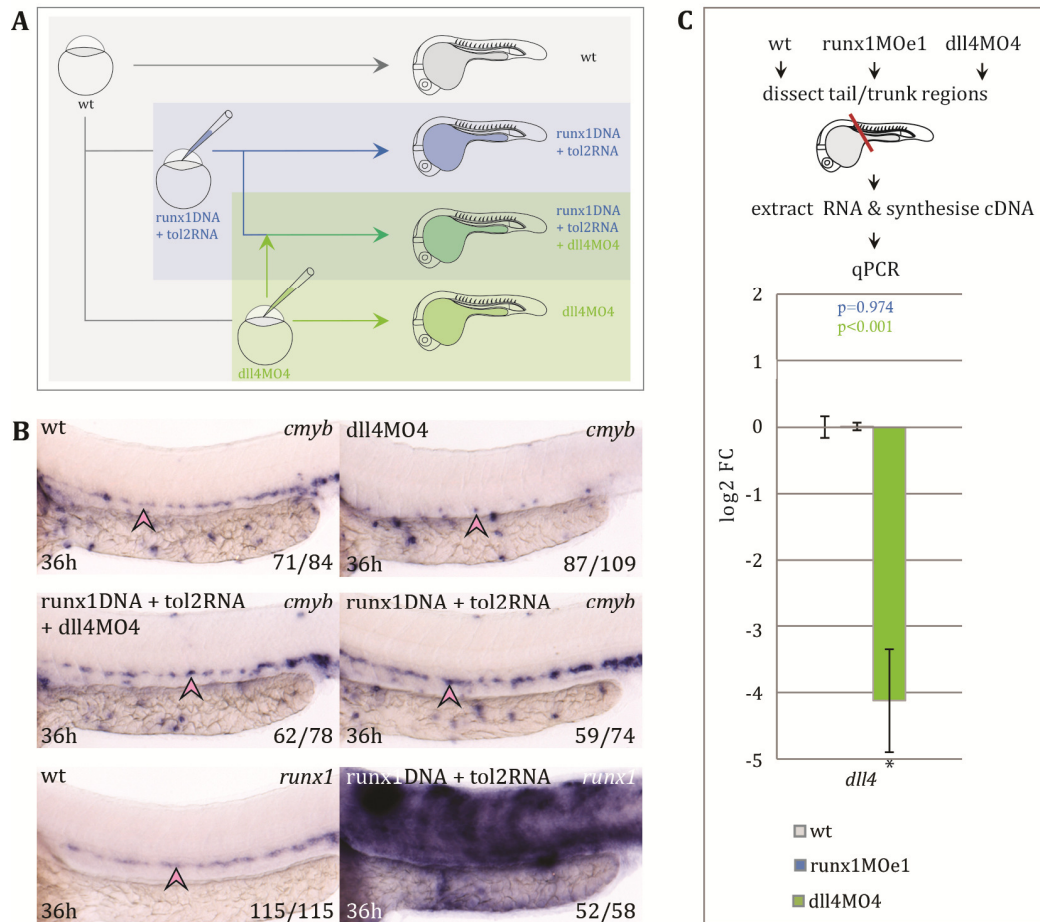


Figure 3-7. The *dll4* HSC phenotype is rescued by *runx1* overexpression but *dll4* is not affected in *runx1* morphants.

A Schematic representation of the experiment: wt embryos were injected with runx1DNA and tol2RNA constructs, dll4MO4 was injected into wt embryos or embryos injected with the constructs, resulting in 4 batches of embryos. **B** *Dll4* morphants show a severe down-regulation of *cmyb* expression in the dorsal aorta, that is rescued upon co-injection with runx1DNA and tol2RNA constructs. *Cmyb* levels in embryos injected with runx1DNA and tol2RNA constructs are comparable to wt siblings, if slightly up-regulated. Embryos injected with runx1DNA + tol2RNA constructs show vast up-regulation of *runx1* transcript, indicating that the constructs are working. Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235). **C** Embryos were injected with runx1MOe1 or dll4MO4 at the 1 cell stage, trunk/tail regions were dissected at 26 hpf, RNA extracted, cDNA synthesized and qPCR conducted: *dll4* is significantly down-regulated in dll4MO4 but is not differently expressed in runx1MOe1. Technical triplicate values were pooled from two independent experiments, normalized to *g6pd*, log2 converted and wt values set to 0 for comparison. Error bars are indicative of the standard deviation, asterisks indicate statistical significance for $p < 0.05$.

be included in the qPCR experiment. The qPCR data reveals that expression of *dll4* is only significantly affected in the *dll4* ($p < 0.01$) but not *runx1* morphants (Figure 3-7-C). The absence of a change in *dll4* expression in *runx1e1MO* suggests that *runx1* is not regulating *dll4* at the time of HSC emergence in zebrafish trunk/tail tissues, while the reduction in *dll4* expression in *dll4MO4* likely indicates de-stabilisation of the *dll4* RNA by *dll4MO4*. Therefore, this cannot be interpreted as an auto-regulatory mechanism of *dll4* with the experimental setup conducted here. It does however confirm that *dll4MO4* is active on *dll4* RNA.

In conclusion, data presented here shows that *dll4* is required for *runx1* expression from its onset in the DA, and that all HSC markers assessed are reduced in *dll4* morphants. Reduction of *cmyb* expression in the absence of *dll4* can be rescued by *runx1* overexpression, showing that the observed *dll4* HSC phenotype predominantly acts through regulation of *runx1*, without a feedback loop.

3.5 *Dll4* is not required for vascular specification in the zebrafish embryo

A loss of the haematopoietic markers *Runx1* and *Gata2* has been observed at E9.5 in *Dll4*^{-/-} null mice, however, it has not been possible to establish a direct requirement for *dll4* in haematopoiesis due to the severe vascular defects in these animals (Duarte et al., 2004, Krebs et al., 2004, Benedito et al., 2008, Robert-Moreno et al., 2008). In contrast, *dll4* morphant zebrafish exhibit arterial hyperbranching while only minor effects are observed in arterial specification (Leslie et al., 2007, Siekmann and Lawson, 2007, Hogan et al., 2009, Bridge et al., 2012). The extent of the arterial abnormalities in the

zebrafish at the time of HSC emergence (26 hpf) needed to be established in order to evaluate the mechanism of the HSC phenotype. It has previously been observed that although the absence of *dll4* causes severe arterial angiogenic defects in zebrafish embryos, no major effects can be observed on the gene expression patterns in the DA at earlier stages (Hogan et al., 2009). The authors did not observe a change in expression of arterial markers *vegfc* and *hey2 (gridlock)* at 26 hpf, while major angiogenic hyperbranching defects were observed in the same morphants at 72 hpf. Here, the hyperbranching effect was confirmed at 72 hpf (Figure 3-8-A), manifesting itself through supernumary branches due to increased numbers of tip cells (Siekman and Lawson, 2007, Hogan et al., 2009, Krueger et al., 2011). In addition, the absence of an effect on the arterial and neuronal expression of *hey2 (gridlock)* in embryos injected with *dll4*MO1 or *dll4*MO4 (Figure 3-8-B) confirmed previous observations (Hogan et al., 2009). The assessment of the arterial programme at 26 hpf was expanded to include *efnb2a* and *flt1 (vegfr1)*, neither of which showed compromised expression in either *dll4* morphant (Figure 3-8-C&D). Levels of expression of *hey2* and *flt1* in the intersomitic vessels were comparable to uninjected wt siblings, and their patterning does not appear to be affected at this early timepoint either. The diameter of the DA appears to be slightly reduced in *dll4* morphants likely due to increased migration of DA cells into the intersomitic vessels, as previously suggested (Leslie et al., 2007, Bridge et al., 2012). This reduction in diameter however does not impede lumenisation or circulation in the axial vessels at 26 hpf, 36 hpf or 4 dpf (data not shown and Bridge et al, 2012). Taken together, I and others (Leslie et al., 2007, Hogan et al., 2009) have shown that arterial patterning in the absence of *dll4* is affected in a minor way that does not appear to impede arterial functioning in the zebrafish at 26 hpf. This arterial effect is unlikely to have an influence on the observed HSC phenotype, as the reduction in *runx1* positive

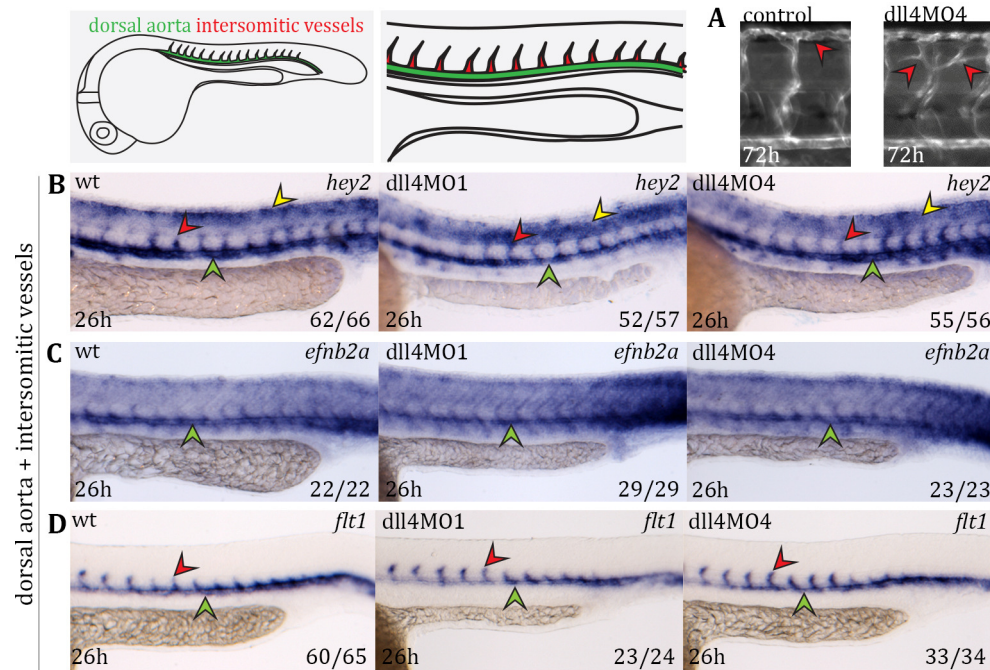


Figure 3-8. Loss of *dll4* activity affects arterial patterning in a minor way at the time of HSC emergence.

Dll4 morphants exhibit an arterial hyperbranching phenotype from 72 hpf onwards (A). At 26 hpf, no defects can be observed in overall DA patterning (B, C, D) or ISV branching (B, D), apart from a slight narrowing of diameter. Arrows indicate DA (green), ISVs (red) and NT (yellow). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

cells (at 24 hpf, Figure 3-5-A) precedes any described and observed arterial phenotype (Leslie et al., 2007, Siekmann and Lawson, 2007, Hogan et al., 2009, Sacilotto et al., 2013).

Hogan and colleagues (2009) have described that the expression of *flt4* (*vegfr3*) is affected in *dll4* morphants at 26 hpf, but not 24 hpf, in the arterial but not venous compartment (Hogan et al., 2009). Here, a very minor up-regulation in expression of *flt4* in the intersomitic vessels (ISVs) is indeed observed at the latter timepoint, but not in the posterior cardinal vein (PCV) (Figure 3-9-A), suggesting that venous development is normal in the absence of *dll4*. In order to test this hypothesis further, more genes

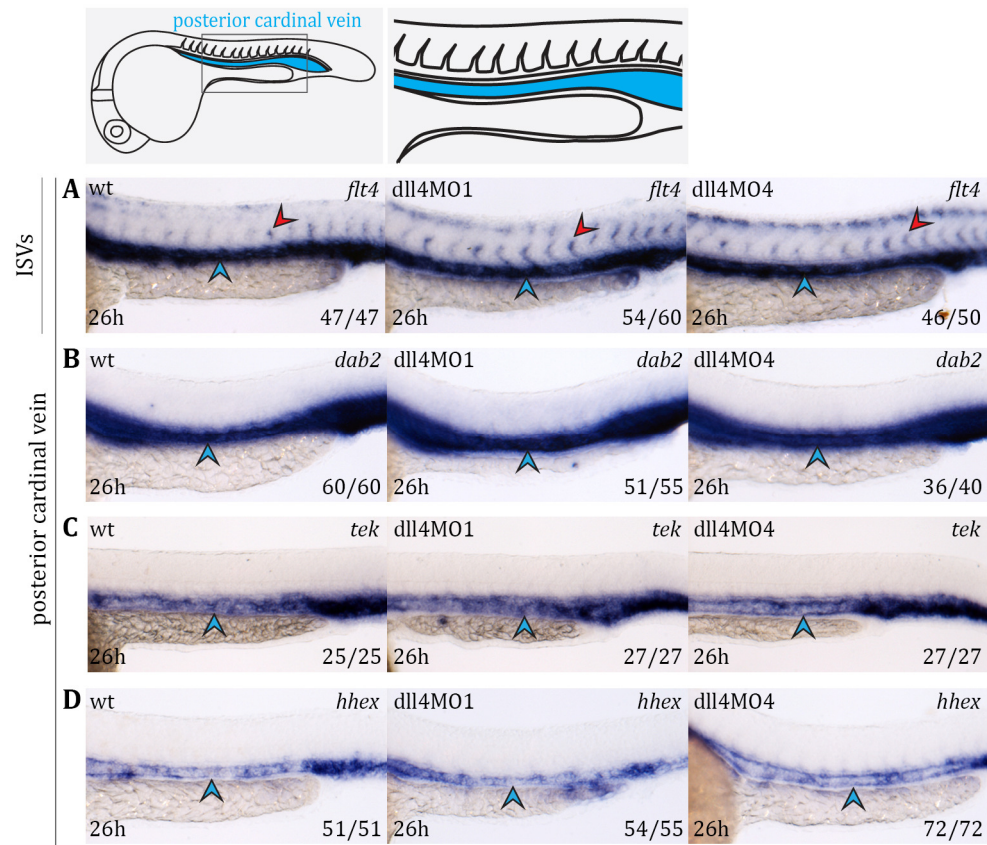


Figure 3-9. The venous programme is not affected by loss of *dll4* activity at the time of HSC emergence.

A-D. Markers preferentially expressed in venous endothelium are not affected in the absence of *dll4*. Expression of *flt4* (*vegfr3*, **A**), *dab2* (**B**), *tek* (*tie2*, **C**), and *hhex* (**D**) in the PCV is not changed in *dll4* morphants. *Flt4* (*vegfr3*) expression in the intersomitic vessels is slightly up-regulated (**A**). Arrows indicate ISVs (red) and PCV (blue). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

expressed predominantly in the PCV were assessed. No effect on *dab2*, *tek* (*tie2*) or *hhex* could be observed in *dll4* morphants, indicating that venous gene expression patterns are largely unaffected in the absence of *dll4*. Structurally, the PCV appears normal at 26 hpf, as circulation is normal in these embryos (data not shown), indicating a competent axial vasculature. Taken together, the data presented here propose a minor role of *dll4* in arterial and none in venous development, with a normally functioning vasculature.

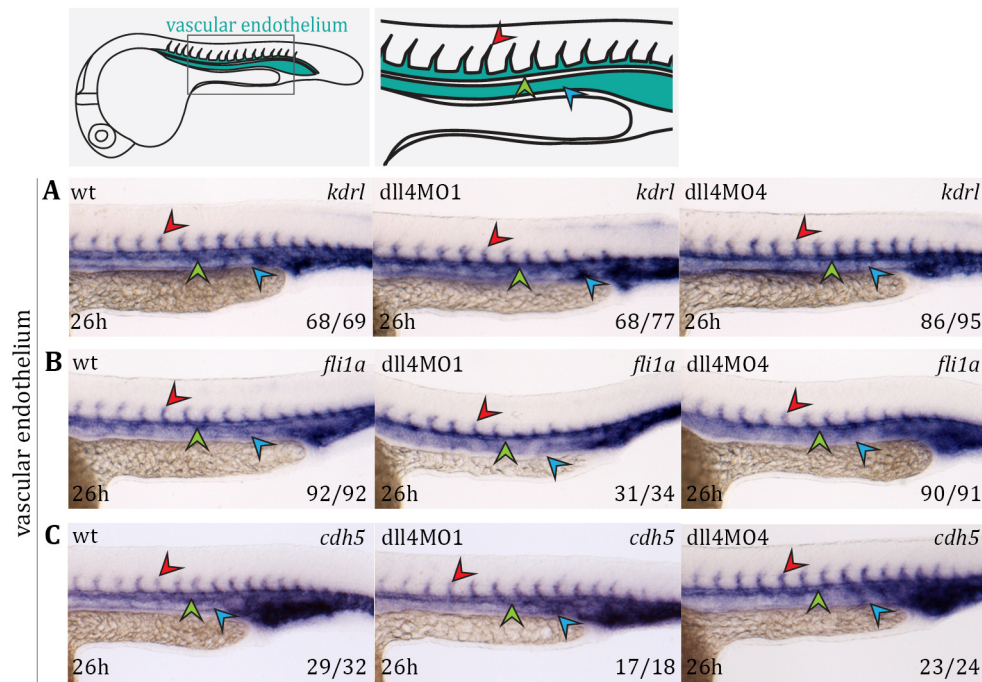


Figure 3-10. The endothelial programme is established appropriately in *dll4* morphants.

Pan-endothelial markers *kdr1* (*flk1/vegfr2*) (A), *fli1a* (B) and *cdh5* (*VE-cadherin*) (C) are expressed at normal levels and in normal patterns in the DA, PCV and ISVs in *dll4* morphants. Arrows indicate DA (green), ISVs (red) and PCV (blue). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

To further test the extent of the defects on vascular patterning and expression in the absence of *dll4*, expression of pan-endothelial markers was monitored. Expression levels and patterns of *kdr1* (*flk1/vegfr2*), *fli1a* and *cdh5* (*VE-cadherin*) were unchanged in *dll4* morphants in DA, PCV and ISVs (Figure 3-10), consistent with the results obtained for arterial and venous patterning. As for arterial markers, ISV sprouting is not adversely affected in the absence of *dll4*, nor is the separation of DA and PCV, as indicated by the presence of normal circulation and the clear staining of the arterio-venous boundaries at this early stage (Figure 3-10).

No evidence of the hyperbranching phenotype can be observed at 26 hpf, in keeping with the previously proposed phenotypic onset at 28-32 hpf (Hogan et al., 2009), after which the ISVs show progressively worsening branching abnormalities, peaking around 72 hpf (Hogan et al., 2009). This suggests a functionally competent set of axial vessels in the absence of *dll4* in *Danio rerio*, initially. As the genetic programme of the arterial and venous endothelium is mainly intact in the *dll4* morphants, the zebrafish provides an ideal model to study the HSC programme in the absence of *dll4*, without the compromising effect of a defective vasculature.

3.6 Loss of *dll4* results in reduction of all HSC-derived lineages

In the absence of HSCs, all HSC-derived lineages are expected to be absent. In the absence of transplantation assays in the fish, the functional loss of HSCs tends to be determined by loss of all later HSC-derived cell populations. Definitive HSC progeny are first observed between 3.5 and 4 dpf in the haematopoietic organs of the zebrafish: lymphocytes in the thymus (Bertrand et al., 2008, Kissa et al., 2008), and myelocytes (Galloway et al., 2005), thrombocytes (Lin et al., 2005) and erythrocytes (Zhang and Rodaway, 2007, Jin et al., 2009) in the caudal haematopoietic tissue (CHT). Haematopoietic stem/progenitor cells (HS(P)Cs) also reside in the CHT, the zebrafish tissue equivalent of the foetal liver, at this timepoint (Jin et al., 2009).

In order to assess if numbers of HS(P)Cs in the CHT are reduced in *dll4* morphants, the Tg(itga2b:gfp) transgenic line was employed to visualise these cells at 4 dpf (Lin et al., 2005). The *itga2b* (*CD41*) transgene is expressed in two distinct cell populations, the

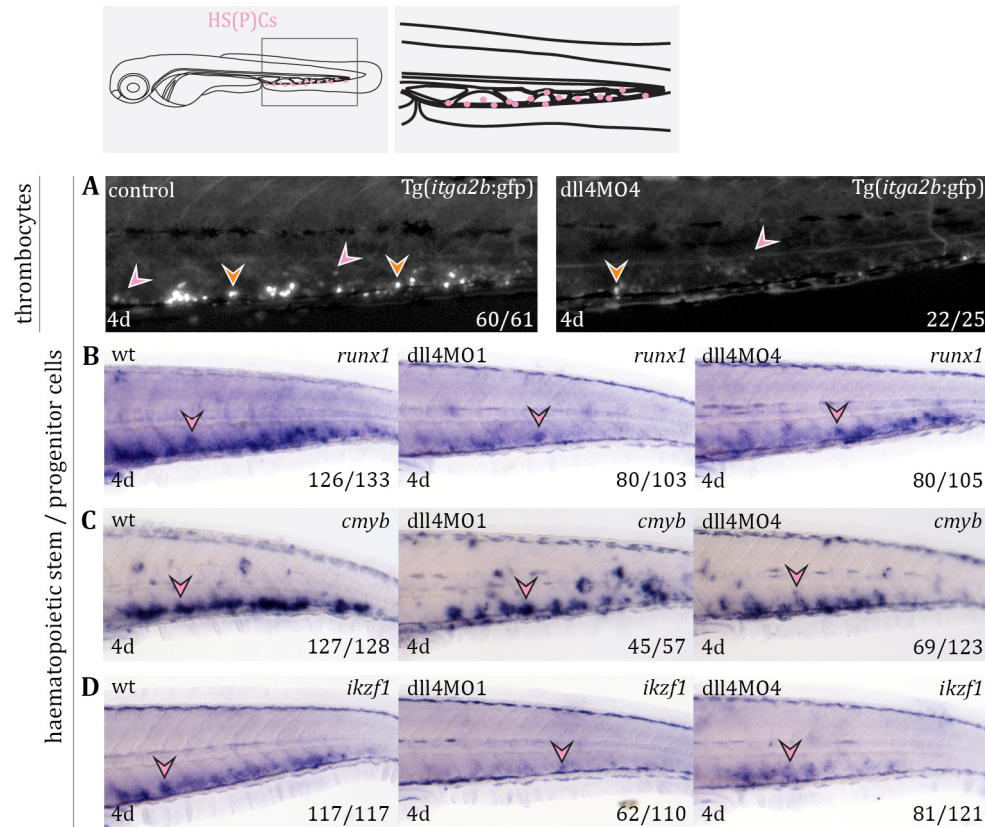


Figure 3-11. Number of HS(P)Cs are reduced in the CHT of *dll4* morphants.

Tg(itga2b:gfp) zebrafish injected with *dll4MO4* show a reduction in thrombocytes (gfp^{high} cells) and HSCs (gfp^{low} cells) as compared to uninjected siblings (A). The reduction in *runx1* (B), *cmyb* (C) and *ikzf1* (*ikaros*) (D) expression marks a reduction of both HS(P)Cs and cells of the myeloid lineages in *dll4* morphants in the CHT at 4 dpf. Arrows indicate HSC derivatives (orange) and HS(P)Cs (pink). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

HS(P)Cs (gfp^{low}) and the thrombocytes (gfp^{high}), which are distinguished by their respective levels of transgene expression (Lin et al., 2005, Bertrand et al., 2008). In *dll4* morphants, both the gfp^{low} and the gfp^{high} cell populations are reduced in number, indicating a reduction in HS(P)Cs (pink arrows, Figure 3-11) and HS(P)C-derived thrombocytes (orange arrows), respectively. HS(P)Cs also express *runx1*, *cmyb* and *ikzf1* (*ikaros*), albeit these markers are also expressed in other definitive blood lineages in the CHT (Murayama et al., 2006, Jin et al., 2007, Zhang and Rodaway, 2007, Jin et al., 2009).

Assessment of these markers in the CHT does not therefore exclusively highlight HS(P)Cs, but an overall reduction in these markers should be observed if HS(P)C numbers are affected, as the other definitive blood lineages are dependent on functional HS(P)Cs (Jin et al., 2009). Indeed, *runx1* staining in the CHT is reduced at 4 dpf (Figure 3-11-B), indicating that fewer cells are expressing this marker. A reduction in *cmyb* expression is also observed, as indicated by less dense staining for this marker in the CHT of 4 dpf old embryos (Figure 3-11-C), similar to a reduction in *ikzf1* (*ikaros*) staining at the same timepoint (Figure 3-11-D). Taken together, these data suggest that there are fewer HS(P)Cs in *dll4* morphants as compared to uninjected wt siblings, confirming the hypothesis that *dll4* is required for HSC development from an early stage.

To further test the reduction of HS(P)Cs in *dll4* morphants, other HSC-derived lineages were monitored. Definitive erythroid cells are reduced in numbers in the CHT of *dll4* morphants at 4 dpf, as indicated by reduction in the erythroid expressed gene *hbbe1.1* ($\beta e1$ globin) (Figure 3-12-A). Primitive erythropoiesis does not appear to be affected as indicated by normal circulation of primitive blood cells at 26 hpf. At 4 dpf, circulation, which still consists mainly of primitive erythrocytes carrying haemoglobin (Zhang and Rodaway, 2007, Jin et al., 2009), is also normal in the axial vessels, and red blood can be seen circulating through these morphants (data not shown).

Cells of the definitive myeloid lineage also appear reduced in numbers in the CHT of *dll4* morphants. Staining with myeloid markers *mpx* (*mpo*) and *lcp1* (*l plastin*) is reduced in intensity in *dll4* morphants (Figure 3-12-B&C), indicating that the definitive myeloid lineage is not appropriately specified.

To complete the assessment of the HSC-derived lineages in *dll4* morphants, the genetic programme of T-cells was assessed at 4 dpf in the thymus. Of all HSC-derived lineages,

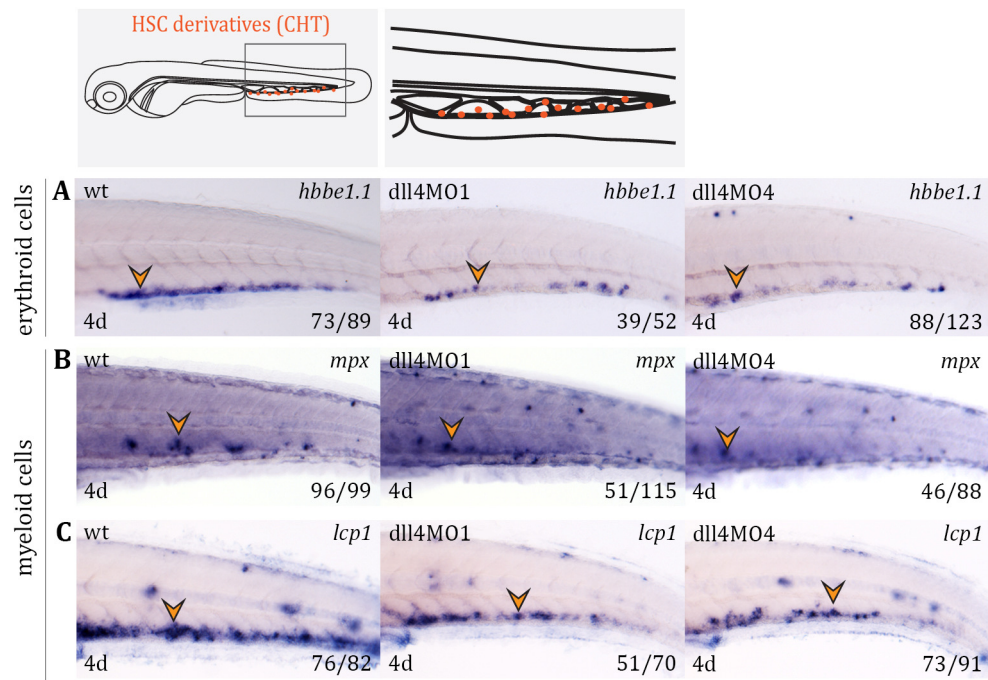


Figure 3-12. The erythroid and myeloid lineages are severely reduced in *dll4* morphants.

A. Erythroid marker *hbbe1.1* ($\beta e1$ globin) is reduced in *dll4MO1* and *dll4MO4*. **B-F.** The myeloid lineage is reduced in *dll4* morphants: myeloid markers *mpx* (*mpo*) (**B**) and *lcp1* (*l* plastin) (**C**) are reduced in both *dll4MO1* and *MO4*. Arrows indicate HSC derivatives (orange). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

the lymphoid lineage appears to be most severely affected in *dll4* morphants, as indicated by near-absence of expression of T-cell marker genes *rag1*, *rag2* and *lck* in embryos injected with *dll4MO1* or *MO4* (Figure 3-13). This severe effect on the lymphoid lineage may be explained by the adverse effect on the thymic epithelial marker *foxn1*, indicating that the thymic anlagen are not specified appropriately, consistent with previous observations in the mouse (Calderón and Boehm, 2012).

Here, the hypothesis that *dll4* is required for HSC emergence has been tested through assessment of the effect on the HSC-derived lineages. All HSC-derived cell types assessed show a reduction in numbers in *dll4* morphants indicating that the loss of *runx1* at 26 hpf is representing the absence of functional HSCs in *dll4* zebrafish morphants.

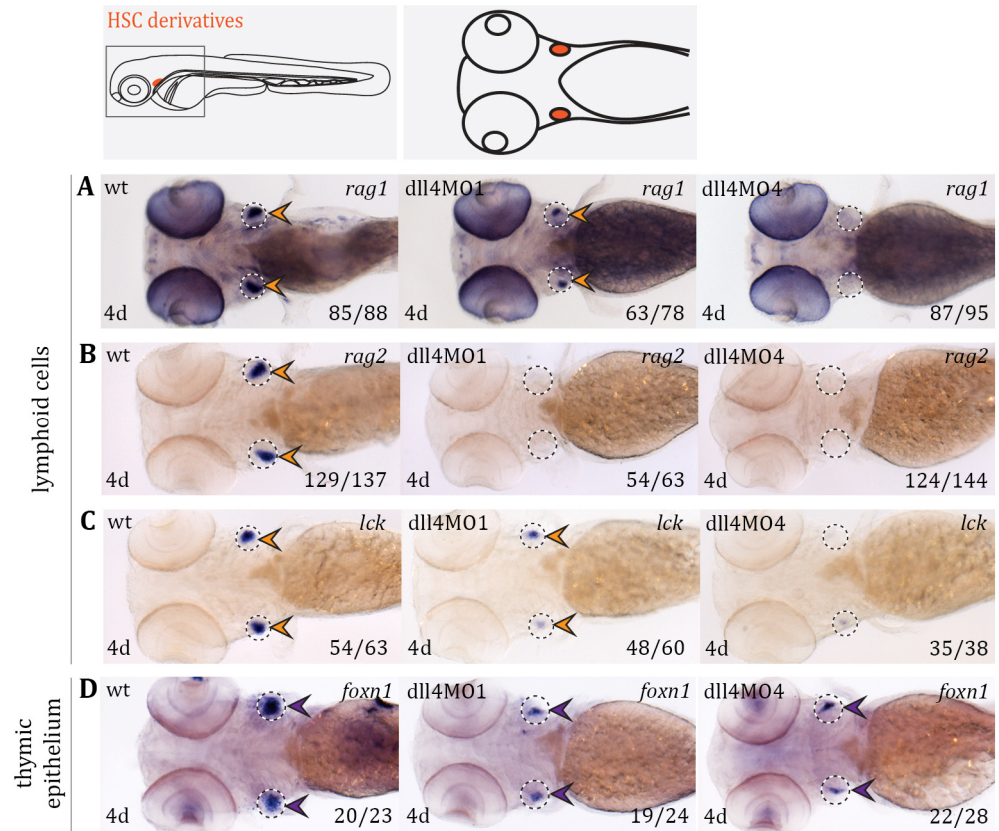


Figure 3-13. Loss of *dll4* causes severe reduction in HSC-derived lymphocytes.

A-C. Assessment of T-cells in *dll4* morphants: T-cell markers *rag1* (**A**), *rag2* (**B**) and *lck* (**C**) are severely reduced if not absent in both *dll4* morphants. Thymic epithelial marker *foxn1* (**D**) is also reduced. Ventral view, anterior to the left, arrows indicate T-cells (orange) and thymic epithelium (purple). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

3.7 Discussion

3.7.1 Overview

The objective of this chapter was to identify the most likely Notch ligand for a role in early HSC development in the zebrafish. To this end, the expression patterns for all known Notch ligands have here been established at 26 hpf and 36 hpf. Only *dlc* and *dll4* were found to be expressed in the dorsal aorta at 26 hpf, and none were expressed at 36 hpf in this tissue. *Dll4* was chosen as the most promising Notch ligand to study in the context of HSC emergence, based on its relatively restricted expression to the DA and ISVs at 26 hpf, and the lack of previous studies in this context.

I have here shown for the first time that *dll4* is required for HSC emergence. This is based on the severe reduction of *runx1* expression in the DA of *dll4* morphants from 24 hpf onwards, which is the earliest described phenotype of *dll4* in the zebrafish to date. I have further shown that reduction in *runx1* in the absence of *dll4* is unlikely due to arterial mis-specification as the reduction in *runx1* positive cells in the DA precedes any described and observed phenotypes (Leslie et al., 2007, Siekmann and Lawson, 2007, Hogan et al., 2009, Sacilotto et al., 2013). The initial reduction in *runx1* staining is also not the result of a delay of the onset of the HSC programme, as *runx1* staining assessed until the time of EHT (approximately 36 hpf) is reduced. Other HSC markers *cmyb* and *ikzf1* are also reduced in the *dll4* morphants, and the *cmyb* phenotype has here been rescued through overexpression of *runx1*. Consistent with the hypothesis that *dll4* is required for the specification of HSCs in the zebrafish embryo, I show that HS(P)Cs as well as all HSC-derived lineages are reduced in these embryos. I provide evidence here

that *dll4* is absolutely required for *runx1* expression and therefore HSC specification in the zebrafish embryo.

Data presented in this chapter have highlighted three major areas of discussion, which will be considered here: firstly the low number of Notch receptors and ligands expressed in the dorsal aorta in the zebrafish; secondly the absence of a requirement for *dll4* in the zebrafish for arterial specification prior to an effect observed on the HSC programme, in contrast to observations made in the mouse; and finally, the novel observation that *dll4* is required for HSC emergence, independently of its role in vascular development.

3.7.2 *Dll4* is the best Notch ligand candidate for a role in HSC specification in the zebrafish

The Notch receptors and ligands are expressed in a highly dynamic fashion across time and tissues during development of different species (Fehon et al., 1991, Kooh et al., 1993, Williams et al., 1995) and, although their expression has been studied in much detail, no conclusive study has yet been conducted that assessed their expression at the time of HSC emergence and EHT in the zebrafish. Here, all known Notch ligands have been assessed for their expression in posterior zebrafish tissues by WISH, in order to determine those most likely to have a regulatory role in HSC development.

Despite the focus here on the posterior tissues, it should be noted that the majority of the Notch receptors and ligands are also expressed in the neural tissues of the head (Figure 3-1, Figure 3-2 and Appendix Figure 8-1), consistent with the established roles of Notch signalling in the developing (reviewed in Pierfelice et al., 2011) and adult (reviewed in Alberi et al., 2013) vertebrate brain.

The *jagged* family of Notch ligands has previously been implicated in HSC emergence in other model organisms, where they are also expressed at high levels in the DA (Kumano et al., 2003, Hadland et al., 2004, Richard et al., 2013). This is different in the zebrafish: the expression analysis conducted here reveals that *jagged* orthologues are only expressed at low levels in the zebrafish at the time of HSC specification, if at all (Figure 3-1 (p. 83) and Appendix Table 8-2 (p. 239)), consistent with previous reports (Thisse et al., 2001, Thisse and Thisse, 2005, Zecchin et al., 2005, Liu et al., 2007). Although this expression does not preclude a role for the *jaggeds* in HSC specification in the zebrafish, the absence of clear expression in the DA at high levels makes them less promising candidates.

In this context, only two Notch receptors and two ligands are expressed at high levels in the DA: *notch1b* (Thisse and Thisse, 2005, Geudens et al., 2010, Sacilotto et al., 2013), *notch3* (Burns et al., 2005, Patterson et al., 2005, Bussmann et al., 2007, Patterson et al., 2007), *dlc* (Smithers et al., 2000, Lawson et al., 2001, Burns et al., 2005, Patterson et al., 2007, Qian et al., 2007) and *dll4* (Leslie et al., 2007, Hogan et al., 2008, Wang et al., 2011) (). Interestingly, these four Notch components are expressed in comparable timeframes, and none appear to be expressed at 36 hpf, indicating that the contribution of Notch to arterial development and definitive haematopoiesis is likely before 36 hpf, consistent with previous reports (Leslie et al., 2007, Hogan et al., 2009, Clements et al., 2011). However, with an expression analysis based on WISH, as conducted here, it is not possible to exclude low-level basal activity of these genes.

The initial aim of this chapter was to determine the most promising candidate Notch ligand for a regulatory role in HSC emergence. The *Dll1* orthologue *dlc* is clearly expressed in the DA and the ISVs around the time of HSC emergence (Smithers et al.,

2000, Lawson et al., 2001) (see Table 3-1, p. 81). *Dll1* can induce haematopoiesis in hematopoietic precursors, with a skew towards T-cell development in humans and mice, even in the absence of a thymus (Ohishi et al., 2002, Schmitt and Zúñiga-Pflücker, 2002, Coste et al., 2005, Dontje et al., 2006, Zakrzewski et al., 2006). This association with later stages of haematopoiesis, and the embryonic death of *Dll1*^{-/-} deficient mice around the time of HSC development at or before E12 (Hrabě de Angelis et al., 1997), makes it a good candidate for a role in HSC emergence. There are three other *Dll1* paralogues in the zebrafish (Gering and Patient, 2010), which will likely obscure the transferability of any observed *dlc* phenotype. *Dll1* is expressed in a highly dynamic fashion in the somites and CNS, but not the vasculature in the embryonic mouse (Bettenhausen et al., 1995). The postnatal arterial programme however does include expression of *Dll1* (Limbourg et al., 2007). Expression analysis of the *delta* genes in the zebrafish suggests functional divergence of the *Dll1* paralogues (Table 4-1); however, it is possible that roles are partially redundant or compensated for within the *Dll1* paralogues. If *Dll1* is involved in mouse HSC development, it is most likely through its somitic expression (Bettenhausen et al., 1995, Hrabě de Angelis et al., 1997). In the zebrafish, *dlc* has been implicated in HSC emergence, before the DA has formed and through the somitic activity of *dlc* (Clements et al., 2011). This suggests that any role of *Dll1* and/or *dlc* in HSC development will be early and not through its arterial activity, and the somitic contribution likely precludes an analysis of the arterial activity of *dlc* in this context, in the absence of a conditional strategy. Therefore, the role of *dlc* in HSC specification was not addressed here.

In contrast to *dlc*, *dll4* has no paralogues in the zebrafish, potentially enabling cross-species comparisons of its function in development. Tissue-specific expression of *dll4* is

highly conserved in the DA, between initial arterial gene induction to the time when HSCs first appear, both in mouse (Shutter et al., 2000, Mailhos et al., 2001, Kumano et al., 2003, Duarte et al., 2004, Gale et al., 2004, Robert-Moreno et al., 2005) and in zebrafish (Leslie et al., 2007, Siekmann and Lawson, 2007). Furthermore, *dll4* is not expressed in neighbouring tissues (Shutter et al., 2000, Mailhos et al., 2001, Kumano et al., 2003, Duarte et al., 2004, Gale et al., 2004, Robert-Moreno et al., 2005, Leslie et al., 2007, Siekmann and Lawson, 2007), making analysis of the tissue contribution easier.

However, it is not possible to exclude a contribution of *dll4* activity through the nervous system. *Dll4* is expressed in the neural tissues (Shutter et al., 2000, Mailhos et al., 2001, Leslie et al., 2007) and while these tissues have not yet been implicated in HSC specification in the zebrafish, there is growing evidence that signalling cues from the nervous system play a vital role in HSC emergence in the mouse (Méndez-Ferrer et al., 2008, Peeters et al., 2008, Fitch et al., 2012). While it is assumed that the here observed requirement in HSC specification is due to *dll4*'s arterial rather than neural activity, a neural signalling role can therefore not be excluded until direct evidence is gathered.

This assumption is further supported by the observation that all arterial cells still express *dll4* at 26 hpf, similar to 24 hpf (Leslie et al., 2007), just before expression of *dll4* fades in an anterior to posterior fashion. Expression cannot be detected in the vasculature by whole mount *in situ* hybridisation from 34 hpf onwards, indicating that expression in this tissue, if any, lies below the threshold required for WISH. Despite its presumed absence in the vasculature, *dll4* is clearly still expressed at 36 hpf in the whole embryo as indicated by PCR. In order to determine vascular expression at this stage, PCR on FACS sorted arterial cells would be necessary. This is consistent with the findings of Siekman and Lawson (2007), who reported an absence of *dll4* expression in the dorsal

aorta and intersomitic vessels at 36 hpf. Consequently, it is not consistent with data presented by Wang et al. (2011), which shows *dll4* expression in the DA and ISVs at 36 hpf. The reason for this discrepancy is not clear, however it could be due to differences in embryonic staging, or differences in probe design. Nonetheless, it is clear that *dll4* expression is down-regulated in the dorsal aorta of the zebrafish embryo, just after initiation of definitive haematopoiesis (Leslie et al., 2007, Wang et al., 2011), consistent with previous observations in other models that down-regulation of Notch ligands upon *runx1* expression is required for haematopoiesis (Richard et al., 2013).

3.7.3 How severe is the vascular phenotype and why does it differ from the mouse?

It was hypothesised in this Chapter that *dll4*'s arterial contribution does not interfere with HSC specification. This hypothesis was based on previous, limited observations, showing that arterial markers *hey2* (*gridlock*) and *vegfc* are not affected in 26 hpf *dll4* morphants while *flt4* (*vegfr3*) is to a minor extent, indicating largely normal arterial development in the *dll4* morphants at 26 hpf. The effect on *flt4* in the absence of *dll4* is only observed at 26 hpf, while at 24 hpf expression is normal (Hogan et al., 2009), at the same time as first effects on *runx1* expression are observed in the DA of these morphants. Data presented here extends the analysis of vascular markers and supports this hypothesis together with the absence of an effect of *dll4* on haemangioblast programming (data not shown).

Hogan et al. (2009) have previously observed increased expression of *flt4* (*vegfr3*) in *dll4* morphants. Here, differential expression of *flt4* is also observed. Consistent with their findings, no change in venous expression of *flt4* is here observed at 26 hpf. The

arterial expression of *flt4* however is affected: while Hogan et al. (2009) describe a down-regulation of *flt4* in the dorsal aorta, here, a slight up-regulation in *flt4* expression in the ISVs is observed (Figure 3-9-A). Despite the discrepancy between these findings, it appears that *flt4* expression is affected in *dll4* morphants within the arterial compartment, in a very minor manner. The down-regulation of *flt4* in the DA in the absence of *dll4* is not detectable at 24 hpf and appears highly temporally controlled (Hogan et al., 2009), therefore it is possible that the observed phenotypes vary due to minor staging differences of the morphant embryos.

As well as being largely consistent with the published zebrafish literature, findings presented here are partially consistent with the data obtained from the mouse: angioblasts migrate and aggregate normally to form the dorsal aorta in *dll4*^{-/-} null mice, and the onset of vasculogenesis is not disturbed, as in zebrafish (Duarte et al., 2004, Leslie et al., 2007, Siekmann and Lawson, 2007, Hogan et al., 2009). A smaller lumen of the dorsal aortae is also consistently observed across mouse and zebrafish, and has been shown to be due to increased migration of aortic cells to the primordial veins in the mouse and to the intersomitic vessels in the zebrafish (Leslie et al., 2007, Benedito et al., 2008, Bridge et al., 2012). It has been proposed that this increased migration is due to increased responsiveness of *Dll4*^{-/-} aortic cells to the angiogenic factors in both species, and not due to an earlier requirement in the angioblasts or to haemodynamic forces (Benedito et al., 2008, Hogan et al., 2009). However, while zebrafish *dll4* morphants are perfectly viable, mice exhibit embryonic death at or before E10.5, due to later disruption of arterial and venous development, showing loosely attached endothelial cells and abnormalities in basement membrane integrity (Duarte et al., 2004, Krebs et al., 2004, Leslie et al., 2007, Siekmann and Lawson, 2007, Benedito et al., 2008, Hogan et al.,

2009). While basement membrane integrity and attachment of endothelial cells are unlikely to be observable with the techniques employed here, the pronounced arterial atrophy and growth retardation of the DA observed in the mouse (Duarte et al., 2004, Krebs et al., 2004) would be. Analysis conducted here focussed particularly on the detection of arterio-venous shunts and severe effects on circulation caused by underdeveloped vasculature in the *Dll4* mouse mutants (Duarte et al., 2004, Krebs et al., 2004), neither of which were observed. Zebrafish are able to tolerate a non-functional vasculature for longer than mice, due to their smaller size and ability to breathe through their skin, so the absence of embryonic death is not surprising, but the absence of severe defects in the axial vasculature is (Stainier et al., 1996, Leslie et al., 2007, Hogan et al., 2009). It is unlikely that this is due to a lowered phenotypic penetrance because of the knockdown approach, as haploinsufficient mice also show severe defects in arterial specification (Duarte et al., 2004, Gale et al., 2004, Krebs et al., 2004).

Another possible explanation has previously been put forward: the Notch input into arterial specification and angiogenesis might be largely independent from one another and may be using different receptor-ligand combinations in different model species (Siekman and Lawson, 2007). The numerous gene duplications and losses observed amongst the Notch ligands in the zebrafish (Table 3-1, p. 81) and their varied expression further support this hypothesis. Here, *dll4* was chosen as a good candidate gene for a regulatory role in definitive haematopoiesis in the zebrafish, partially on the basis that it is expressed in a similar fashion to the mouse and is the single orthologue to other vertebrate *Dll4* genes. This strategy extrapolates functional conservation from evolutionary conservation, which may be common (Fitch, 2000) but not necessarily always true. If the arterial role of *dll4* is only partially consistent between species, there

is no guarantee that the haematopoietic role is conserved either. However, if it is, then the zebrafish provides a unique opportunity to study it, precisely due to the absence of the arterial phenotype observed in the mouse.

3.7.4 *Dll4* is required for HSC emergence

It was here hypothesised that *dll4* is a regulator of HSC specification, based on the activity of *dll4* in the DA around the time of HSC emergence in the zebrafish (Leslie et al., 2007, Siekmann and Lawson, 2007) and in the mouse (Robert-Moreno et al., 2005), the absence of a severe arterial defect in the zebrafish (Hogan et al., 2009) and the absence of zebrafish paralogues. Data collected in this Chapter supports the hypothesis.

Expression of both *cmyb* and *ikzf1* (*ikaros*) are considered to be downstream of *runx1* (Burns et al., 2005, Gering and Patient, 2005) in the zebrafish at 36 hpf. The loss of *cmyb* expression in the *dll4* morphants is rescued by overexpression of *runx1*, confirming the absence of functional HSCs in these morphants. Intriguingly however, only a reduction in *runx1*, *cmyb* and *ikzf1* is observed at 36 hpf, similar to a reduction in HS(P)Cs and HSC-derived lineages. However, the mere reduction rather than absence of HSCs, HS(P)Cs and HSC-derived cells could indicate that some functionality is left or regained (Jin et al., 2009). This in turn might indicate that there is another unknown factor that can partially compensate for the absence of *dll4* in HSC development. This is likely to be at a later stage, as early *runx1* is nearly absent and observations made here point at a potential second compensatory factor downstream of *runx1*, from 36 hpf onwards.

It has been described before that overexpression of *runx1* induces some *cmyb* expression in the posterior cardinal vein in zebrafish embryos, albeit at very low

frequency (Burns et al., 2005). This ectopic *cmyb* expression could not be assessed here, due to the expression of *cmyb* in myeloid cells (Figure 3-7). It was not possible to distinguish between *cmyb*-expressing myeloid cells in the vicinity of the vein and venous cells ectopically expressing *cmyb* with the techniques employed here. *Runx1* regulates the number of *cmyb* positive cells in the DA (Burns et al., 2005), as confirmed by the up-regulation of *cmyb* staining in the embryos injected with the *runx1* overexpression construct alone. Interestingly, this up-regulation of *cmyb* appears to be restricted to the DA, while *runx1* is clearly ectopically expressed across the embryo. This observation is indicative that only those few cells of the DA have the potential to express *cmyb* and potentially other HSC genes, even if *runx1* is expressed in cells of other tissues. One explanation for this lack of ectopic induction of HSC fate is the heterodimeric complex that RUNX1 works in with CBF β . Mice with targeted disruptions in *Cbfb* show an essentially identical phenotype to *Runx1* knockout mice with embryonic lethality between E11.5 and E13.5 accompanied by complete lack of definitive haematopoiesis (Okuda et al., 1996, Sasaki et al., 1996, Wang et al., 1996a, Wang et al., 1996b). Biochemical studies show that RUNX1 and CBF β form a heterodimer which regulates the expression of target genes by DNA-binding of the complex, facilitated through the DNA-binding domain of the α subunit (Runx) (Ogawa et al., 1993, Wang et al., 1993 and reviewed in Ito, 2008, Mangan and Speck, 2011). Taken together, these observations suggest that RUNX1 and CBF β function together in definitive haematopoietic development *in vivo*, and that both genes are required in HSC specification. Consequently, ectopic expression of either individual gene is unlikely to induce ectopic haematopoiesis in the absence of ectopic expression of its molecular partner. This provides one possible explanation why no severe increase in *cmyb* expressing cells in the DA of embryos injected with the *runx1* overexpression construct were observed

here. Therefore, overexpression of *runx1* alone is unlikely to result in an increase of functional HSCs in the DA or ectopically.

One further piece of evidence supporting the hypothesis that *dll4* is required for definitive haematopoiesis is the reduction of all markers assessed for HS(P)Cs and all HSC-derived lineages. Fewer thrombocytes (Tg(*itga2b:gfp*)), myeloid (*mpx (mpo)*, *lcp1 (lplastin)*) erythroid (*hbbe1.1 (βe1 globin)*) and lymphoid (*rag1*, *rag2*, *lck*) cells are observed in these morphants, indicating that a common progenitor of these cells requires *dll4* function for appropriate development. Taken together with the absence of *runx1* positive cells from the onset of definitive haematopoiesis, this common progenitor is most likely the earliest HSCs, even before undergoing EHT (Kissa and Herbomel, 2010). Nevertheless, the absence of T-cells at 4 dpf in *dll4* morphants could be explained by a requirement for *dll4* during T-cell maturation, as previously described in the mouse (Koch et al., 2008). This role has not been validated in zebrafish, but data presented here show a reduction in the expression of thymic epithelial marker *foxn1*, hinting at a requirement for *dll4* in this context. In the mouse, *Dll4* is required for the haematopoietic function of the thymic epithelium, but not for physiological accessibility of the tissue to T-cell progenitor cells (Calderón and Boehm, 2012). The requirement for *dll4* in thymic specification shown here does not preclude a role for *dll4* in T-cell maturation, although it precludes its analysis *in vivo* in the absence of a conditional or temporal approach.

Considering the effect *dll4* has on later angiogenic development (Leslie et al., 2007, Siekmann and Lawson, 2007, Robert-Moreno et al., 2008, Hogan et al., 2009) and that the CHT forms at least partially through angiogenic processes (Torregroza et al., 2012), it is possible that the reduction of the HSC lineages in the CHT is partially due to mis-

development of this vascular plexus. In the case of a mis-specified CHT, defects in HSC migration and endothelial “cuddling” would be expected (Murayama et al., 2006, Tamplin et al., 2013). Unfortunately, endothelial “cuddling” could not be assessed here, due to the lack of appropriate transgenic HSC lines at the time. HSCs showing migration defects would presumably still be present in the embryo. No ectopic HS(P)C marker expression is observed in *dll4* morphants, but still, migration defects could be masked by the earlier reduction in *runx1* positive cells. Conclusively this implies that *dll4* is required in sequence throughout haemato-vascular development in the zebrafish: initially for HSC emergence and *runx1* expression in the DA, then for angiogenesis (Leslie et al., 2007, Siekmann and Lawson, 2007, Hogan et al., 2009), potentially affecting CHT development, and finally thymic development (Calderón and Boehm, 2012).

In order to address these issues concerning reduction of HSC-derivatives due to CHT development and/or T-cell maturation, an induced or conditional functional assessment would be necessary. Several technologies to achieve this are presently available, including transient methods such as caged morpholinos (Deiters et al., 2010, Shestopalov and Chen, 2011) and ones requiring establishment of a stable line, such as heat-shock technology (Seok et al., 2006) and inducible Cre lines (Jungke et al., 2013). If it is true that *dll4* is required for these processes, then the reduction of HSC-derived cells could not be used as a readout of functional HSCs. However, considering that all observations made here are consistent with a requirement for *dll4* in HSC specification, it is presumed that the reduction in HSC-derived lineages in the CHT is representative of a loss of functional HSCs.

Despite previous reports of absence of *Gata2* and *Runx1* in E9.5 *Dll4*^{-/-} mice (Robert-Moreno et al., 2008), just before death due to severe vascular abnormalities at E10.5

(Krebs et al., 2000, Duarte et al., 2004), the evidence I present here is the first to show a specific requirement for *dll4* in HSC specification, independent of a role in arterial development. Further, the reduction in *runx1* expressing cells in the DA represents the earliest described phenotype for *dll4* in the zebrafish yet.

3.7.5 Conclusions and directions

Here, the previously known role of *dll4* has been expanded from angiogenesis to developmental haematopoiesis by showing a requirement for *dll4* in HSC specification through *runx1* expression, the earliest phenotype for *dll4* described to date in the zebrafish. I have further shown that *dll4* is required for HSC emergence, this is not a transient requirement, as *runx1* expression remains down-regulated in the absence of *dll4*, between 24 hpf and 36 hpf. At the latter stage the assessment of further HSC markers revealed that these also require *dll4* expression, and that the reduction of *cmyb* positive cells in the DA of *dll4* morphants is due to the requirement of *dll4* for *runx1* expression. I have further shown that the regulation of *runx1* by *dll4* is unidirectional and *runx1* does not regulate *dll4* in a feedback loop. The data shown here is consistent with the hypothesis that *dll4* is required for HSC specification.

Chapter 4

Establishing *dll4* targets

Data presented in Chapter 3 show a requirement for *dll4* in HSC specification upstream of *runx1*. HSC specification is arguably the most crucial step in HSC development, but not much is known about the genetic regulatory mechanisms involved upstream of *runx1* (reviewed in Ciau-Uitz et al., 2014). This chapter aims to define those *dll4* targets upstream of or parallel to *runx1* that are most likely to be involved in the process of HSC specification. This is addressed by identification of *dll4* targets using RNA-seq technology followed by a series of gene prioritisation steps to identify the most promising targets for a role in HSC specification.

4.1 Introduction

There are multiple ways to define direct and indirect targets of a gene of interest, including CHIP-seq, microarray, and RNA-seq technologies (reviewed in Hawkins et al., 2010). Chromatin-immunoprecipitation identifies protein-DNA interactions (reviewed in Park, 2009) and are thus limited to DNA binding proteins and their cofactors (Solomon et al., 1988). Methods based on microarray technology are inherently limited by the choice of probes represented in the array, and thus can preclude detection of novel or unexpected genes (Schena et al., 1995). As very little is known about the genetic regulation of *runx1* and *dll4* at the time of HSC emergence in the zebrafish, these methods were not judged appropriate for the question addressed here. Instead, an unbiased next-generation sequencing approach was adopted in the form of RNA-seq with the potential to identify both direct and indirect targets of *dll4* (Figure 4-1-(1)).

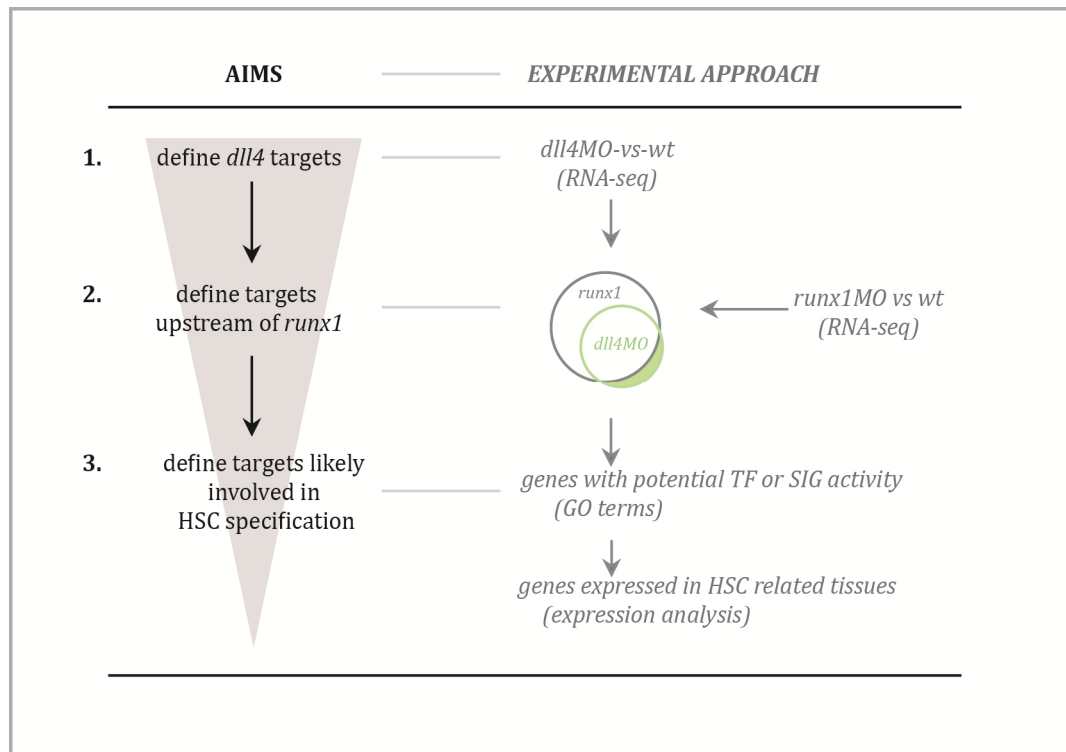


Figure 4-1. Aims and experimental approach taken in Chapter 4.

Chapter 4 has three aims: firstly, to define *dll4* targets through RNA-seq analysis of *dll4*MO-vs-wt. Secondly, to define *dll4* targets upstream of or parallel to *runx1* by comparative analysis with *runx1*MO-vs-wt. Lastly, to define targets likely involved in HSC specification by identification of transcription factors (TF) and signalling molecules (SIG) followed by expression analysis.

With *runx1* being first expressed in the dorsal aorta from 24 hpf (Gering and Patient, 2005), just after initial expression of *dll4* in the DA (Leslie et al., 2007) and having shown in Chapter 3 that reduction of *runx1* in the dorsal aorta is observed at 26 hpf in *dll4* morphants in a largely unaffected dorsal aorta, this timepoint was ideal for the question addressed here. To identify *dll4* targets that are upstream of or parallel to *runx1*, we opted to include a parallel assessment of *runx1* targets in the same experiment, allowing elimination of genes that are targets of both *dll4* and *runx1* from further analysis (Figure 4-1-(2)). Dissection eliminated tissues of the head in which *dll4* and *runx1* are highly expressed (Mucenski et al., 1991, Leslie et al., 2007), although elimination of the spinal

chord which also expresses both genes (Mucenski et al., 1991, Kalev-Zylinska et al., 2002, Leslie et al., 2007, Chapouton et al., 2010) was not attempted.

Next generation sequencing approaches, including RNA-seq, result in long lists of genes that usually have to be prioritised one way or another (reviewed in Moreau and Tranchevent, 2012). Here, emphasis was put on genes with the ability to influence developmental decisions within the presumptive HSC and their activity in the dorsal aorta or closely related or positioned tissues (eg. the immediately adjacent somites) at the appropriate developmental time. Consequently genes were filtered based on the expression activity of transcription factors (TF) and signalling molecules (SIG) which were represented as *dll4* but not *runx1* targets in the RNA-seq output (Figure 4-1-(3)).

4.2 Comparative RNA-seq to establish *dll4* targets upstream of (or parallel to) *runx1*

In order to enrich for HSC-related cells, FACS sorting of dissected trunk/tail tissues of vascular transgenic lines was trialled but resulted in samples of variable RNA quality and quantity (data not shown). No RNA quality issues were encountered with the alternative approach of dissection alone and despite resulting in samples of higher dilution of cells of interest within the samples, higher and more consistent quality was obtained due to drastically reduced processing time. It further enabled us to use higher numbers of embryos and dissected tissue, resulting in higher quantity RNA samples. Therefore dissection of trunk/tail tissues was deemed the most reliable approach available at the time.

The phenotypes of *runx1* and *dll4* morphants are remarkably similar: *runx1* morphants show a reduction in *runx1* positive cells in the dorsal aorta at the time of HSC specification (26/27 hpf, Peterkin and Pinheiro, *unpublished*, Gering and Patient, 2005), and a reduction in *runx1*, *cmyb* and *ikzf1* (*ikaros*) positive cells at the beginning of endothelial-to-haematopoietic transition (EHT, 36 hpf). HSC-derived *rag1* positive thymocytes at 5 dpf are also reduced (Kalev-Zylinska et al., 2002, Gering and Patient, 2005), whereas development of the axial vessels is not affected in *runx1* morphants (Kalev-Zylinska et al., 2002, Burns et al., 2005, Gering and Patient, 2005). While the haematopoietic phenotype and the absence of an arterial phenotype are almost indistinguishable for *runx1* and *dll4* morphants, their intersomitic vessel phenotypes are distinct. *Runx1* morphants show an early (27 hpf) retardation of angiogenic sprouting (Kalev-Zylinska et al., 2002) while *dll4* morphants show hypersprouting after 30 hpf (Siekman and Lawson, 2007, Hogan et al., 2009). Therefore it is likely that *runx1* is not regulated by *dll4* during angiogenesis, as it is for HSC specification. MO experiments knock down gene activity throughout development (Summerton and Weller, 1997), and as *runx1* is expressed in the posterior lateral mesoderm from early somitogenesis (Kalev-Zylinska et al., 2002, Dooley et al., 2005) while *dll4* is only expressed in these cells at high levels from late somitogenesis (Leslie et al., 2007 and Figure 3-3, p. 87), *runx1* targets are expected to be independent of *dll4* regulation at this early timepoint. The extremely similar phenotypes however indicate that *dll4* mainly acts through *runx1* in HSC specification, and a large overlap in targets is therefore expected (Figure 4-2).

Consequently, a comparative approach between *dll4* and *runx1* morphants was here adopted to identify *dll4* targets upstream of and parallel to *runx1*. Two independent

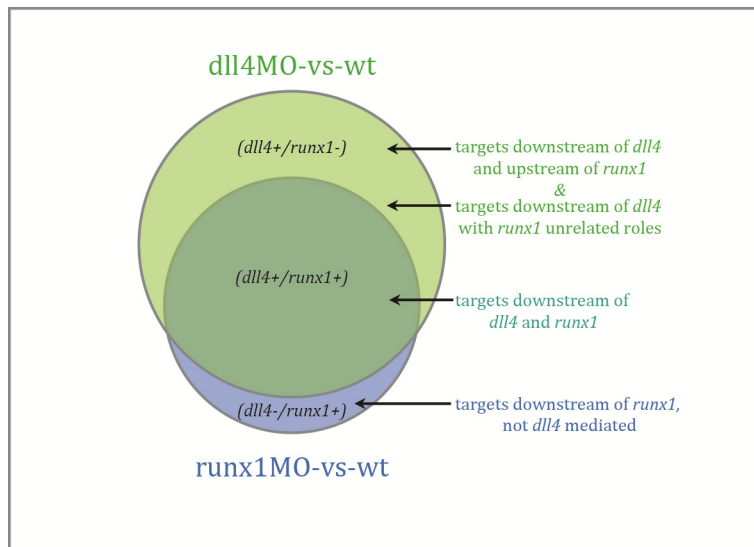


Figure 4-2. Schematic representation of the comparative RNA-seq approach.

When comparing *dll4*MO-vs-wt and *runx1*MO-vs-wt RNA-seq results, targets will fall within three categories: those only represented in *dll4*MO-vs-wt are likely to be targets of *dll4* upstream of *runx1* or not regulated by *runx1*; targets represented in both datasets are interpreted to be downstream of both *dll4* and *runx1*; while a subset of targets represented only in *runx1*MO-vs-wt are *runx1* targets not mediated by *dll4*, most likely in early development and angiogenesis.

morpholinos were chosen to knock down *runx1* activity, *runx1*MOe1 (Gering and Patient, 2005), a splice blocking MO specific to the Exon1-Intron2 boundary upstream of the RUNT domain, which has been shown to be specific to *runx1* and to result in severely reduced levels of wt *runx1* product (Gering and Patient, 2005). Another MO targeting *runx1* was used (*runx1*e6MO), specific to the Intron5-Exon6 boundary, presumably resulting in unstable *runx1* RNA product (Peterkin and Pinheiro, *unpublished*). The *runx1* phenotype observed was consistent across both morpholinos (Kalev-Zylinska et al., 2002, Burns et al., 2005) and unpublished data (Peterkin and Pinheiro), allowing for an RNA-seq experiment to be performed in collaboration.

4.3 Preparing and choosing samples for RNA-seq analysis

Injections and RNA sample preparations for RNA-seq were carried out in collaboration with Dr. Tessa Peterkin and Dr. Philip Pinheiro. Morpholinos injected by each person were rotated in every biological repeat of the experiment to minimise experimenter-related injection effects. *Dll4MO4*, *runx1e1MO* and *runx1e6MO* were injected in the same sitting, and embryos were grown to 26 hpf. Dechorionisation was carried out manually with forceps, and approximately 20 embryos were collected for WISH to control for knockdown quality (Figure 4-4). Embryos were anaesthetised at sub-lethal dose and forceps were used to isolate trunk/tail regions from the head and main portion of the yolk. To reduce statistical bias due to different dissection techniques, all batches of embryos were divided into three separate pools which were dissected by each person, snap-frozen, stored at -80°C and re-pooled when RNA was extracted. RNA was simultaneously extracted from all samples and RNA quality and quantity were assessed immediately afterwards (Figure 4-3).

To assess knockdown quality prior to RNA sequencing, WISH was conducted on all samples. Down-regulation of *runx1* in *runx1* morphants increases from 24 hpf to 27 hpf, thus the latter timepoint was chosen for assessment of knockdown quality, while *runx1* expression was assessed at 26 hpf in *dll4* morphants (Figure 4-4).

Unfortunately, knockdown by *runx1e1MO* was not convincing for any of the 6 biological replicates, so these samples were not submitted for RNA-seq. For all other samples, knockdown was convincing and the best phenotype penetrance for both *dll4MO4* and *runx1MOe6* was observed in Experiments 1, 2, 3 and 4. As *runx1MOe1* was excluded

from the RNA sequencing and only samples for runx1MOe6 were submitted, these will be referred to as runx1MO from here on.

RNA quality assessment by bioanalyzer for these samples revealed high RNA quality (RIN scores between 9.5 and 6.4) with near absence of RNA degradation as indicated by clear bands and peaks (Appendix Figure 8-3, p. 246). All 4 samples were submitted for RNA sequencing based on down-regulation of *runx1* in the morphants and the high quality of the extracted RNA.

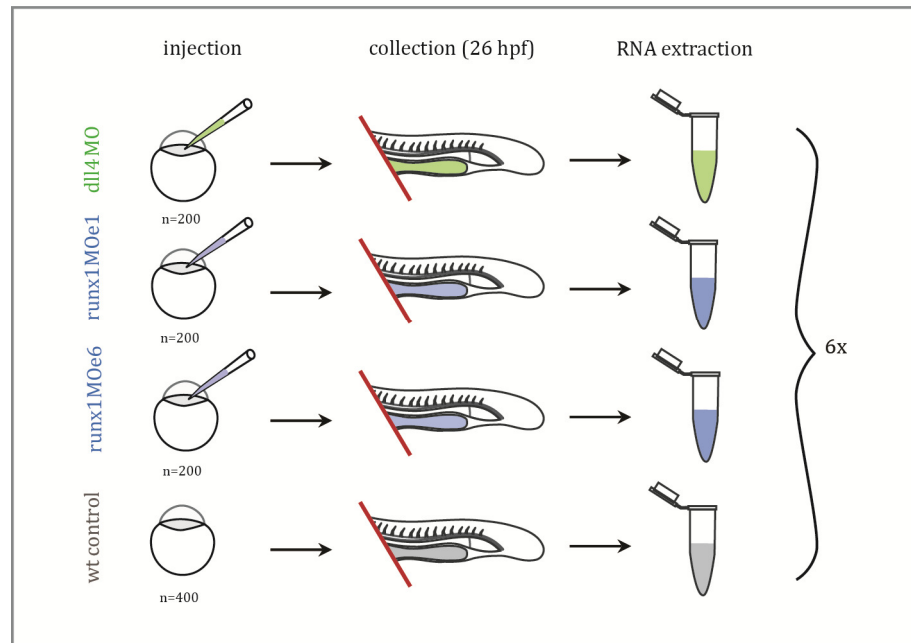


Figure 4-3. Establishing *dll4* targets in the zebrafish.

Experimental setup: *dll4*MO, *runx1*MOe1 and *runx1*MOe6 were injected at the single cell stage. Trunk/tails were isolated from embryos at 26 hpf, including uninjected wt siblings. 200 trunk/tails were collected for RNA extraction for each MO per experiment, 400 were collected for wt controls, in 6 replicates.

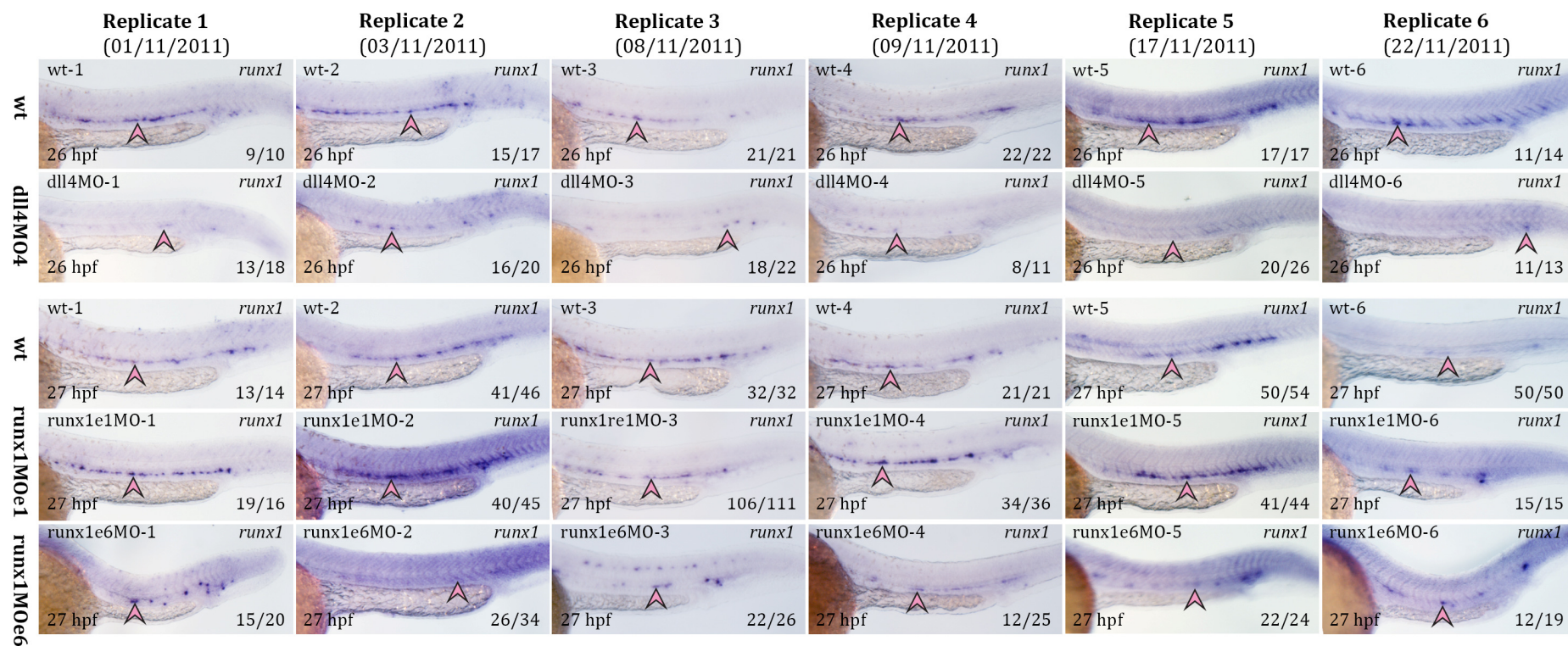


Figure 4-4. WISH to assess knockdown quality for samples submitted for RNA-seq.

Expression of *runx1* (pink arrows) was assessed at 26 hpf and 27 hpf for a subset of embryos from the batches submitted for RNA-sequencing. *Runx1* expression is clearly reduced in all 6 experiments in *dll4MO* and *runx1e6MO* samples. Knockdown was not convincing for any of the *runx1e1MO* samples.

4.4 Quality control and differential gene expression analysis (DE) of RNA-seq output

Illumina RNA sequencing presented in this section was conducted at the Wellcome Trust Centre for Human Genetics (WTCHG) Oxford; processing and analysis of the obtained RNA-seq data was conducted by Dr. Helen Lockstone (WTCHG, Oxford).

Raw expression data of all samples was generated by multiplexing 3 samples into one lane of 50bp paired-end (PE) sequencing on the Illumina HiSeq, yielding circa 30,000 gene readings. Before further analysis, counts were normalised by adjusting for library size using the DESeq package. A log₂ transformation followed this normalisation and gene count distributions were assessed and found to be very similar for most samples, as expected (Appendix Figure 8-4, p. 247).

Several clustering analyses were performed to compare similarities between samples (Figure 4-5 and Appendix Figure 8-4, p. 247). In the hierarchical clustering analysis (Figure 4-5) distance between samples is represented by the length of the connection (Bergkvist et al., 2010), similar to a phylogenetic tree. This analysis shows that the biggest difference within the tree is between the wt and MO clusters, indicating a biological difference between these groups rather than a technical difference, which would be expected to result in samples clustering by experiment (ie. wt-1 with dll4MO-1 and runx1MO-1). MOs cluster together relatively tightly, and the most closely clustering samples are dll4MO-1, dll4MO-2, dll4MO-3 and dll4MO-4 (Figure 4-5-A and Appendix Figure 8-4, p. 247), suggesting that the *dll4* phenotype is highly similar across samples. Despite runx1MO-1 clustering with the wt group, overall the clustering

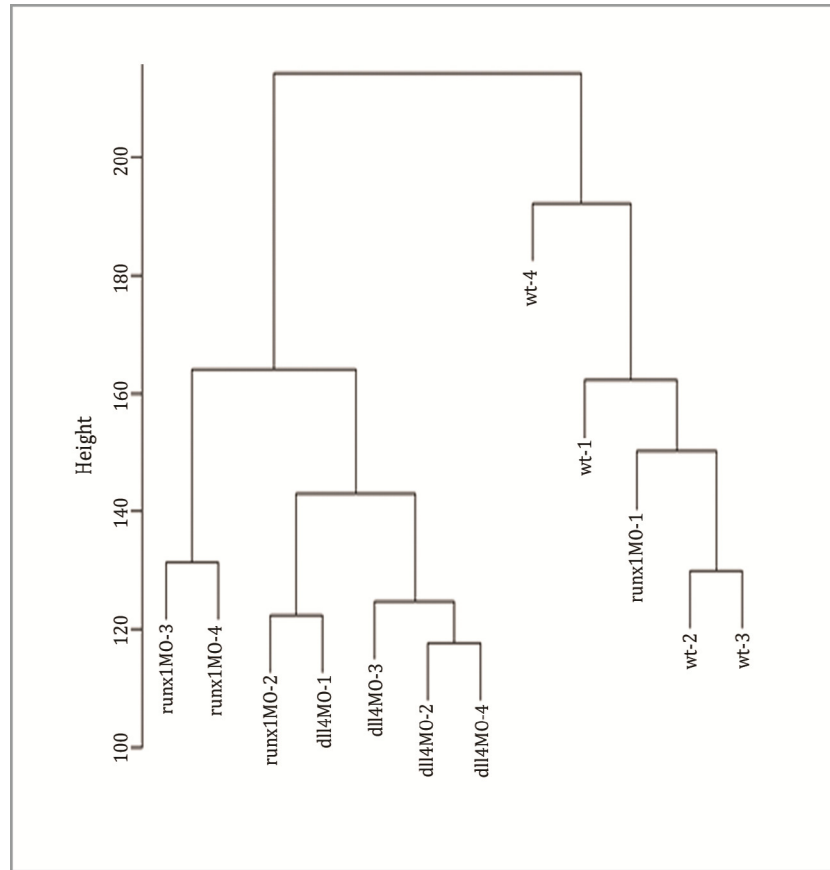


Figure 4-5. Gene count and clustering analyses on RNA-seq samples.

Hierarchical Clustering: this graph represents the relative similarities between RNA-seq datasets submitted, where “Height” is a measure of difference. *Dll4* morphants cluster with each other, *runx1* also cluster but *runx1MO-1* clusters with the wt samples. *Dll4* and *runx1* morphants cluster together away from the wt samples.

analyses show that most wt samples cluster together, suggesting that the overall RNA-seq experiment assigns statistical difference between wt and morphant samples, as observed by WISH.

Overall the clustering analyses show that wt samples segregate from the morpholino groups. It is therefore likely that significant biological differential expression patterns will be represented between wt and morpholino groups. Based on these clustering analyses, all samples were included in the following expression analysis, aiming to

eliminate unexpressed genes from further analysis. For this, genes with fewer than 10 reads on average per sample were considered not expressed and thus excluded from further analysis (Chen et al., 2011a). Application of this filter reduced the number of genes submitted for further analysis from 30,049 to 19,516.

All 19,516 genes that passed the expression filter were included in the following differential expression (DE) analysis. This encompassed pairwise comparisons between the wt and morphant groups using DESeq, resulting in two lists of genes considered differentially expressed: *dll4*MO-vs-wt and *runx1*MO-vs-wt. In this DE analysis, all genes that reached an adjusted p-value (adjp) >0.05 were considered to be differentially expressed, irrespective of their fold change (FC) values (Chen et al., 2011a).

4.5 Validation of the RNA-seq data

The differential expression analysis of the *dll4* RNA-seq dataset yielded 1030 differentially expressed genes in the trunk/tail region of *dll4* morphants as compared to wt siblings. Before conducting further in-depth analysis of these targets, we first attempted to validate some of these targets. This was approached by comparing the DE results with previously established *dll4* targets, as well as by a nanostring experiment on aliquots of the the same samples submitted for RNA-seq.

Only a single gene has been described previously to be differentially expressed in *dll4* morphants at 26 hpf : *fms-related tyrosine kinase 4 (flt4)*, also known as *vascular endothelial growth factor receptor 3 (VEGFR3)* or *expando*, has been shown to be down-regulated in the dorsal aorta at 26 hpf (Hogan et al., 2009). In the DE analysis of the RNA-seq dataset, *flt4* was not represented as differentially expressed in *dll4* morphants

(Figure 4-6-F). In order to address the reason for the absence of an effect on *flt4*, the variation within the samples was assessed by representing DE data from the RNA-seq output as boxplots (Figure 4-6). In this way, a down-regulation was observed (Figure 4-6-B) although it was not significant. This allows a trend to be observed, even if it lies below the statistically significant level.

I have established in Chapter 3 that *runx1* is regulated by *dll4* as early as 24 hpf (see Section 3.4, p. 90), however this was not represented in the RNA-seq experiment: *Runx1* reads were less than 10 per sample and therefore were filtered out of the differentially expressed gene lists. When considering expression boxplots for *runx1*, the difference between *dll4* morphants and wt samples are exceedingly minor, despite showing a slight, non-significant down-regulation of *runx1* in *dll4*MO samples (Figure 4-6-A). This indicates that differential expression of *runx1* lies below the detection threshold of the experiment, despite having been shown to be present in the samples by WISH (Figure 4-4, p. 126).

While there is an absence of genes regulated by *dll4* at 26 hpf in the literature, a few genes have been shown not to be under the control of *dll4* just prior to this timepoint. Expression of *vascular endothelial growth factor c* (*vegfc*) has been described to be unaffected by *dll4*MO at 24 hpf as compared to wildtype siblings (Hogan et al., 2009). Consistently, *vegfc* is not significantly differentially expressed in the RNA-seq experiment conducted here, and no trend towards up- or downregulation can be seen from the boxplots (Figure 4-6-C&F).

Similarly, lack of differential expression of *figf* (*vegfd*) in the RNA-seq dataset (Figure 4-6-D&F) is consistent with previous reports of *figf* not being differentially expressed in

the tailbud of *dll4* morphants (Hogan et al., 2009). It has also been reported that the arterial expression of *hey2* (*gridlock*) is unaltered in *dll4*MO injected zebrafish embryos at 24 hpf (Hogan et al., 2009) as well as 26 hpf (Figure 3-8-E, p. 97). This is reflected in the absence of change in *hey2* expression in *dll4*MO-vs-wt (Figure 4-6-E&F), however, a not significant downregulation is indicated in the boxplots. *Hey2* is expressed across many tissues at this developmental stage, and it is not possible to say if this sub-threshold down-regulation is tissue-specific or not (Figure 3-8-E, p. 97).

Runx1 targets in the zebrafish have been only been described at later timepoints (Burns et al., 2005, Gering and Patient, 2005), precluding validation of the *runx1* target dataset in a similar fashion.

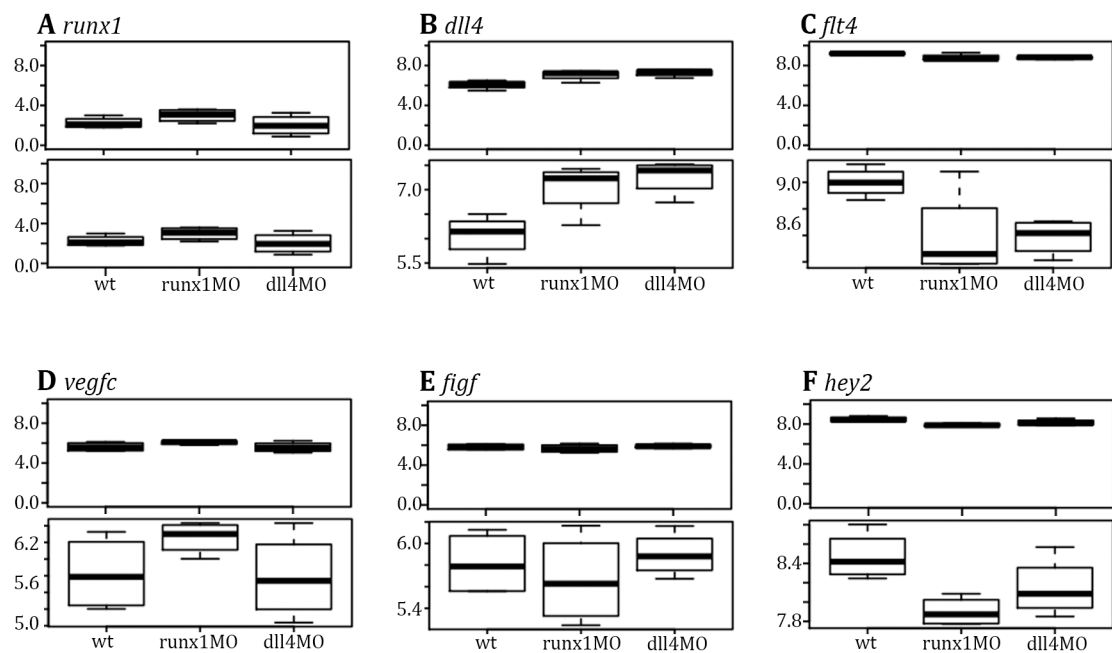


Figure 4-6. Gene expression boxplots.

Expression of genes of interest that were not considered differentially expressed in each of the 3 experimental groups. Expression is represented in two plots for each gene: upper plots are on the same y axis scale (expression level) to enable comparison to other genes, lower plots have customised y-axis scales to enable comparison between groups.

4.5.1 Some genes described as *dll4* targets in other models and developmental timepoints are also represented in the RNA-seq dataset

Before further assessment of the *dll4* targets in the zebrafish identified here, genes that have been described as *dll4* targets in other models were considered, as an alternative validation of the dataset.

CXCR4 has previously been described to be a *DLL4* target in human umbilical vein endothelial cells (HUVECs) and to be suppressed by *DLL4* in transduced cells (Williams et al., 2008). *CXCR4* is represented in the integrated molecular interaction database (IMiD) (Balaji et al., 2012) to undergo a molecular interaction with *DLL4*. Interestingly, *cxcr4a* is significantly down-regulated upon loss of *dll4* activity in the RNA-seq dataset ($\log_2FC_{(dll4MO-vs-wt)}=-1.19$; $p_{adj}(dll4MO-vs-wt)=0.003$), indicating a conserved regulatory function of *dll4* on *Cxcr4*. Williams et al. (2008) employed an overexpression assay whereas *dll4* was down-regulated here, indicating that appropriate levels of *dll4* are required for normal *Cxcr4* expression or that *dll4* can act as an activator and repressor of *Cxcr4* in different developmental contexts.

The integrated molecular interaction database (IMiD) (Balaji et al., 2012) suggests an interaction of *dll4* with *fibronectin 1* based on previously described observations in *Xenopus (Silurana) tropicalis* (Skuli et al., 2009). This is a physical protein-protein interaction (Skuli et al., 2009) and it can therefore not necessarily be expected that this interaction also holds up on the transcriptional level that was assessed by the RNA-seq experiment carried out here. However, zebrafish *fibronectin 1b* (*fn1b*) is significantly up-regulated in the RNA-seq dataset ($\log_2FC_{(dll4MO-vs-wt)}=1.33$; $p_{adj}(dll4MO-vs-wt)<0.001$), indicating *dll4* is suppressing *fn1b* expression in the zebrafish trunk/tail region.

Additional confidence in the *dll4* RNA-seq dataset was provided by comparative analysis with a dataset of *dll4* targets in HUVECs (unpublished deep cap analysis of gene expression (CAGE) analysis of an overexpression experiment, kindly provided by Prof. Adrian Harris (WIMM, Oxford)): 66.6% (n=70) of *dll4* targets not differentially expressed in *runx1* morphants are also considered to be *dll4* targets in the HUVEC dataset and have at least one splice isoform that behaves in the CAGE assay as predicted from the RNA-seq dataset (Appendix Table 8-4, p. 252), indicating that these are not only likely true *dll4* targets, but also that their regulation by *dll4* is conserved between zebrafish and human.

4.5.2 Validation of the RNA-seq experiment using nanostring

Due to the absence of published targets of *dll4* in the zebrafish at this early timepoint, we decided to employ nanostring technology in an attempt to validate the RNA-seq dataset on the same samples (in collaboration with Dr. Tessa Peterkin). Nanostring technology was chosen for two reasons: with only 2 µl of the original RNA sample available, nanostring technology has the sensitivity to assess the differential expression of ~100 probes, and, secondly, a zebrafish nanostring probe set independently designed for blood- and HSC- related probes was available within the lab.

Nanostring technology is based on the microarray concept, using molecular barcodes and microscopic imaging for detection of up to several hundred unique transcripts in a single hybridisation reaction (Nam and Davidson, 2012). This is possible through unique barcodes (colour-coded) attached to individual target-specific probes which correspond to a single gene of interest (Xu and Cleaver, 2011, Nam and Davidson, 2012).

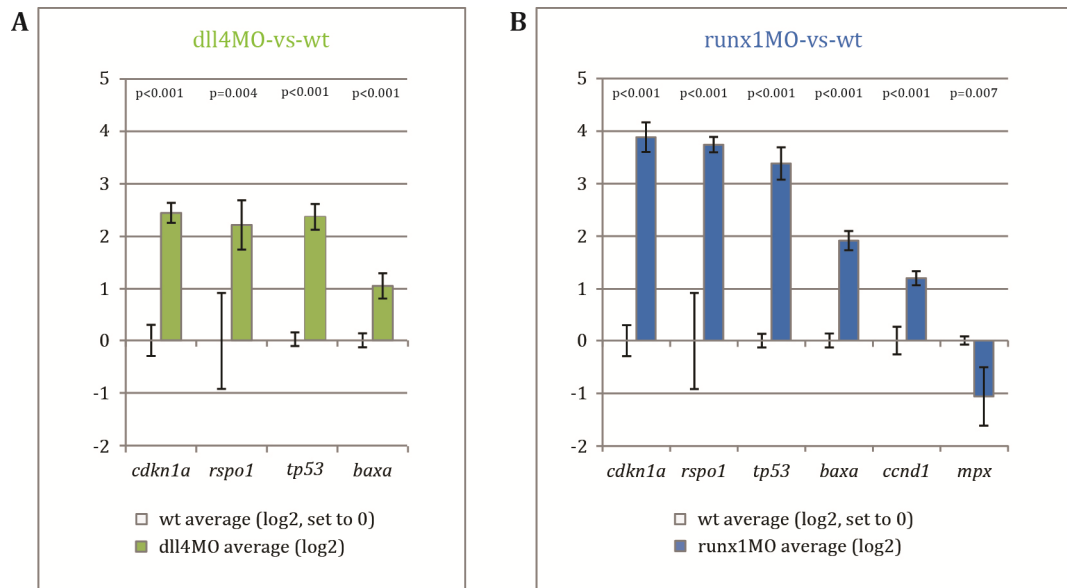


Figure 4-7. Nanostring analysis reveals differentially expressed genes similar to RNA-seq.

Nanostring analysis of the RNA-samples submitted for RNA-sequencing. Only a few genes are significantly differentially expressed in *dll4MO-vs-wt* (**A**) and *runx1MO-vs-wt* (**B**), and these are differentially expressed in both samples with the exception of *ccnd1* (*cyclinD1*) and *mpx* (*mpo*) which are only differentially expressed in *runx1MO-vs-wt* (**B**). Graphs show log2 fold change values with wt log2 values set to 0 for comparison.

The probeset we used was not specifically designed to validate the RNA-seq experiment and consequently contained a majority of probes for genes that were not differentially expressed in the RNA-seq dataset. However, using this dataset allowed us to assess genes considered differentially expressed in the RNA-seq experiment while also assessing genes that were not. Data presented in Figure 4-7 indicate that there are only a few genes differentially expressed in *runx1MO-vs-wt* and *dll4MO-vs-wt*, as suggested by nanostring. All genes differentially expressed in *dll4MO-vs-wt* are also differentially expressed in *runx1MO-vs-wt*, while conversely *ccnd1* (*cyclin D1*) and *mpx* (*mpo*) are differentially expressed in *runx1MO-vs-wt* but not in *dll4MO-vs-wt*.

Only a quarter of genes differentially expressed in the RNA-seq experiment and represented in the nanostring dataset (10/42, 24%) are also differentially expressed in

the nanostring experiment, and 16% of the overall probeset are differentially expressed in the nanostring but not the RNA-seq experiment (21/133). This observation implies that false positive *dll4* targets are represented in at least one of the analyses, but most likely both. Furthermore, this discrepancy also indicates that there are false negatives represented in the datasets. Consequently, in order to establish true *dll4* targets, further validation is necessary, to exclude the possibility of classifying false positives as *dll4* targets. This has been done in further detail in Chapter 5, on best candidate genes. The full list of the nanostring probeset, the analysis and comparison to the RNA-seq are given in Appendix Table 8-3 (p. 249).

4.6 Identifying best *dll4* candidate targets for a role in HSC specification

4.6.1 Comparative analysis of the two RNA-seq datasets reveals potential *dll4* target genes upstream of *runx1*

When comparing *dll4*MO-vs-wt with *runx1*MO-vs-wt, 100% (n=808) of genes common to both datasets (*dll4*⁺/*runx1*⁺) change in the same direction, ie. genes up-regulated in *dll4*MO are also up-regulated in *runx1*MO (analysis not shown), suggesting that these datasets are highly comparable and that little noise is present. This consistent behaviour of gene expression across the two morphants further supports the hypothesis tested in this Chapter: *Dll4* acts upstream of *runx1*. The primary objective for this project was to identify the mechanism through which *dll4* regulates *runx1*, those *dll4* targets that are believed to qualify in this category were further investigated by identifying those differentially expressed in the *dll4*MO-vs-wt dataset only (*dll4*⁺/*runx1*⁻, Figure 4-8).

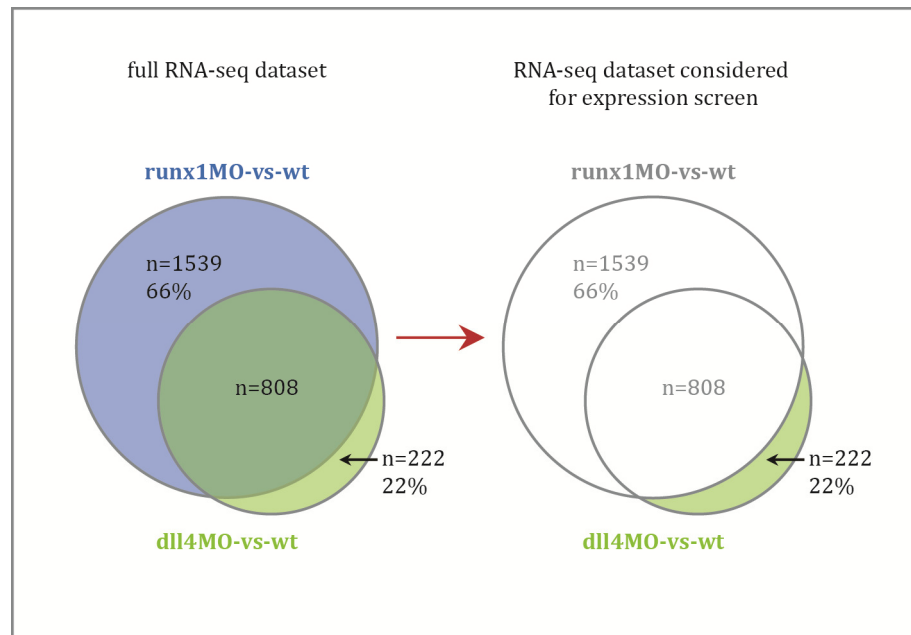


Figure 4-8. Defining the RNA-seq data subset specific to *dll4*MO-vs-wt.

The datasets of differentially expressed genes in *runx1*MO and *dll4*MO were compared for common differentially expressed genes and all genes “specifically” differentially expressed in the *dll4*MO dataset, but not the *runx1*MO dataset, were considered for further analysis.

This comparative analysis yielded 222 *dll4* target genes (Figure 4-8 and Appendix Table 8-4 (p. 252)), 35% of which (n=78) are up-regulated while 65% (n=144) are down-regulated in *dll4*MO as compared to wt. By definition, none of these genes are differentially expressed in the *runx1* morphant dataset.

4.6.2 Identification of *dll4* target genes with likely transcription factor and signalling activity

Signalling molecules and transcription factors are regulators of developmental processes. To determine which of the 222 targets might act as either transcription factors or signalling molecules, two approaches were taken: GO-term analysis (Gene Ontology Consortium, 2009) carried out by Dr. Jie Zuo (WIMM, Oxford) for

“transcription factor and DNA binding” activity (TF, n=17) and “signalling pathway” activity (SIG, n=29). GO term analysis is inherently restricted to its database consisting of previously described molecular function (Plessis et al., 2011, Škunca et al., 2012). To expand this list, I added genes that are named after the functional domains considered to be likely to have TF activity. Combined, these analyses yielded a total of 28 genes with potential transcription factor activity (TF, Figure 4-9).

Previously uncharacterised genes are also of interest, as no functions have yet been assigned and no previous work has been conducted with these genes. Here, previously “uncharacterised” genes were defined as genes that have a non-descriptive gene name in the zv. 9 genome annotation at the time of the RNA-seq project (n=40). These genes may be genes that have only been identified by genomic or EST sequencing projects and have been assigned a temporary name as the conventional nomenclature cannot be applied due to the lack of information at the time of naming (ZFIN, 2014). These genes are being

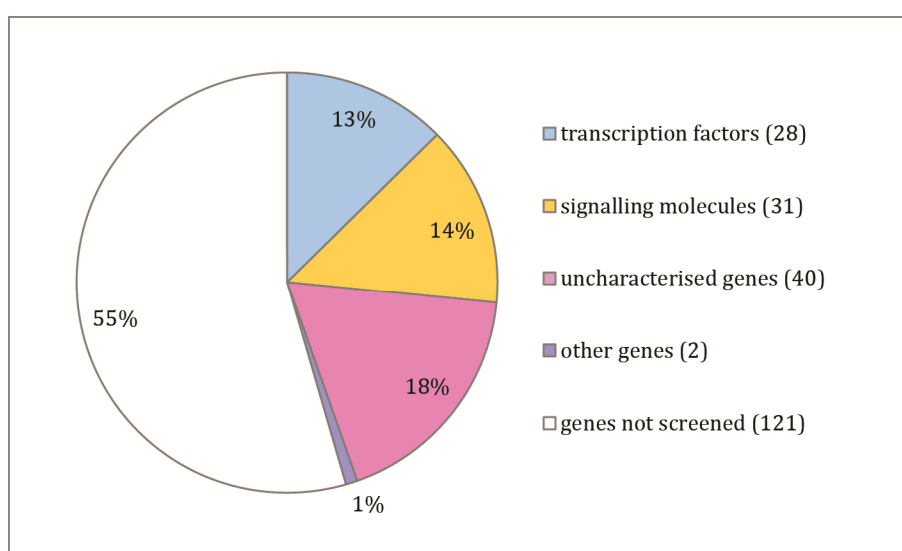


Figure 4-9. Identifying the most promising RNA-seq data subset by molecular function.

Targets defined in Figure 4-8 were divided into potential transcription factors, signalling molecules and uncharacterised genes, as well as “other genes”. Total number of genes included in these sub-classifications are given in parentheses.

renamed as more information becomes available, as is the case for some of the genes investigated here (the original names are given after the new names in parentheses in Appendix Table 8-4, p. 252). On this basis, uncharacterised genes have also been included as candidate genes as they cannot be excluded from having TF activity or acting within a signalling pathway. Finally, an additional (small) group of genes was included in the expression screen, classified as “other” (n=2). These do not fall under any of the above categories but are the most extremely differentially expressed genes in the dataset.

Accordingly, a total of 101 genes were identified to fall within at least one of the above categories (Figure 4-9) and were considered to be good candidates for a likely role in HSC specification downstream of *dll4* but upstream of or independent of *runx1*. This amounts to 45% of the overall 222 genes identified to be differentially expressed in *dll4*MO but not *runx1*MO (*dll4*⁺/*runx1*⁻).

4.6.3 Expression profiling reveals best candidate genes for a role in HSC specification

Transcription factors and signalling molecules need to be active in HSC-related tissues at the right developmental time in order to influence HSC specification. One indication of gene activity is RNA expression analysis by WISH (Jin and Lloyd, 1997, Yee et al., 2005). Genes defined as promising candidates for a role in HSC specification downstream of *dll4* but not *runx1* have here been subjected to RNA WISH. Ninety-nine genes were screened for their expression pattern at the time of HSC emergence. For 2 genes (*bmp1a* and *si:dkeyp-51b9.3* (UG05)), technical difficulties precluded probe creation thus preventing expression pattern analysis.

Here, results of the expression screen are presented by tissue of interest, namely any sites of haematopoiesis as well as tissues with established influence on HSC development.

Only very few genes (n=2, 2%) assessed in the expression screen were clearly expressed in the dorsal aorta: *cldn5b* (SIG) and *foxc1b* (TF) (Figure 4-10-A, blue arrows). Both genes have previously been described to be expressed in arterial endothelium in the zebrafish (Skarie and Link, 2009, De Val and Black, 2009, Xie et al., 2010, Wang et al., 2014), indicating that although the primary objective of the expression screen was achieved by identifying these two genes, it was not possible to identify genes that were not previously known to be clearly expressed in the DA. Nonetheless, *foxc1b* and *cldn5b* both qualify under the original conditions set for identification of potential candidates with high potential for a role in HSC specification downstream of *dll4*.

Zfp36 (TF) is the only gene found here to be expressed in blood cells, most likely in the myeloid lineage (Figure 4-10-B, red arrow). At the developmental stage the RNA-seq experiment and the expression screen were conducted, HSCs are just being specified, meaning that all blood at this developmental timepoint is of primitive origin, indicating that this gene is mainly involved in primitive blood specification and has thus been excluded from further analysis.

The posterior blood island (PBI) is the forming vascular plexus which will later in embryonic development be termed caudal haematopoietic tissue (CHT, foetal liver equivalent) (reviewed in Chen and Zon, 2009). The PBI is the location in which erythromyeloid progenitors (EMPs) arise in the embryo between 26-36 hpf (Bertrand et al., 2007). 12% of all genes assessed here are expressed in this tissue (Figure 4-10-C, pink arrows). As the PBI has blood and vascular identity (Jin et al., 2007), it is perhaps not

surprising that many genes expressed in the PBI are also expressed in vascular regions (Figure 4-10-C, green arrows); while the high percentage of genes also being expressed in the neural tube is possibly more unexpected (yellow arrows). Only one gene is expressed in the somites in addition to the PBI (orange arrows).

Although only 2 genes screened are clearly expressed in the DA, more than a quarter of all genes screened (27%) are expressed in what is here referred to as the “*vascular region*” (Figure 4-10-C/D, green arrows). This expression pattern is not limited to endothelial cells, but is clearly restricted to the ventral part of the trunk/tail regions of the zebrafish, the region encompassing the dorsal aorta, posterior cardinal vein and surrounding tissues. More than a quarter of the *dll4* target genes assessed for their expression pattern here show this expression, and considering that this expression encompasses the region of HSC specification, *zinc finger and AT hook domain containing (zfat)* has been chosen as a representative of this group for further analysis in Chapter 5. *Zfat* is not only of interest because of its expression but also its previous implication in haematopoiesis (Tsunoda et al., 2010). Most genes expressed in this fashion are also expressed in other tissues, most predominantly the neural tube and somites (Figure 4-10-D, yellow and orange arrowheads).

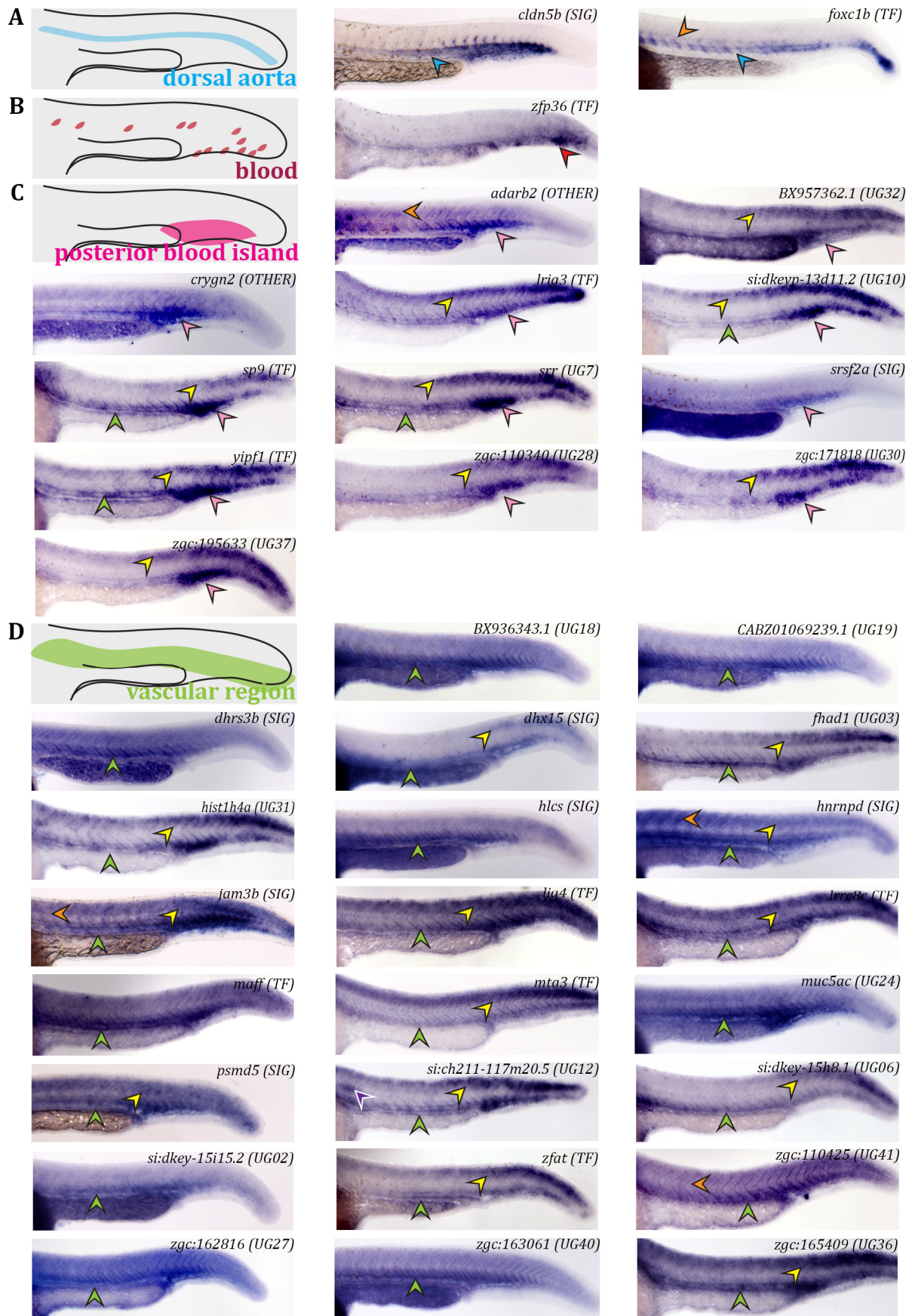
Another tissue of high interest in the context of HSC specification are the somites as Vegf signalling from the somites is required for HSC development (Gering and Patient, 2005, Ciau-Uitz et al., 2010, Clements et al., 2011, Leung et al., 2013) and the somites have recently been shown to give rise to endothelial cells in the zebrafish (Nguyen et al., 2014). 19% of genes screened (n=19) are expressed in the somites, most of which are also expressed in other tissues (orange arrows in Figure 4-10-C/D/E). A particularly interesting example is *forkhead box D1 (foxd1)* due to its highly restricted expression in

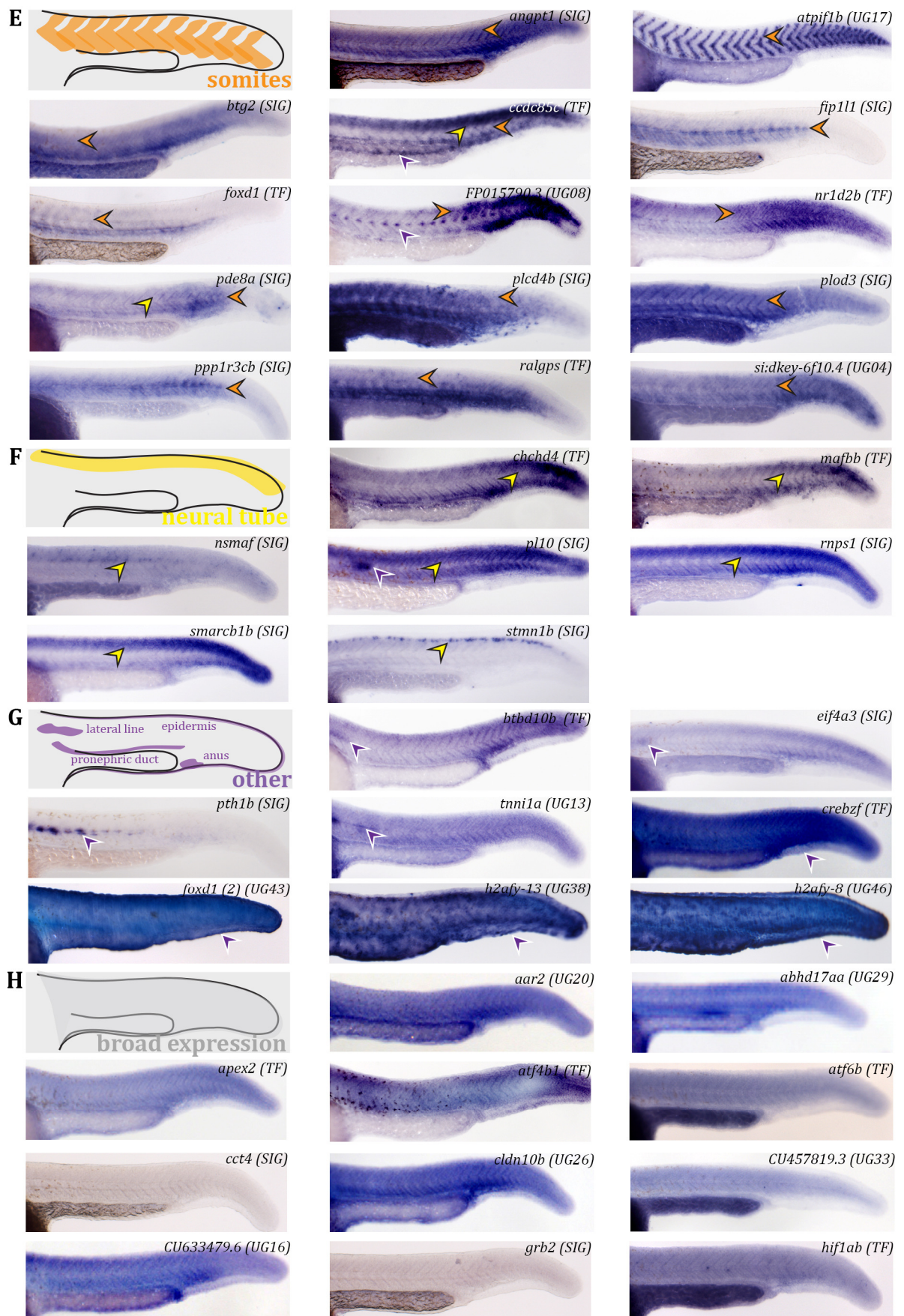
the sclerotome, immediately adjacent to the DA. The sclerotome has been proposed to be a signalling source influencing HSC specification but the exact pathway involved is still unclear (Clements et al., 2011). *Foxd1* has been analysed further for a role in HSC development in Chapter 5.

Although the neural tissues have not been shown to have a distinctive role in HSC development, both *runx1* and *dll4* are expressed in a subset of neurons, and it is therefore not surprising that genes expressed in this tissue are widely represented within the expression screen conducted here (yellow arrows in Figure 4-10-C/D/E/F).

With a total of 31% (n=31) of genes expressed there, neural tissues represent the tissue with the most genes expressed in this screen. Although the contribution of the neural tissues to the HSC programme is not clear, gene regulatory pathways required for HSC development are also employed in neural tissues (Kalev-Zylinska et al., 2002, Muroyama et al., 2005, Yoshikawa et al., 2007).

Dll4 targets are also expressed in other tissues, most prominently the lateral line (7%, n=7), the ectoderm (4%, n=4) and *ccdc85c* is expressed in the pronephric duct (purple arrows in Figure 4-10-D/E/F/G). *Dll4* expression can be discerned by WISH in the pronephric duct at 30 hpf (Geudens et al., 2010) but not at 26 hpf (Figure 3-2-I, p. 85), indicating that *dll4* expression in this tissue initiates around 26 hpf, but not yet at high enough levels to be detectable by WISH. Similarly, *dll4* has not been observed to be expressed in the lateral line or the ectoderm, however, the expression of *dll4* targets in these tissues indicate a role of *dll4* in these tissues, likely requiring low level *dll4* activity or cross tissue signalling.





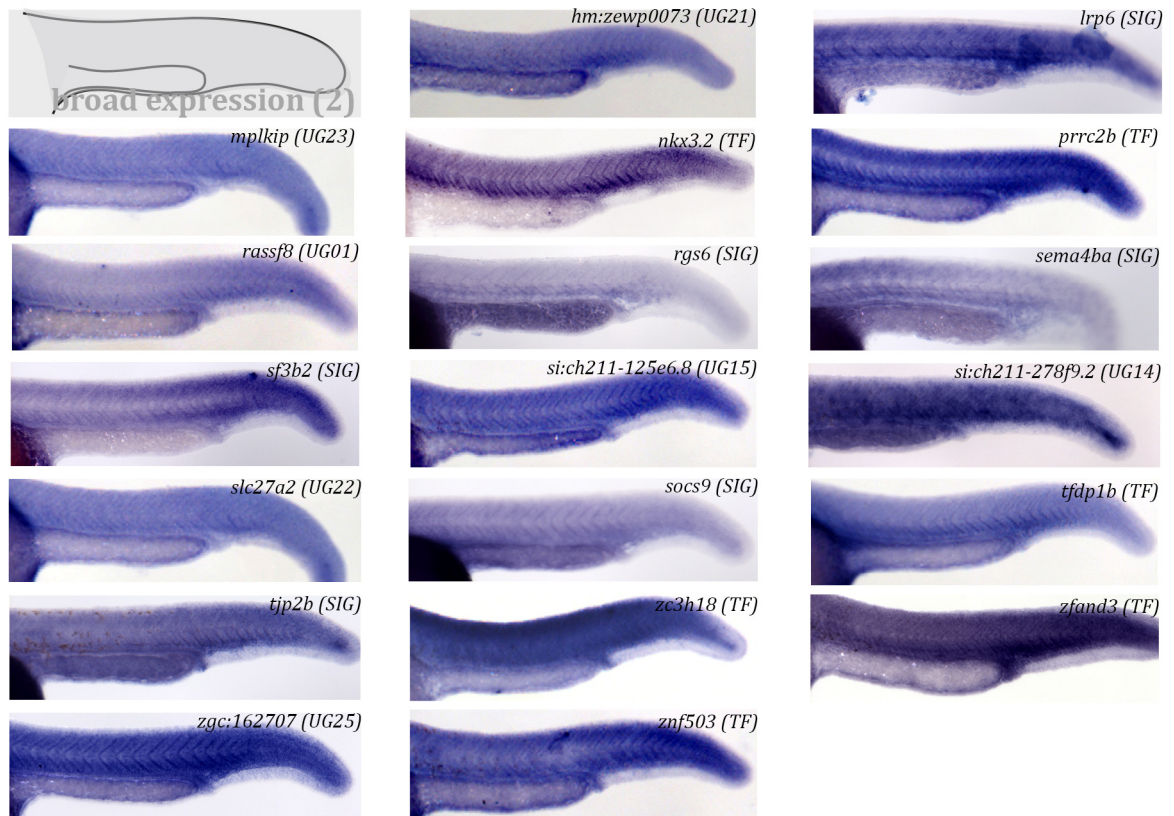


Figure 4-10. Expression patterns of *dll4* targets.

WISH for genes included in the expression screen expressed in the DA (blue), blood (red), the PBI (pink), vascular region (green), somites (orange), neural tube (yellow) and other tissues (purple). Genes are often expressed in more than one tissue and have thus been listed here in order of priority (A-G). Only two genes are expressed in the DA (A) and only one in blood (B). Genes expressed in the PBI are often also expressed in the somites, neural tube or vascular region (C), as are genes expressed (D). Some genes are only expressed in the somites (E) and neural tube (F). Other tissues represented in the expression screen are the lateral line and ectoderm (G). Some genes included in the expression screen show no restricted expression pattern to any tissues in the trunk/tail region (H). Classification of each gene is given in parenthesis behind the gene name. Pictures show trunk/tail regions of embryos at 26 hpf.

A large subset of *dll4* targets is expressed in a broad fashion, in many cases in addition to tissue-specific expression. In total, 77% (n=77) of all genes assessed here show broad expression (Figure 4-12, p. 155), 30 of which (30% total) are not expressed in a tissue-restricted manner (Figure 4-10-G).

4.7 Discussion

4.7.1 Overview

In Chapter 3, the requirement of *dll4* upstream of *runx1* in HSC specification was established. Here, *dll4* targets with the potential for a role in HSC specification have been identified. A succession of filters has been employed, firstly by identifying targets of *dll4* potentially upstream of *runx1*. Molecular function was predicted, and TFs and signalling molecules were subjected to an expression screen around the time of HSC specification. Previously uncharacterised genes (UG) were also included. Most genes screened here are active in HSC related tissues but none have been identified that are expressed exclusively in HSCs or their precursors.

The experiments conducted in this Chapter have highlighted four major areas of debate which will be discussed here. Firstly, high throughput technologies such as RNA-seq create large datasets; the challenge lies in the analysis of the data rather than the acquisition thereof (Cooper and Shendure, 2011). This ranges from the analysis of raw data and differential expression analysis, to the experimental design. Secondly, the lack of availability of appropriate technology to purify the cells of interest leads to a highly diluted sample, which requires a strategy for identification of the genes of interest.

Thirdly, the choice and implementation of filters, and the favouring of RNA quality over cell purity are highly subjective, and there is a multitude of other approaches, not used here, to arrive at a reduced list of candidate genes. Finally, the expression screen conducted here presented more genes with restricted expression patterns than expected which supports the choice of filters applied.

4.7.2 Not all *runx1* targets are regulated by *dll4*

The large overlap between *dll4*MO-vs-wt and *runx1*MO-vs-wt was predicted, based on the close regulatory relationship established in Chapter 3 and the phenotypic similarity, indicating that the main activity of *dll4* at and prior to 26 hpf in the zebrafish trunk/tail tissues is mediated through *runx1*. More unexpected however was the large percentage of genes regulated by *runx1* but not *dll4*, indicating that not all of *runx1*'s actions are regulated by *dll4*. There are two explanations for this: firstly, despite the highly similar phenotypes of *runx1* and *dll4*, there appear to be differences in their role in angiogenesis: *Runx1* is required for initial ISV sprouting (Kalev-Zylinska et al., 2002, Burns et al., 2005) while *dll4* is required later for appropriate tip cell identity (Leslie et al., 2007, Siekmann and Lawson, 2007). This points to a *dll4*-independent role of *runx1* in early angiogenesis, and it is likely that *runx1* targets involved in this process are represented amongst the *runx1* specific targets. A second explanation for these “*runx1* specific” genes is their involvement in earlier development, when *dll4* is not active. Despite assessment of *runx1* and *dll4* targets at 26 hpf, using traditional morpholinos to cause gene specific knockdown results in knockdown of the gene of interest throughout development (Summerton, 2007). *Runx1* is expressed from early somitogenesis onwards in the posterior lateral mesoderm in the zebrafish (Kalev-Zylinska et al., 2002,

Patterson et al., 2005), while *dll4* expression cannot be detected by WISH before the 16-18 somite stages but by PCR at very low levels (Leslie et al., 2007 and Figure 3-3-A&F, p. 87). This indicates that *runx1* does not need *dll4* to initiate expression, but becomes dependent on it later in the context of HSC development. *Runx1* is required for primitive blood development (Lacaud et al., 2002) and primitive erythropoiesis in mice (Yokomizo et al., 2008), zebrafish (Kalev-Zylinska et al., 2002) and *Xenopus* (Tracey et al., 1998), supporting this hypothesis. Therefore, the data presented in this Chapter provide novel insights into the regulation of *runx1* when it is dependent on *dll4* in the context of HSC specification, but not during earlier development.

4.7.3 Dissection of trunk/tail tissues causes high dilution of HSC precursors within the samples

Despite the reduction of non-haematopoietic tissues achieved by dissection, dilution still affects the samples. This in turn makes detection of HSC-specific genes difficult. A higher level of purification is desirable, while maintaining RNA integrity and quality; ideally through purification of HSC precursors from embryos, which unfortunately was not possible with the tools available at the time. An available alternative was the isolation of endothelial cells from transgenic fish lines, although the need for cell-sorting affects RNA quality. In our case, RNA-quality was prioritized over dilution.

We investigated the option of cell sorting prior to RNA extraction for RNA-sequencing, but judged it not reliable enough with regard to RNA quality and quantity obtained. Even if it has been possible to obtain RNA of high quality and quantity after FACS sorting, the lack of a haematopoietic-specific transgenic line currently prevents us from specifically extracting HSCs and HSC precursors in the dorsal aorta. With the lack of a transgenic line

enabling the specific isolation of this cell population, there will always be “contamination” by cells from other tissues within the sample.

One potential consequence of dilution is the absence of any known HSC expressed genes, most importantly *runx1*. *Runx1* was expressed below the threshold of 10 reads per sample on average in the RNA-seq dataset and it was therefore not possible to include *runx1* in the DE analysis. As *runx1* served as the single most important positive control, this is a worrying observation. However, convincing knockdown of *runx1* has been assessed and confirmed by WISH for all samples prior to RNA-sequencing, indicating that the change in *runx1* expression was not detectable in the RNA sample rather than not present biologically.

It is most likely that this lack of detection is due to the dilution of the cells of interest within the sample. At 26 hpf *runx1* is expressed in cells in the ventral DA and at low levels in the Rohon-Beard neurons as well as in neural tissue in the head (Kalev-Zylinska et al., 2002), implying that in the dissected trunk/tail tissue used for RNA extraction there are very few *runx1* positive cells present. This indicates that we were extremely unlikely to detect novel genes expressed at similar levels to *runx1* in the expression screen.

As *runx1* and *dll4* are both expressed in the neural tissues of the trunk/tail region (Mucenski et al., 1991, Kalev-Zylinska et al., 2002, Leslie et al., 2007, Chapouton et al., 2010), the RNA-seq experiment conducted here was also expected to have a large population of differentially expressed genes in this tissue. This is clearly the case as seen by the large percentage of genes expressed in the neural tube in the expression screen (Figure 4-10, p. 144). This inclusion of *dll4* and *runx1* targets in other tissues in the RNA-

seq dataset was another reason for prioritising the expression screen over general target validation.

HSC development is a process that is affected and regulated by signalling pathways from other tissues (Gering and Patient, 2005, Ciau-Uitz et al., 2010, Clements et al., 2011, Leung et al., 2013). Including whole trunk/tail dissected tissues in the samples allows for assessment of those pathways that are regulated by *dll4* and *runx1*, an angle that would not be possible through HSC purification. For example, several requirements of signalling from the somites have been shown in the context of HSC development (Gering and Patient, 2005, Ciau-Uitz et al., 2010, Clements et al., 2011), and this signalling involving the somites may not be unidirectional. As *dll4* is a part of the Notch signalling pathway, which acts between neighbouring cells, classically of non-identical tissues (Lai, 2004, Bray, 2006), it is possible that the role of *dll4* in HSC specification is not direct within the HSCs but potentially through the surrounding tissues, including the immediate surrounding somites (Gering and Patient, 2010). Thus, components of the Notch pathway have been shown to be required in both cell autonomous and non-autonomous manners (Hadland et al., 2004, Robert-Moreno et al., 2005), indicating that *dll4* may act in a similar way.

In conclusion, with the state of the technology at the given time, we consider the dissection approach to be the most reliable if not the most specific available. We have been aware of the dilution issue at all stages of the experiment and have opted for a GO-term analysis followed by an expression screen ahead of target validation with the aim of identifying genes active in the appropriate tissues before validating these as targets.

4.7.4 Approaches for gene prioritisation

When conducting high throughput sequencing analyses such as RNA-seq or CHIP-seq, the data output is almost invariably a long list of genes (Cooper and Shendure, 2011, Moreau and Tranchevent, 2012). In fact, it has been argued that the biggest challenge in next-generation sequencing is not the data acquisition per se but the biological interpretation of the output (Cooper and Shendure, 2011). Consequently, the tools available for gene prioritisation are many (reviewed in Moreau and Tranchevent, 2012).

Here, computational as well as medium and small scale experimental prioritisation have been combined in 6 consecutive gene prioritisation steps performed on the gene list created by the DE analysis (Figure 4-11).

The first filter identified *dll4* targets not regulated by *runx1* (Figure 5 17-(1)), narrowing the RNA-seq output down to 22% of the original dataset. A recent study has used a highly similar approach to identify co-dependent targets of *gata5* and *gata6* by including a double-morphant in the experimental design followed by comparative analysis of the tree datasets (Novikov and Evans, 2013), a highly effective way of prioritisation especially when datasets are highly comparable.

The second filter prioritised genes based on their molecular function. This strategy relies on known association with a given molecular function, and is thus only applicable to genes and functions previously annotated in the GO database (Gene Ontology Consortium, 2009). Similarly to pathway analyses (Khatri et al., 2012) and protein-protein interactions (Balaji et al., 2012) it excludes previously uncharacterised genes from further analysis, by definition. Considering the dataset presented here, this would exclude 18% of genes that were cleared through filter 1. As there are still many

zebrafish genes that have only been identified by large-scale sequencing projects (Bradford et al., 2011), and some poorly assembled genomic regions remain (Howe et al., 2013), it can be problematic to reliably identify vertebrate orthologues (Eckfeldt CE et al., 2005), which these analyses rely on (Gene Ontology Consortium, 2009, Balaji et al., 2012, Khatri et al., 2012). Here, these issues have been circumvented by including those uncharacterised genes in the expression screen. Assuming that these uncharacterised genes follow a similar distribution to the rest of the dataset, a percentage of

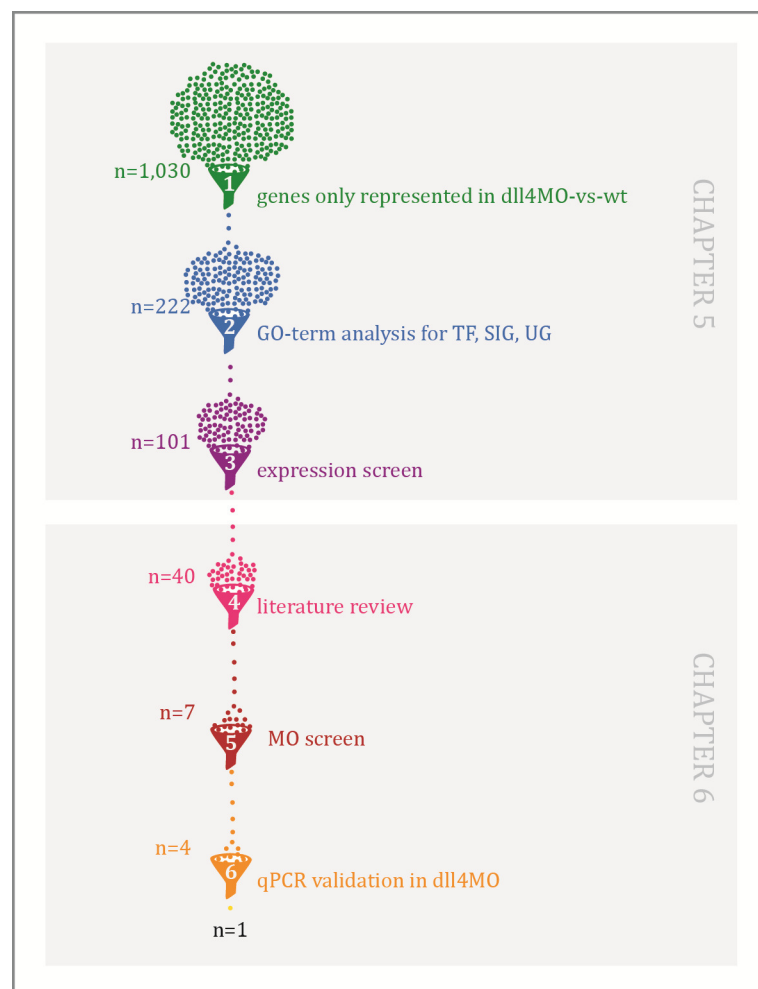


Figure 4-11. A succession of filters prioritises the RNA-seq dataset systematically.

Six successive filters have been applied to the RNA-seq dataset to narrow down potential *dll4* target genes methodically. Numbers indicate how many genes have been submitted to the filter at any given stage.

approximately 30% would be expected to have either transcription factor or signalling activity (Figure 4-9, p. 137) which is equivalent to an additional 12 candidate genes. Since the time of analysis, several of these genes have been annotated further and are now considered to be likely transcription factors, such as *foxd1* (2) or *fhad1*, acting as a proof of concept for inclusion of these “*uncharacterised*” genes.

Likewise, all 222 genes considered for the next filtering step have been subjected to manual evaluation of their gene names, sometimes allowing deduction of molecular function. This approach enabled inclusion of genes such as *foxc1b* which were classified as neither having transcription factor nor DNA binding activity in the GO database at the time of assessment, although belonging to the *forkhead-box* family of genes, whose members are known to have transcription factor activity (Coffer and Burgering, 2004, Katoh and Katoh, 2004).

Following prioritisation of the candidate gene list by molecular function, I aimed to determine the spatio-temporal gene activity of the best candidate genes. For a gene to be involved in any developmental process, it needs to be expressed at the appropriate time and in the appropriate place in development. Analysis of the expression patterns of candidate genes addresses both these questions and has here been chosen as the third filter for DE genes. Expression screens in zebrafish embryos were originally based on random probes and were conducted to define genes with restricted expression patterns (Kudoh et al., 2001, Tamme et al., 2001). Later they were adapted to become an approach for gene discovery (Yoda et al., 2003) and prioritisation of gene lists of interest (Kawahara et al., 2012). With just under 76,000 zebrafish gene expression patterns available (ZFIN, 2014), expression screens for gene lists of interest have been conducted using this published data (Novikov and Evans, 2013, Tixier et al., 2013). This approach is

highly time and cost efficient as it does not involve any experimentation, however it is restricted to previous knowledge. Further caveats are the highly dynamic temporal expression of some genes during development (Steiner et al., 2014) and the poor quality of some of the data submitted. With these limitations in mind, an unbiased experimental expression screen was conducted here, enabling assessment of all genes of interest at the appropriate timepoint (Kawahara et al., 2012). Nevertheless comparison with published gene expression data (Bradford et al., 2011, ZFIN, 2014) was used as a means of validation for the probes created here, and was consistent in more than 95% of cases.

4.7.5 The expression screen revealed more spatially restricted genes than expected

The expression screen conducted in this chapter was more successful at identifying genes with tissue restricted expression patterns than previously reported. Zebrafish expression screens on a random selection of probes have detected genes not spatially restricted in the range between 70-80% (Kudoh et al., 2001, Tamme et al., 2001, Yoda et al., 2003), similar to observations in *Xenopus* (Gawantka et al., 1998). In an expression screen aimed at the identification of novel posteriorly expressed genes, posterior specific cDNA libraries were used, but only 18% of probes were restricted to these tissues (Yoda et al., 2003). In a zebrafish *in situ* hybridisation screen with 60 randomly picked clones/probes, just under 70% of genes showed ubiquitous expression at all 7 stages of development assessed, while 15% were not visibly expressed at all (Tamme et al., 2001). In yet another expression screen based on random selection of probes from a cDNA library, under 15% of genes screened were considered restricted in at least one of four developmental stages (Kudoh et al., 2001). Interestingly however, approximately

75% of these restricted genes were putative transcription factors or signalling molecules (Kudoh et al., 2001). This observation could provide an explanation why only 30% (30/99) of genes screened for their expression patterns here are considered to be “ubiquitous” (Figure 4-10-H, p. 144), where “ubiquitous” expression is defined here as broad expression without any tissue specific restriction at 26 hpf in trunk/tail regions. Conversely, the observation that spatio-temporally restricted genes are enriched for transcription factors and signalling molecules (Kudoh et al., 2001) and the enrichment of restricted expression in the screen conducted here (70%) over the expected average of 10-20% (Kudoh et al., 2001, Tamme et al., 2001, Yoda et al., 2003), might indicate that the previous enrichment for TF and SIG was successful.

Figure 4-12 shows a summary of the expression screen. The low number of genes expressed in arterial and blood cells stands out, as does the complete absence of genes expressed in HSCs. The absence of HSC markers is perhaps not surprising, as the DE analysis of the RNA-seq data was not able to identify *runx1* as differentially expressed and all *runx1* targets were excluded from expression analysis. This is most likely due to low expression of *runx1* in a very small subset of cells within the overall sample. If *runx1* cannot be identified, then it is expected that any genes with a similar expression pattern will also be missed. This should be taken into account for the experimental design of any future experiments aiming to identify genes expressed in HSCs. It seems the dilution factor is too high in the trunk/tail dissections and that for an experiment with such an aim, the dilution of HSCs within the overall sample needs to be reduced. In the context of this project however this should not prove detrimental: as the aim was to identify the regulatory network involved in HSC specification downstream of *dll4*, targets expressed in the DA and adjacent tissues are good candidates.

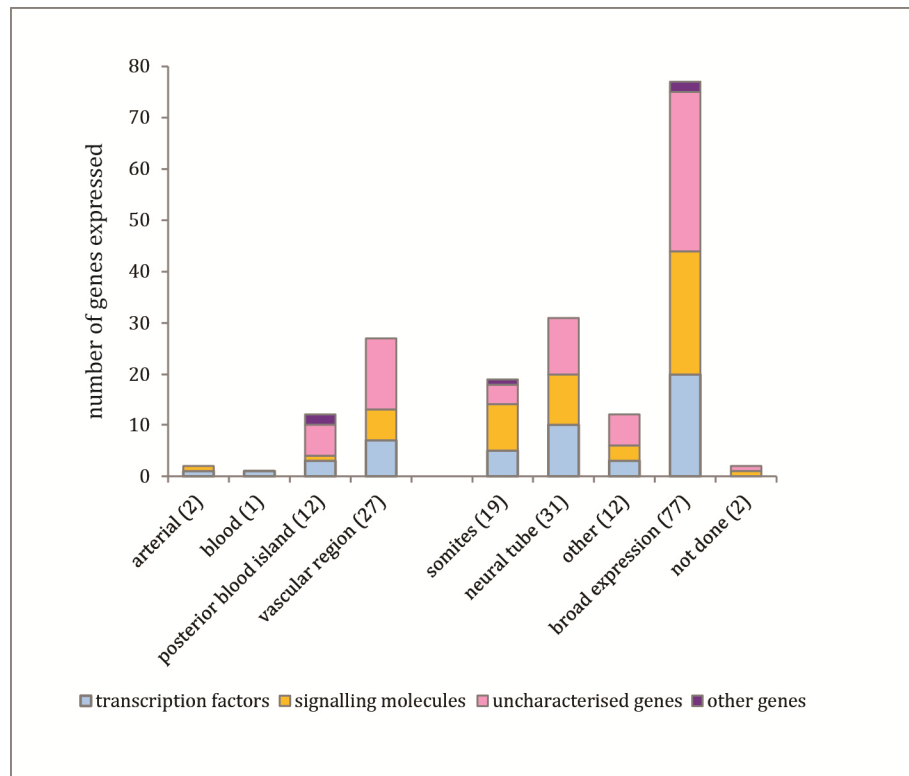


Figure 4-12. Summary of the expression screen by gene classification.

Genes included in the expression screen are represented by their expression in different tissues related to haematopoiesis or classified as “other” or “broad”. A gene may appear in several of these classes if it is expressed in several tissues. Total numbers of genes represented expressed within each tissue are given in parentheses.

Considering the known expression of genes throughout zebrafish development, 760 have been described to be expressed in the neural tube, as compared to 212 in blood and only 141 in the dorsal aorta (ZFIN, 2014). As a result, it might not be surprising that the neural tube was the tissue with the most genes expressed in this screen. Another observation made here is that genes generally tend to be expressed in more than one tissue, indicating that these regulatory genes are involved in development of several structures, and that the same regulatory mechanisms are employed in different contexts in development (reviewed in Pires-daSilva and Sommer, 2003).

4.7.6 Conclusions and directions

Here, *dll4* targets have been identified using RNA-seq technology and have been prioritised for candidates most likely involved in HSC specification upstream or independently of *runx1*. The choice and implementation of filters is highly subjective, and there is a multitude of other approaches to arrive at a limited list of candidate genes. However, the specific successive filters chosen here, seem to have been highly effective at identification of promising spatiotemporally restricted candidate genes, as the proportion of restricted expression patterns was considerably higher than expected from randomly selected genes (Kudoh et al., 2001, Tamme et al., 2001, Yoda et al., 2003).

Having identified 69 potential *dll4* targets upstream of or parallel to *runx1* with spatially restricted expression at the time of HSC emergence, these will be further narrowed down in Chapter 5, by critical evaluation of the expression patterns described here as well as previously described roles in haematopoiesis. These best candidates were validated as *dll4* targets by qPCR and then subjected to a MO screen for functional analysis of their role in HSC specification in Chapter 5.

Chapter 5

Functional assessment of candidate *dll4* targets

The expression screen conducted in Chapter 4 was aimed at discovering *dll4* targets that are active in tissues related to HSC development at the time of HSC specification. The three tissues of particular interest were the dorsal aorta, the “vascular region” and the somites, in order of priority. Here, the genes considered most promising candidates for a role as haematopoietic regulators downstream of *dll4* were assessed for their functionality in the context of HSC and vascular development by means of a MO screen.

5.1 Introduction

The two arterial expressed genes *cldn5b* and *foxc1b* were included in the functional screen based on their expression patterns. *Foxd1* is restricted to the sclerotome, the region of the somites that is immediately adjacent to the DA and has been implicated as an important signalling centre in HSC development (Clements et al., 2011). Amongst the genes expressed in the vascular region, *zfat* is a transcription factor that has previously been suggested to be involved in definitive haematopoiesis in the mouse (Koyanagi et al., 2008, Tsunoda et al., 2010). *Hif1ab* is broadly expressed, but is known to have an important role in HSC development (Imanirad and Dzierzak, 2013). Two additional genes were included in the screen: *hes6* and *her8a*. These were represented in the RNA-seq experiment as *dll4* targets, but also in the *runx1*MO-vs-wt dataset. Members of the *hes/hey* family are known downstream effectors of Notch signalling (reviewed in Kageyama et al., 2007) but a role downstream of *runx1* has not previously been described. Vertebrate orthologues have been assessed for all genes to ensure appropriate literature review.

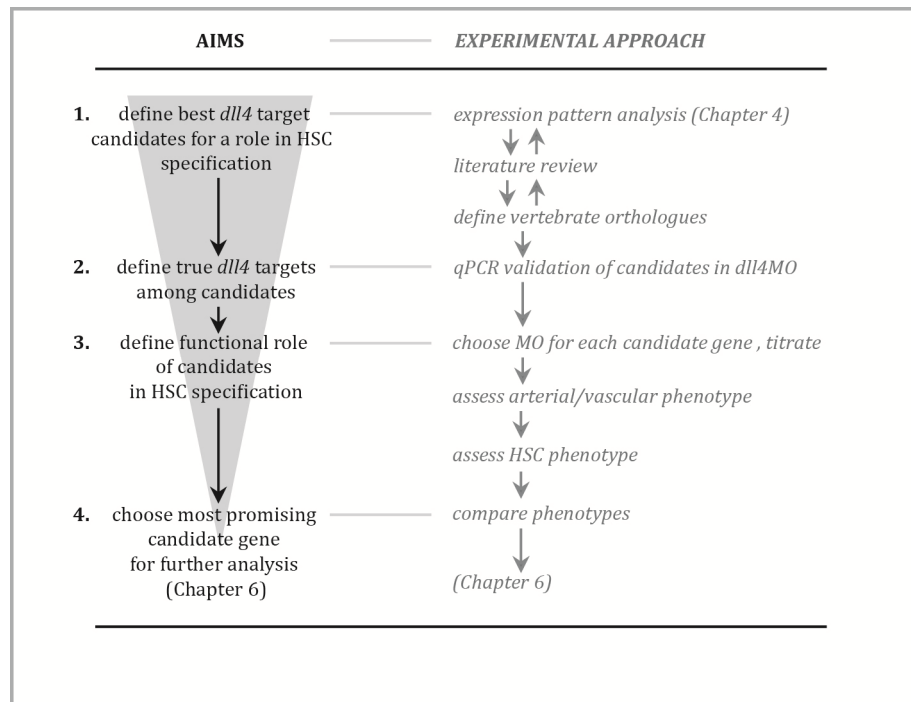


Figure 5-1. Aims and experimental approach taken in Chapter 5.

In Chapter 5, best candidate genes assessed for expression in Chapter 4 were screened for their role in HSC development. Firstly, best candidate genes were defined by evaluating the expression pattern analysis with the help of a literature review, then these best candidates were validated as true *dll4* targets by qPCR, followed by definition of their functional role in HSC specification through a MO screen. Finally, the most promising target was chosen for further in depth analysis of its HSC phenotype in Chapter 6.

5.2 Designing a “perfect” morpholino screen

The aim of this Chapter is to identify the single best candidate gene for a role upstream of *runx1* in HSC emergence through the use of a functional screen. To date, morpholinos are the only established means of functional analysis in the F0 generation in the zebrafish, they are highly specific and stable (Summerton and Weller, 1997, Summerton, 2007), thus providing the tool of choice for the functional screen conducted here. Despite the many examples of MO screens in the zebrafish, there is no perfect screen design suitable for every question addressed (Bedell et al., 2011).

The MO screen was designed to preliminarily address the HSC phenotype of the 7 best candidate genes (Figure 5-2): the use of a single previously published and characterised morpholino in a single experiment was considered sufficient in this context. However, because morpholino dose is important to elicit a specific phenotypic response without causing overt non-specific effects (Nasevicius and Ekker, 2000, Robu et al., 2007, Bedell et al., 2011), all MOs were titrated for an effect on HSC emergence using *runx1* expression at 26 hpf in the absence of overall gross morphological defects as the assay (Appendix Figure 8-6, p. 258).

HSCs first appear in the zebrafish as *runx1* positive cells at around 24 hpf to 26 hpf (Burns et al., 2005, Gering and Patient, 2005). *Runx1* expression and HSC emergence is dependent on appropriate arterial specification, so an arterial marker was also assessed for all morphants. Pan-endothelial and venous markers were also used, where morphant numbers permitted. Where an effect on *runx1* was observed and none on the vascular markers, further analysis of the HSC programme was conducted. Additional HSC markers were assessed at 36 hpf, when the endothelial to haematopoietic transition (EHT) has initiated (Gering and Patient, 2005, Kissa and Herbomel, 2010). HSC-derived lineages were also assessed.

It is absolutely necessary to validate MO effects after initial screening in order to exclude non-specific off-target effects (Bedell et al., 2011). In the context of the screen conducted here, validation was only conducted for the best candidate gene chosen for further analysis in Chapter 6.

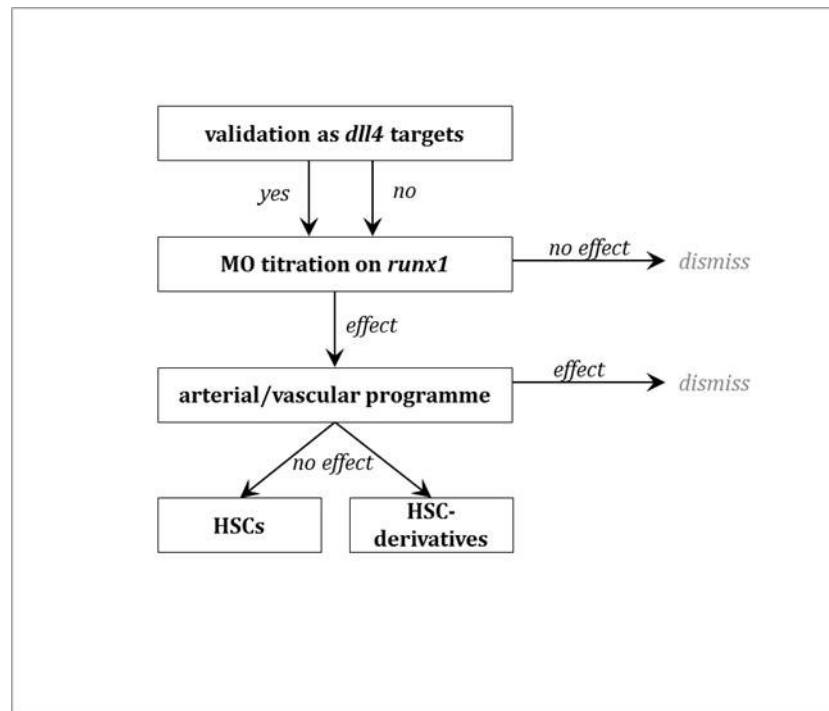


Figure 5-2. Morpholino screen design.

All genes were subjected to target validation analysis by qPCR and MOs were titrated on *runx1* expression at 26 hpf. All MOs with an effect on *runx1* were then assessed for the arterial programme and, in the case of no major effects, markers for HSCs (36 hpf) and HSC-derivatives (4 dpf) were assessed in these morphants.

5.3 Validating best candidate *dll4* target genes

Prior to functional analysis of the 7 best candidate genes, they were subjected to validation as *dll4* targets: a qPCR experiment was set up with comparable conditions to the RNA-seq experiment to assess whether these genes are significantly differentially expressed in the trunk/tail regions of *dll4* morphant embryos. Dll4MO4 was injected into wt embryos and trunk/tails were dissected at 26 hpf for RNA extraction (Figure 5-3-A). Data shown in Figure 5-3-B is representative of three biological repeats.

Tp53 was used as a positive control in the absence of a published *dll4* target consistent with the RNA-seq data. *Tp53* is amongst the top 50 (fold change = 4.5, log2 fold change =

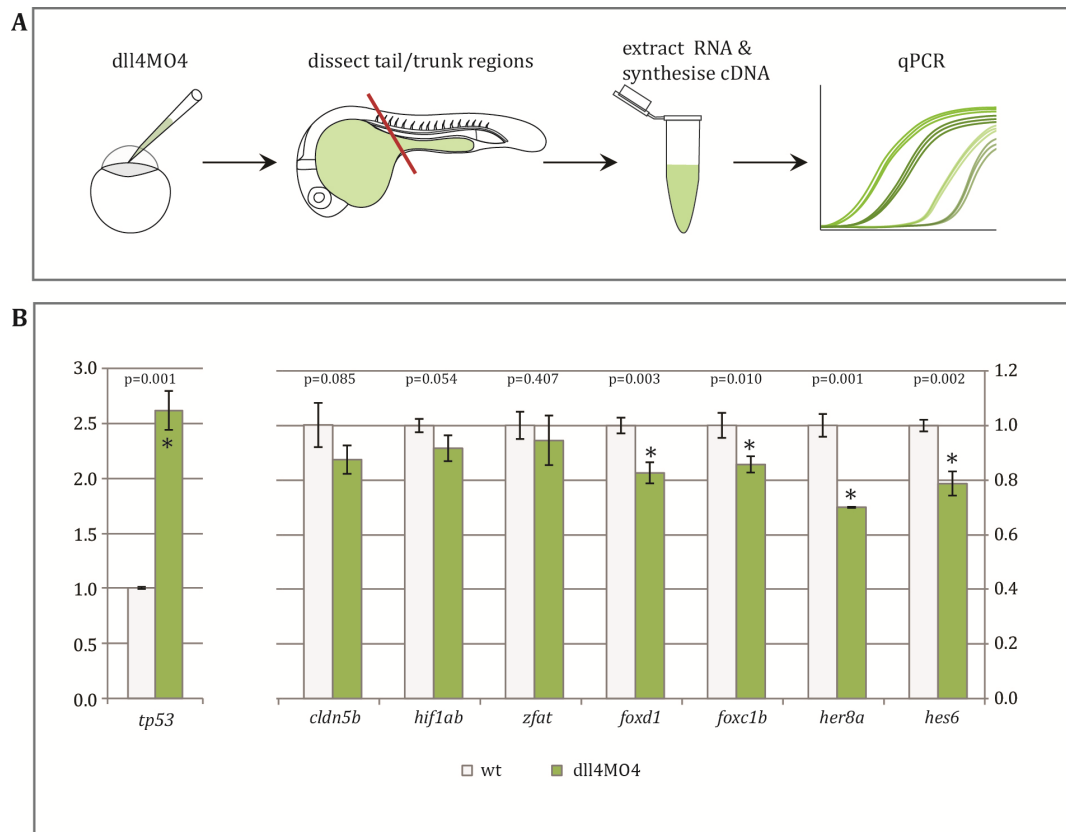


Figure 5-3. Validation of candidate *dll4* target genes by qPCR.

Schematic representation of experimental procedure (**A**): Dll4MO was injected and embryos grown to 26 hpf when trunk/tail tissues were dissected and RNA collected from the samples. cDNA was synthesized and qPCR run in triplicate (**A**). 3 out of 7 tested genes were not significantly differentially expressed in *dll4* morphants as compared to wt siblings but *foxd1*, *foxc1b*, *her8a* and *hes6* were down-regulated (**B**). One representative experiment of three independent biological replicates is shown, error bars are representative of the SEM, asterisks indicate significantly differentially expressed samples, all readings were normalized to *g6pd* and *rpl13a*.

2.17) most differentially expressed genes within both the *runx1* and *dll4* RNA-seq datasets, and has been proposed to be differentially affected in most morphants (Robu et al., 2007). Up-regulation of *tp53* in *dll4*MO4 observed in the RNA-seq dataset was reproduced by qPCR (Figure 5-3-B). All seven genes assessed by qPCR show a down-regulation in the *dll4* morphants, although only those of *foxd1* ($p=0.003$), *foxc1b* ($p=0.010$), *hes6* ($p=0.001$) and *her8a* ($p=0.002$) are statistically significant, supporting

the hypothesis that that these four genes are regulated by *dll4* in the zebrafish trunk/tail region. Thus, they have here been assessed for their functional role in HSC specification downstream of *dll4*.

Cldn5b, *zfat* and *hif1ab* could not be validated as *dll4* targets in the trunk/tail of the zebrafish. They were chosen among the best candidate genes for a role in HSC specification due to expression in the DA in the case of *cldn5b*, and their known roles in HSC development in the case of *zfat* and *hif1ab*. Consequently, they are still valid candidates for a role in HSC specification, albeit they appear not to be regulated by *dll4*. Consequently, they have also been assessed for their functional role in this context.

5.4 *Claudin5b* is required for arterial specification and consequently haematopoietic stem cell emergence

Claudins are tight junction (TJ) proteins and *Cldn5* is specific to endothelial cell to endothelial cell junctions (Morita et al., 1999, Xu and Cleaver, 2011); *cldn5b* is expressed in the arterial endothelium in the zebrafish (Xie et al., 2010, Wang et al., 2014), but its role in development has not been assessed.

*Cldn5b*MO caused severe disruption of circulation, manifested in delayed onset, accompanied by blood pooling over the yolk and in the tail veins (data not shown). Circulation is dependent on appropriate arterio-venous specification and a fully lumenised and competent vessel network (Schuermann et al., 2014), indicating that *cldn5b*MO abrogates appropriate vessel development, consistent with previous reports that *Cldn5* is required for vascular integrity (Burek et al., 2010, Yuan et al., 2012, Hata et al., 2014).

Runx1 expression is reduced in *cldn5b* morphants (Figure 5-4-A), most likely due to arterial mis-specification, as indicated by the up-regulation in *dll4* levels towards the anterior in both ISVs and the DA (Figure 5-4-A, red & green arrows, respectively). Note the up-regulation of *runx1* and *dll4* expression in the neural tissues (Figure 5-4, yellow arrows). Due to the mis-expression of *dll4* and the effect on circulation, two assumptions are made here: firstly, that other arterial and vascular genes are also mis-expressed in these morphants and that this is a reflection of a true mis-specification of arterial development (Schuermann et al., 2014). Consequently, the HSC phenotype is likely to be a secondary effect due to the mis-specification of arterial endothelium and *cldn5b* has not been analysed in further detail.

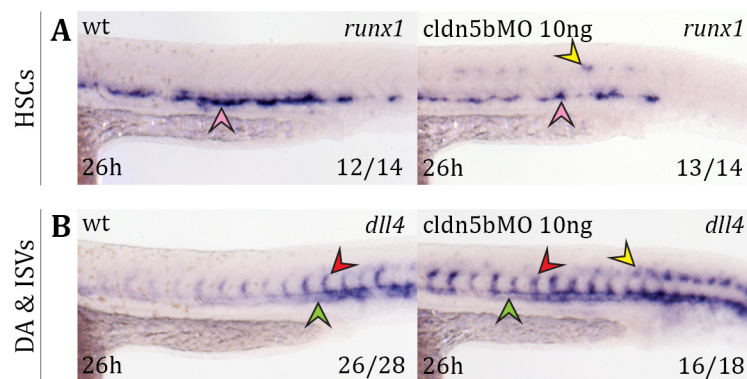


Figure 5-4. *Cldn5b* is required for appropriate arterial and HSC specification.

Runx1 positive cells are reduced in *cldn5b* morphants (pink arrows) (A). *Cldn5b* morphants show disturbed arterial development as evidenced by anterior expansion of *dll4* expression in ISVs (red arrows) and DA (green arrows) and up-regulation in *dll4* expression levels (B). Note expression of *runx1* (A) and *dll4* (B) in neurons in *cldn5bMO* (yellow arrows). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

5.5 Hypoxia-inducible factor 1 alpha (*hif1ab*) is required for HSC cell emergence and vascular specification

HIF genes and hypoxia are known to be required for haematopoietic development (reviewed in Imanirad and Dzierzak, 2013). HIF1A has been shown to directly interact with the RUNT domain of RUNX1 in leukaemic U937 cells (Peng et al., 2008). Overexpression of *Runx1* inhibited DNA binding and transcriptional activity of *HIF1A*, while knockdown of *Runx1* activity resulted in increased *HIF1A* activity as measured by an increase in mRNA levels of HIF1A target genes *VEGF* and *Glut-1* (Peng et al., 2008). Conversely, suppression of *HIF1A* levels by si-RNA increased RUNX1 DNA binding and transcriptional activity (Peng et al., 2008).

Expression of HSC marker *runx1* was down-regulated in the DA of *hif1ab* morphants at 26 hpf (Figure 5-5-A), indicating an absence or a delay in onset of the HSC programme. To address this, *cmyb* expression was assessed at 36 hpf and proved to be reduced in the morphants (Figure 5-5-B), indicating a requirement within the HSC programme for *hif1ab*, rather than in timing. This is confirmed by the reduction of T-cells (*rag1*) in the thymus at 4 dpf (Figure 5-5-D), suggesting a functional requirement of *hif1ab* in HSC development as early as 26 hpf. The other HSC-derived lineages are not affected, as demonstrated by myeloid marker *mpx* (*mpo*) and erythroid marker *hbbe1.1* (*βe1 globin*) (Figure 5-5-E&F), which could be due to a lineage skew or a compensatory mechanism in these lineages through other genes.

Despite normal circulation (data not shown), expression levels of arterial marker *dll4* are reduced in *hif1ab* morphants (Figure 5-5-C), indicating a minor defect in arterial specification. This indicates that *hif1ab* is required for appropriate arterial development

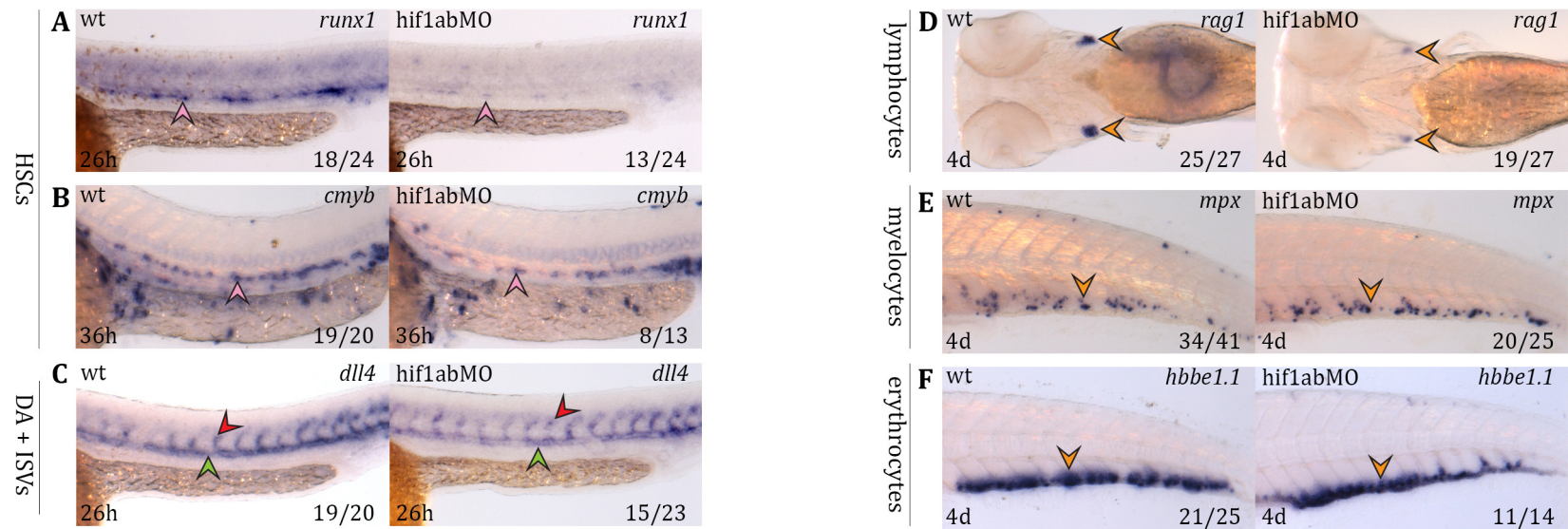


Figure 5-5. MO screen to assess the potential role of *hif1ab* in HSC development.

The number of *runx1* positive cells in the DA is reduced if not absent in *hif1ab* morphants at 26hpf (**A**) as is the number of *cmyb* positive cells at 36hpf (**B**). *Dll4* expression is reduced in the *hif1ab* morphants (**C**). *Rag1* staining at 4dpf is severely reduced; however *mpx* (*mpo*) and *hbbe1.1* (*βe1 globin*) levels are comparable to wt siblings (**E & F**). Arrows indicate: HSCs (pink), DA (green), ISVs (red), and HSC derivatives (orange). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

but not for overall structural integrity of the vessels. It can therefore not be ruled out that the observed definitive haematopoietic phenotype could be a consequence of these arterial defects. Further assessment of the vascular and haematopoietic programmes are required to elucidate the role of *hif1ab* in these tissues.

5.6 Zinc finger and AT hook domain containing (*zfat*) gene is required for HSC emergence

Zfat promotes progenitor cell differentiation by direct binding to Tal1, Lmo2 and Gata1 (Tsunoda et al., 2010). It has previously been established that Lmo2 forms a complex with Tal1 (Scl) (Larson et al., 1996; El Omari et al., 2011) and Gata1 (Osada et al., 1997; El Omari et al., 2011) which is likely to be directly targeted by Zfat during haematopoietic development (Tsunoda et al., 2010). All three genes in this complex are well known to have crucial functions in primitive (Onodera et al., 1997; Patterson et al., 2007) and definitive haematopoiesis (Pevny et al., 1991; Robb et al., 1996; Yamada et al., 1998), rendering *zfat* an interesting gene for a putative role in definitive haematopoiesis.

In *zfat* morphants, *runx1* expression was nearly absent in the DA at 26hpf (Figure 5-6-A), however, no effect can be seen on the expression of *cmyb* at 36hpf while a slight up-regulation in the number of cells expressing *ikzf1* (*ikaros*) is observed (Figure 5-6-B&C). This is intriguing as the early reduction of *runx1* positive cells appears compensated for later. At 4 dpf T-cells are slightly down-regulated in the thymus (Figure 5-6-E), while numbers of erythroid cells appear more affected than the T-cells (H), whereas the myeloid lineage is not affected by the knockdown of *zfat* activity (G).

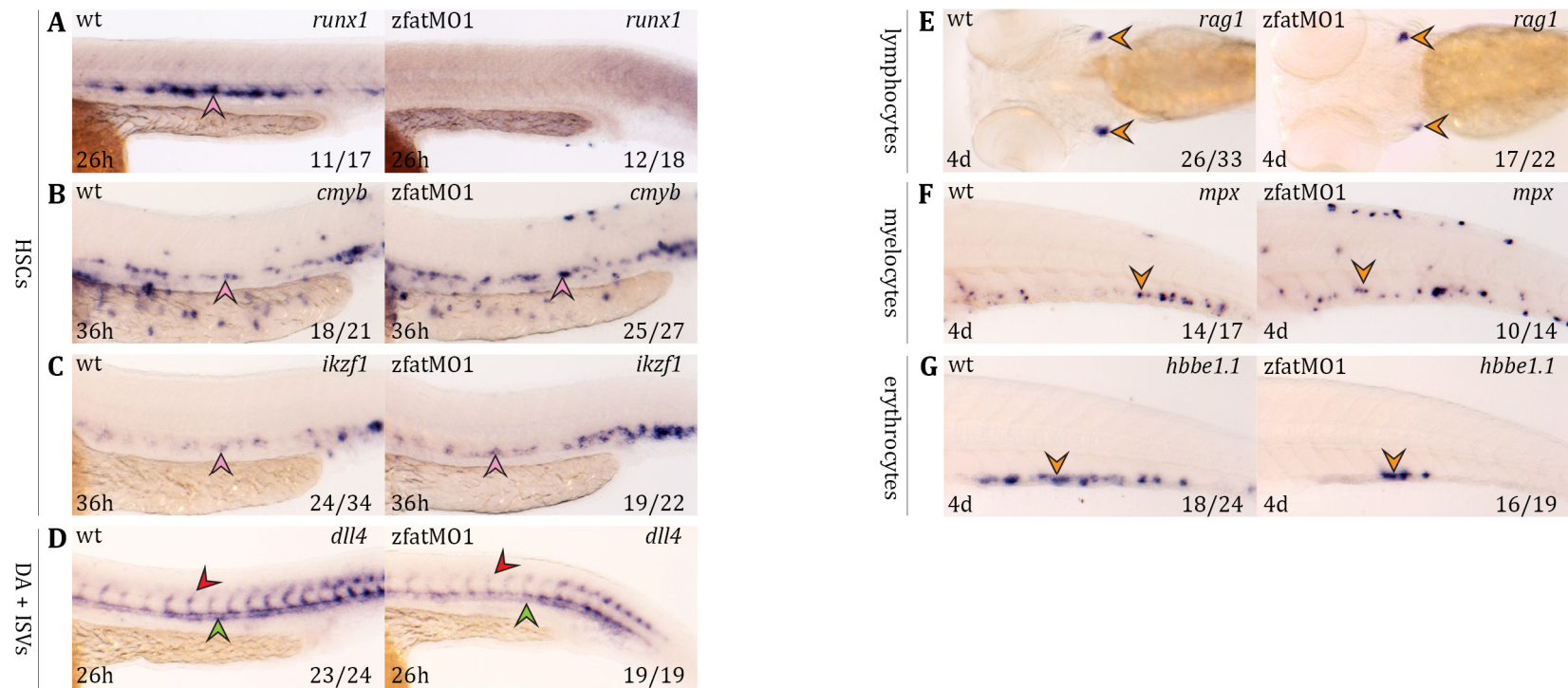


Figure 5-6. MO screen to assess the potential role of *zfat* in HSC development.

Expression of *runx1* in the DA at 26h is reduced if not absent in *zfat* morphants* (A), while expression of HSC markers *cmyb* and *ikzf* at 36 hpf are not affected (B) or slightly up-regulated (C) in *zfatM01* injected embryos, respectively. Expression of arterial marker *dll4* is reduced in these morphants (D). HSC-derived lineages are slightly reduced in the case of the lymphoid (*rag1*) (E) and erythroid (*hbbe1.1* ($\beta e1$ globin)) (G) while the myeloid lineage (*mpx*) is not affected (F). Arrows indicate: HSCs (pink), DA (green), ISVs (red), and HSC derivatives (orange). *note that *runx1* data is also shown in Appendix Figure 8-6-C, p.249). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

This phenotype might at least be partially due to a mis-specification of arterial gene expression, as *dll4* is down-regulated in the anterior part of the DA in *zfat* morphants (Figure 5-6-D). Circulation is normal in these embryos (data not shown), indicating only a minor effect of *zfat* on vascular development (Schuermann et al., 2014). Nevertheless, in keeping with the screen design, *zfat* has not been analysed in more detail.

5.7 *Forkhead box D1 (foxd1)* is required for arterio-venous specification and haematopoietic stem cell emergence

Foxd1 is expressed in the somitic subpopulation immediately adjacent to the DA, the sclerotome (Figure 4-10, p. 144). Furthermore, it is amongst the genes with the strongest differential expression in the RNA-seq experiment, and has been validated by qPCR to be a *dll4* target in the trunk/tail regions of zebrafish embryos (Figure 5-3, p. 162). Very little is known about the role of *foxd1* in development, apart from an association with cranio-facial development (Balikova et al., 2010), and there have been no reports linking *foxd1* to haematopoiesis, making *foxd1* a very interesting candidate gene.

Runx1 positive cells in the DA are clearly reduced in *foxd1* morphants at 26 hpf (Figure 5-7-A). At 36 hpf *ikzf1* (*ikaros*) positive cells are reduced while the HSC marker *cmyb* does not appear to be affected (Figure 5-7-B&C). *Rag1* positive T-cells are reduced in *foxd1* morphants in the thymus at 4 dpf, while myeloid (*mpx* (*mpo*)) and erythroid (*hbbe1.1* ($\beta e1$ globin)) lineages are not affected (Figure 5-7-H-J).

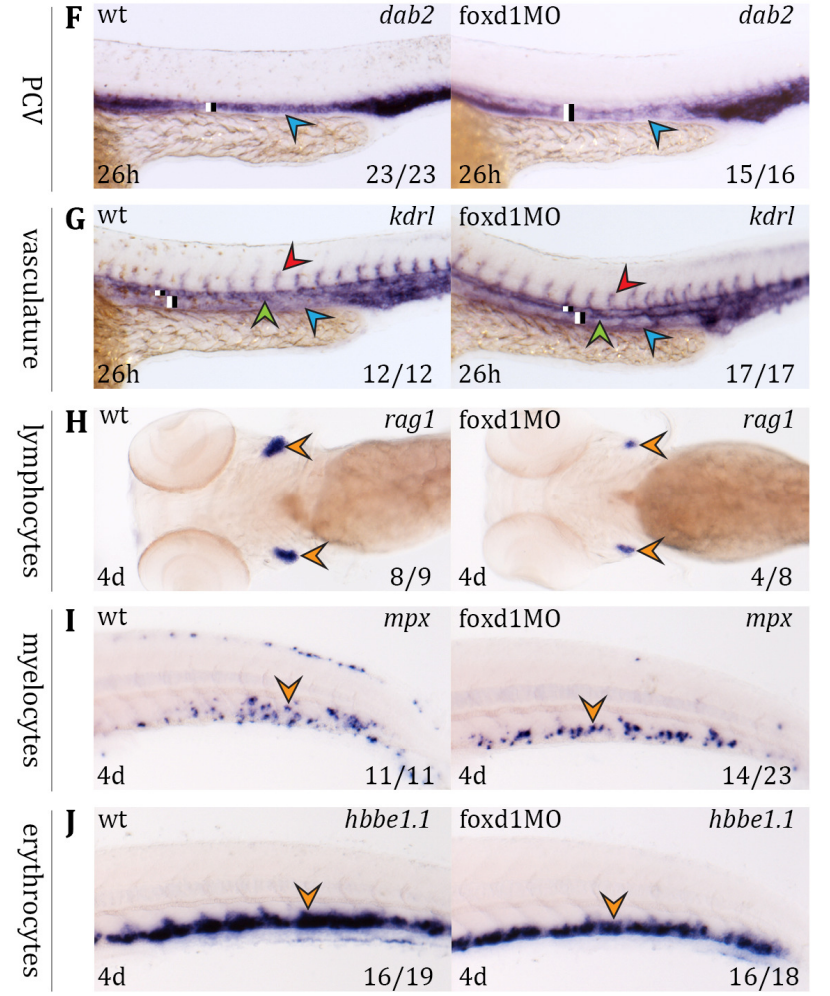
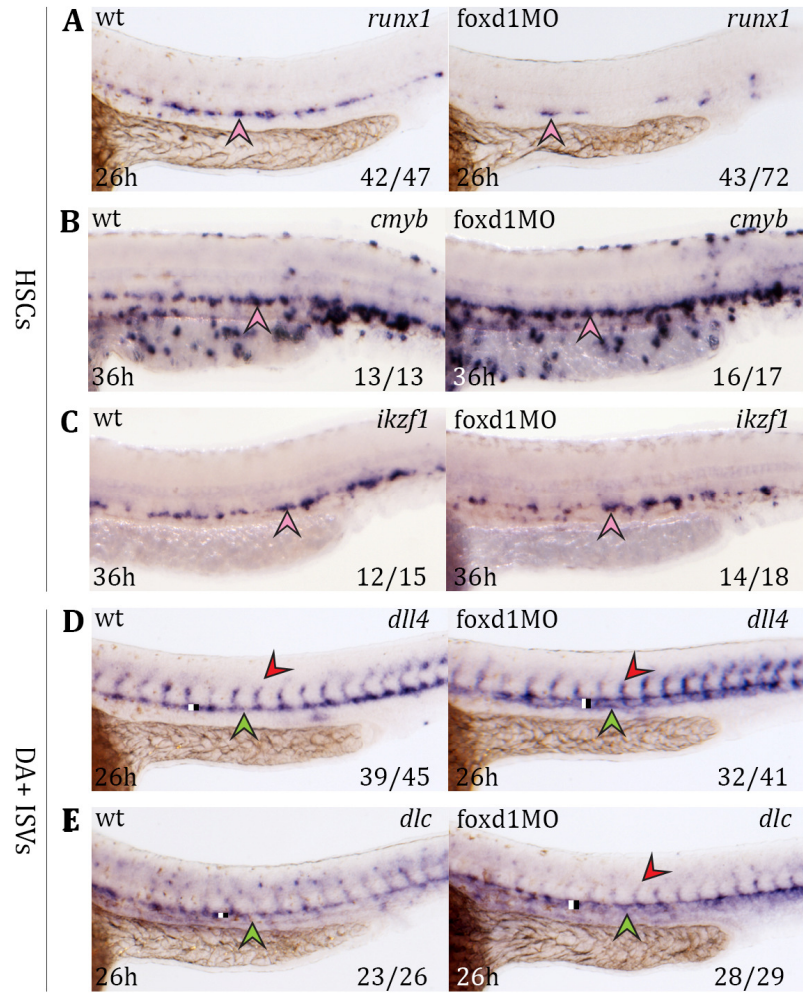


Figure 5-7. MO screen to assess the potential role of *foxd1* in HSC development.

In *foxd1* morphants *runx1* positive cells are reduced in the DA at 26 hpf (A). At 36 hpf, the HSC marker *cmyb* is not affected (B) while there are less *ikzf1* (*ikaros*) positive cells in the DA (C). Expression of arterial markers *dll4* and *dlc* is expanded in the DA (black/white bar) at 26 hpf (D, E), this is also true for venous expression of *dab2* (bar, F). The pan-endothelial marker *kdrl* (*flk1/vegfr2*) is not affected (bar, G), as is expression of *dll4*, *dlc* and *kdrl* in the ISVs (D, E, G). *Rag1* expression in the thymus is reduced in *foxd1* morphants (H) while there is no change observable for the myeloid marker *mpx* (*mpo*) (I) and erythroid marker *hbbe1.1* (*βe1 globin*) (J). Arrows indicate: HSCs (pink), DA (green), ISVs (red), PCV (blue) and HSC derivatives (orange). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

In order to investigate whether the observed down-regulation of *runx1* at 26 hpf is specific to a requirement for *foxd1* in HSC emergence or is secondary to arterial defects, the vascular programme was assessed. *dll4* and *dlc* are expanded in a dorso-ventral fashion (Figure 5-7-D&E, bar), suggesting that more cells have undergone arterial specification in these morphants.

This expansion of expression is only observed in the DA, but not in the ISVs. The venous programme is correspondingly affected, with *dab2* being expanded in a similar fashion (Figure 5-7-F, bar). The pan-endothelial marker *kdrl* (*flk1/vegfr2*) is not affected, and DA and PCV can clearly be distinguished within the *kdrl* positive population, neither of which seems to be expanded (Figure 5-7-G, bar). This expression indicates that *foxd1* might restrain the expression of arterial and vascular markers to their respective tissues, while not affecting overall pan-endothelial structure. No defects in circulation were observed, indicating a functional vasculature (Schuermann et al., 2014). With the observed defects in arterial and venous development, it is not possible to conclude whether the effect of *foxd1* on HSC development is specific or secondary, from the

experiments presented here. Thus, despite presenting a very interesting phenotype, *foxd1* has not been investigated further for its role in HSC development.

5.8 *Hes6* and *her8a* are required for HSC emergence

The transcriptional repressors of the *Hairy and Enhancer of Split* (*hes/hey*) and *Hes-related* (*her/hrt*) basic helix-loop-helix (bHLH) DNA-binding protein families are known to be directly activated by Notch signalling (Lai, 2004, Bray, 2006, Gering and Patient, 2010). Two bHLH genes are differentially expressed in the *dll4* morphant RNA-seq dataset: *hes6* and *her8a*. Interestingly, it has previously been implied that *hes6* and *her8a* are closely related (Chung et al., 2011b, Webb et al., 2011). Despite evidence that mammalian / vertebrate *hes6* genes are not regulated by Notch signalling (Kageyama et al., 2007), *her8a* has been shown to be activated through *suppressor of hairless* (*SuH*) regulated Notch signalling (Chung et al., 2011b).

5.8.1 *Hes6* is required for HSC development

HSC markers *runx1* and *cmyb* are reduced at 26 hpf and 36 hpf upon *hes6* activity knockdown (Figure 5-8-A&B), suggesting a requirement for *hes6* in HSC specification. Arterial development was assessed to rule out arterial mis-specification: expression of *dlc* appears normal (Figure 5-8-C), as does expression of venous marker *dab2* (Figure 5-8-D). *Kdrl* (*flk1/vegfr2*) also shows no effect on vascular patterning (Figure 5-8-E), indicating the absence of an effect on vascular development in *hes6* morphants. This suggests that the observed effect on HSC development is likely specific to a role for *hes6* in HSC development and secondary to arterial mis-specification. The reduction in HSCs

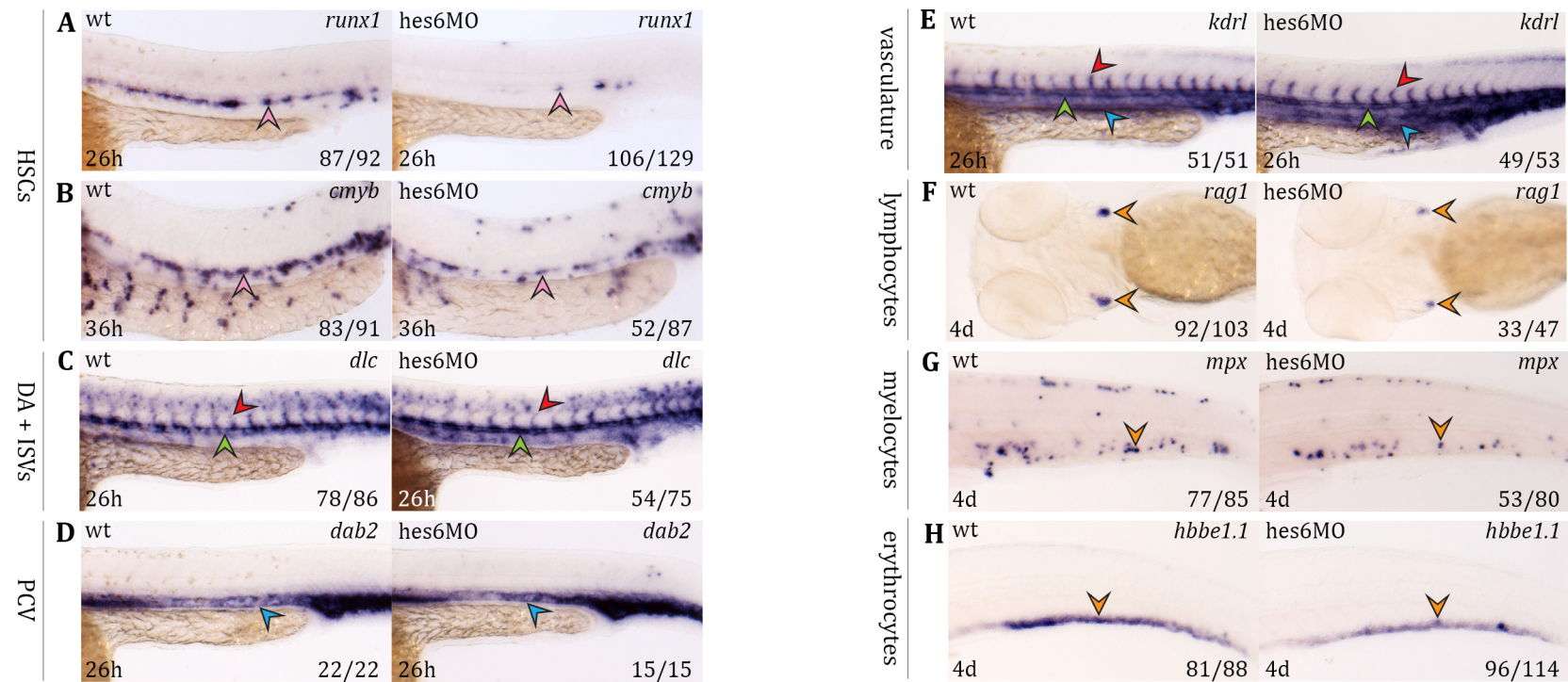


Figure 5-8. MO screen to assess the potential role of *hes6* in HSC development.

Runx1 positive cells are severely reduced in *hes6*MO injected embryos as compared to wt siblings at 26 hpf (A). At 36 hpf, fewer cells are expressing HSC marker *cmyb* (B) in *hes6* morphants as compared to wt siblings. Arterial expressed gene *dlc* is expressed in the DA and ISVs at comparative levels and patterns to wt siblings at 26 hpf (C). Venous expressed gene *dab2* is not affected in *hes6* morphants (D), neither is pan-endothelial marker *kdr1* (*flk1/vegfr2*) (E). T-cell marker *rag1* (F) is expressed at lower levels in the thymus of 4 dpf *hes6*MO injected embryos. Numbers of *mpx* (*mpo*) positive cells in the CHT of *hes6*MO injected embryos are reduced as compared to wt siblings (G). Levels of *hbbe1.1* ($\beta e1$ globin) expression are also reduced in *hes6* morphants (H). Arrows indicate: HSCs (pink), DA (green), ISVs (red), PCV (blue) and HSC derivatives (orange). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

appears to be maintained through development, as *rag1* staining in the thymus is reduced in *hes6* morphants (Figure 5-8-F), indicating a reduction in HSC-derived T-cells. The other HSC-derived definitive erythroid (*hbbe1.1* ($\beta e1$ globin)) and myeloid (*mpx* (*mpo*)) blood lineages are also reduced in the *hes6* morphants (Figure 5-8-G&H).

Consequently, it appears that *hes6* is at least partially required for HSC specification, calling for a more detailed analysis of the *hes6* phenotype. In this context, experiments conducted for the MO screen shown above were repeated at least once, to confirm the observed phenotype. Biological repeats confirmed all initial observations made and numbers in Figure 5-8 thus represent all experiments conducted. The MO screen analysis was then expanded to include further markers for the tissues assessed, with mostly a single biological replicate.

When assessing the HSC phenotype of *hes6* morphants in more detail, it appears that *hes6* might be skewing expression of HSC markers: the reduction of *runx1* in *hes6* morphants at 36 hpf is minor, as is the reduction of *gfi1aa* expressing cells (Figure 5-9-A&B). Unexpectedly though, *ikzf1* (*ikaros*) seems to be slightly up-regulated at 36 hpf (Figure 5-9-C) in contrast to the large reduction in *cmyb* positive cells (Figure 5-8-B). All markers used to assess the presence of HS(P)Cs in the CHT are reduced (Figure 5-9-I-L), including *ikzf1* (*ikaros*).

Assessment of DA development was also extended to address the possibility of an arterial mis-specification causing the observed HSC phenotype. Patterning of the DA appears to be unaffected in *hes6* morphants for all markers used for assessment, eg. *dll4*, *hey2* (*gridlock*) and *tbx20* (*hrt*), while there appear to be minor effects on the expression of these genes in the ISVs (Figure 5-9, red arrows). Levels of *dll4*, *hey2* (*gridlock*) and *tbx20* (*hrt*) are slightly reduced in the ISVs, but *kdrl* (*flk1/vegfr2*), *fli1a* and *dlc* are

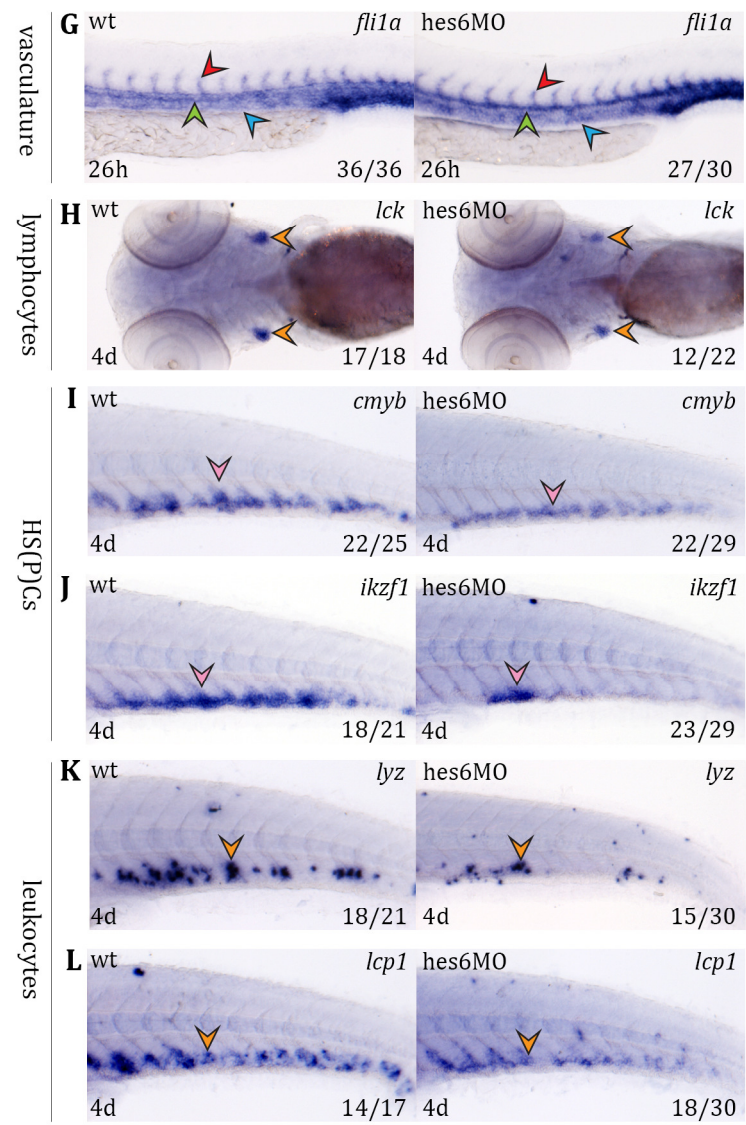
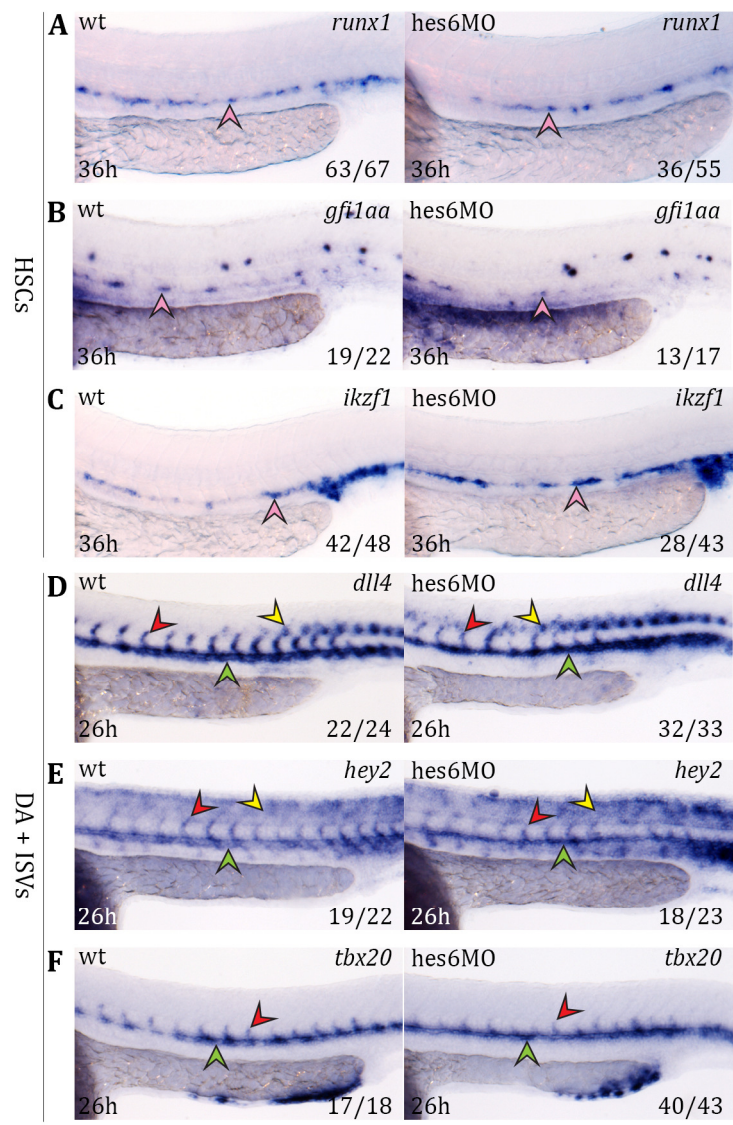


Figure 5-9. Extended phenotypic analysis of *hes6* morphants.

At 36 hpf, fewer cells are expressing HSC markers *runx1* (A) and *gfi1aa* (B) in the DA of *hes6* morphants as compared to wt siblings, while HSC marker *ikzf1* (*ikaros*) appears increased (C). Arterial patterning is not affected in *hes6* morphants as evidenced by appropriate expression of *dll4* (D), *hey2* (*gridlock*) (E), *tbx20* (*hrt*) (F) in the DA and ISVs at 26 hpf. Note the increased expression of *dll4* (D) and *hey2* (*gridlock*) (E) in neural cells. Expression of *fli1a* is not affected in *hes6* morphants (G). T-cell marker *lck* (H) is reduced in *hes6*MO injected embryos. Cells expressing *cmyb* (I) and *ikzf1* (*ikaros*) (J) are reduced in the CHT of 4 dpf *hes6* morphants, as are cells expressing leukocyte makers *lyz* (*lysozyme c*) (K) and *lcp1* (*l plastin*) (L). Arrows indicate: HSCs (pink), DA (green), ISVs (red), PCV (blue), HSC derivatives (orange) and neurons (yellow). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

expressed at wt levels in all vessels of *hes6* morphants (Figure 5-8 and Figure 5-9), suggesting that intersomitic vessels are present in *hes6* morphants but that their arterial character is partially compromised.

Dll4 expression in *hes6* morphant neural tissues is expanded more anteriorly, as compared to wt siblings and expression intensity is increased in these cells, similar to the increased intensity observed for *hey2* (*gridlock*) expression in the neural tube (Figure 5-9). Markers for HSC derivatives are all reduced, consistent with the hypothesis that *hes6* is required for HSC specification. There appear to be reduced numbers of T-cells in *hes6* morphants as indicated by *rag1* and *lck* expression. The erythroid (*hbbe1.1* (*βe1 globin*)) and myeloid (*mpx* (*mpo*), *lyz* (*lysozyme c*), *lcp1* (*l plastin*)) lineages are reduced in *hes6* morphants (Figure 5-8-G&H and Figure 5-9-K&L), as are HS(P)Cs (Figure 5-9-I&J), consistent with a requirement for *hes6* during HSC specification.

In conclusion, it appears that *hes6* is required for HSC emergence while minimally affecting development of the axial vessels.

5.8.2 *Her8a* is required for HSC development

In *her8a* morphants *runx1* positive cells are markedly reduced at 26 hpf (Figure 5-10-A). Expression of HSC marker *cmyb* is also reduced at 36 hpf (Figure 5-10-B), while the dorsal aorta maintains its structural integrity and specification as indicated by normal circulation in these embryos at all timepoints assessed (26 hpf, 36 hpf and 4 dpf, data not shown). Normal vascular development is supported by the wt-like expression of arterial expressed *flt1* (*vegfr1*) and venous expressed *dab2* (Figure 5-10-C&D). These observations suggest that *her8a* is required for HSC specification while not being required for vascular development.

Consistent with a proposed role for *her8a* in HSC specification, HSC derivatives of all lineages appear to be reduced in *her8a* morphants. The T-cell marker *rag1* is drastically reduced in *her8a* morphants (Figure 5-10-E). *Mpx* (*mpo*) is also clearly reduced (Figure 5-10-G), indicating an effect on the myeloid lineage. *Hbbe1.1* (*βe1 globin*) is reduced, although the erythroid lineage is not affected as severely (Figure 5-10-F).

The phenotype observed for *her8a* in the initial MO screen specified *her8a* as a good candidate gene for a role in HSC specification downstream of *dll4*. Biological replicates were obtained for the data collected in the screen in order to confirm this phenotype. All data presented in Figure 5-10 was reproduced at least once, and all replicates showed the same phenotype.

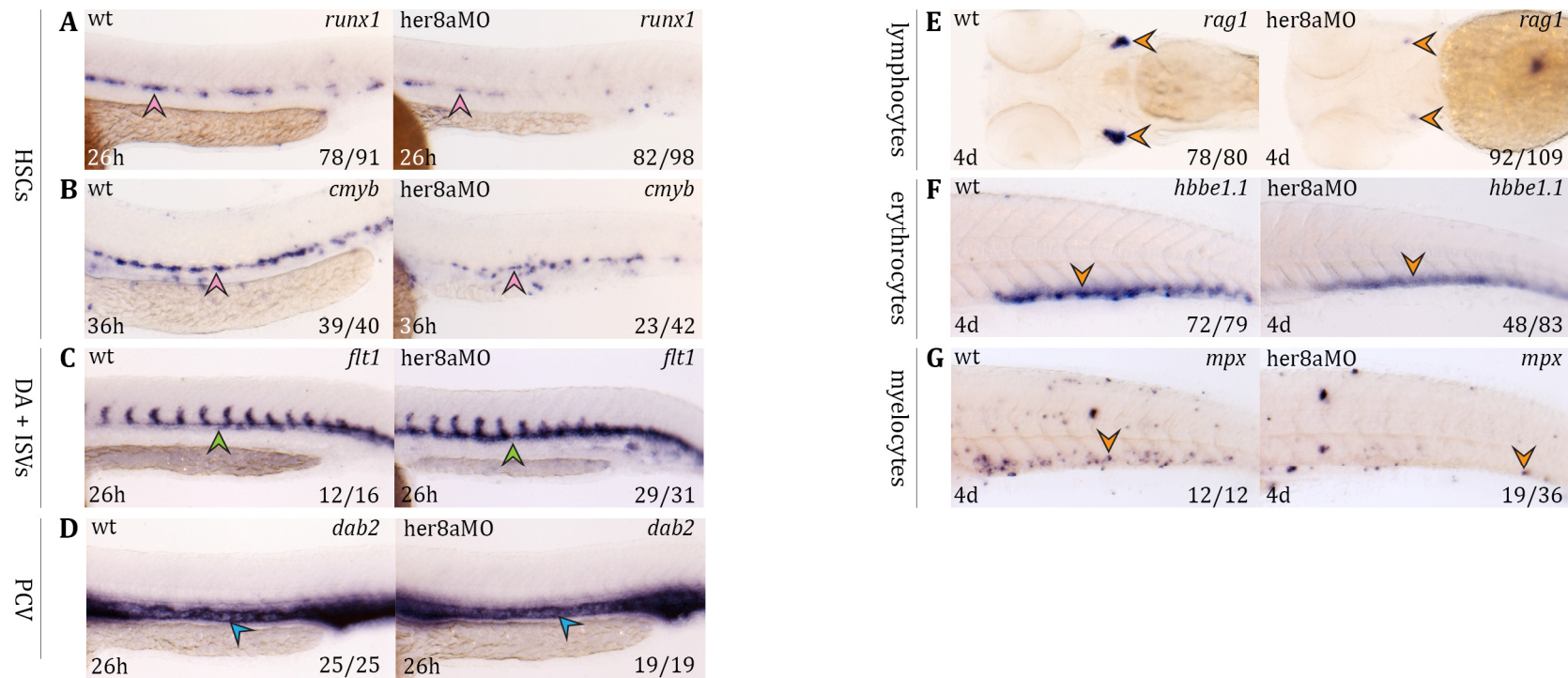


Figure 5-10. MO screen to assess the potential role of *her8a* in HSC development.

runx1 positive cells are markedly reduced in *her8a* morphant embryos at 26 hpf (**A**) as are *cmyb* positive cells at 36 hpf (**B**). No effect on the dorsal aorta and posterior cardinal vein can be observed, as *flt1* (*vegfr1*) (**C**) and *dab2* (**D**) are expressed normally. T-cells are highly reduced in *her8a* morphants (*rag1*) (**E**). A slight reduction of definitive erythrocytes is observed at 4 dpf as indicated by a reduction in *hbbe1.1* (*βe1 globin*) in the CHT (**F**). The definitive myeloid lineage is also reduced in *her8a* morphants indicated by a reduction of *mpx* (*mpo*) staining in the CHT (**G**). Arrows indicate: HSCs (pink), DA (green), ISVs (red), PCV (blue) and HSC derivatives (orange). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

Numbers presented in Figure 5-10 represent the total number of replicates in all experiments. Assessment was extended to include more markers, to confirm the role of *her8a* in HSC specification.

HSC markers *runx1* and *ikzf1* (*ikaros*), are reduced in *her8a* morphants at 36 hpf (Figure 5-11-A&B). Further, *foxn1* expression is not changed in the *her8a* morphants at 4 dpf (Figure 5-11-H), indicating that the observed reduction in T-cells (Figure 5-10-E) is not due to an absence or mis-specification of the thymic epithelium.

Interestingly though, despite no visible effects on expression of arterial genes *flt1* (*vegfr1*), *dll4*, *hey2* (*gridlock*) and *tbx20* (*hrt*), *her8a* is required for the arterial restricted expression of *dlc*, which is ectopically expressed in the PCV in *her8a* morphants (Figure 5-11-C). Pan-endothelial marker *kdrl* (*flk1/vegfr2*) is also up-regulated in the PCV while expression levels in the DA and ISVs are comparable to those of wt siblings (Figure 5-11-G).

Taken together this data suggests that *her8a* is required for HSC specification. Although it appears that *her8a* has a role in vascular development as well, the data presented here suggests that this is in venous identity rather than arterial, indicating that the effect of *her8a* on HSC specification is likely specific, although further analyses would be necessary to confirm this.

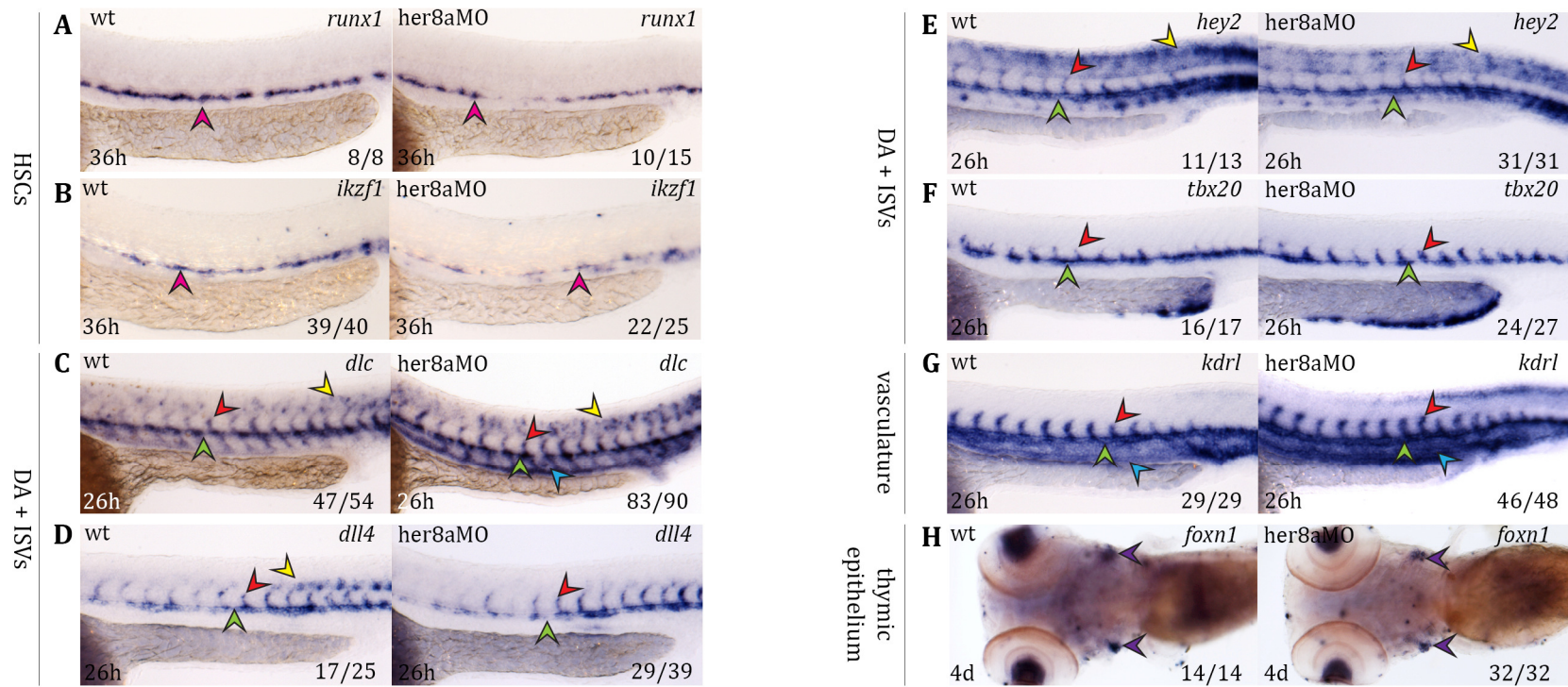


Figure 5-11. Extended phenotypic analysis of *her8a* morphants.

HSC markers *runx1* (A) and *ikzf1* (*ikaros*) (B) are reduced in *her8a* morphant embryos at 36 hpf, as compared to wt siblings. Overall structural development of the dorsal aorta is not affected in *her8a* morphants as indicated by normal arterial expression of *dlc* (C), *dll4* (D), *hey2* (*gridlock*) (E) and *tbx20* (*hrt*) (F). Intersomitic vessels are developing normally at 26 hpf (C-F). Note expansion of *dlc* into the vein in *her8a* morphants (C) and the up-regulation of markers expressed in the neural tissues (C - E). Arterial expression of pan-endothelial marker *kdr1* (*flk1/vegfr2*) is not affected while its venous expression is increased (G). Expression of *foxn1* is not affected in the thymic epithelium (H). Arrows indicate: HSCs (pink), DA (green), ISVs (red), PCV (blue), HSC derivatives (orange) and neurons (yellow). * Note that data shown in panel (A) is also represented in (Appendix Figure 8-8, p. 259). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

5.9 *Forkhead box C1b* (*foxc1b*) is required for haematopoietic stem cell emergence but not vascular specification

Foxc1b is a transcription factor expressed in the DA and the immediately adjacent somitic sclerotome in the zebrafish embryo (Figure 4-10, p. 144; De Val et al., 2008, Skarie and Link, 2009). Despite its expression in the DA, *foxc1b* is not required for arterial specification and patterning on its own but in conjunction with its paralogue *foxc1a* (De Val et al., 2008). *Foxc1* has been suggested to be an important downstream effector of Notch signalling (Seo et al., 2006, Hayashi and Kume, 2008), making it a very good candidate gene for a regulatory role in HSC development.

Runx1 positive cells are reduced in the DA of *foxc1b* morphants at 26 hpf (Figure 5-12-A), indicating a role for *foxc1b* in HSC specification. Intriguingly, however, at 36 hpf *cmyb* positive cells are increased, indicating more cells with an HSC-like expression profile (Figure 5-12-B). These increased numbers of cells expressing HSC markers do not seem to be functional, as HSC-derived lineages are reduced rather than increased. Expression of T-cell marker *rag1* is clearly reduced, if not absent in morphant embryos (Figure 5-12-F). There are also fewer cells expressing erythroid marker *gata1a* and myeloid marker *mpx* (*mpo*) in the CHT of 4 dpf morphants (Figure 5-12-G&H).

This effect of *foxc1b* on the HSC programme appears likely to be specific to a requirement of *foxc1b* in HSC development as no phenotypic effect on the arterial programme (*flt1* (*vegfr1*)) was observed in this initial screen (Figure 5-12-C), and

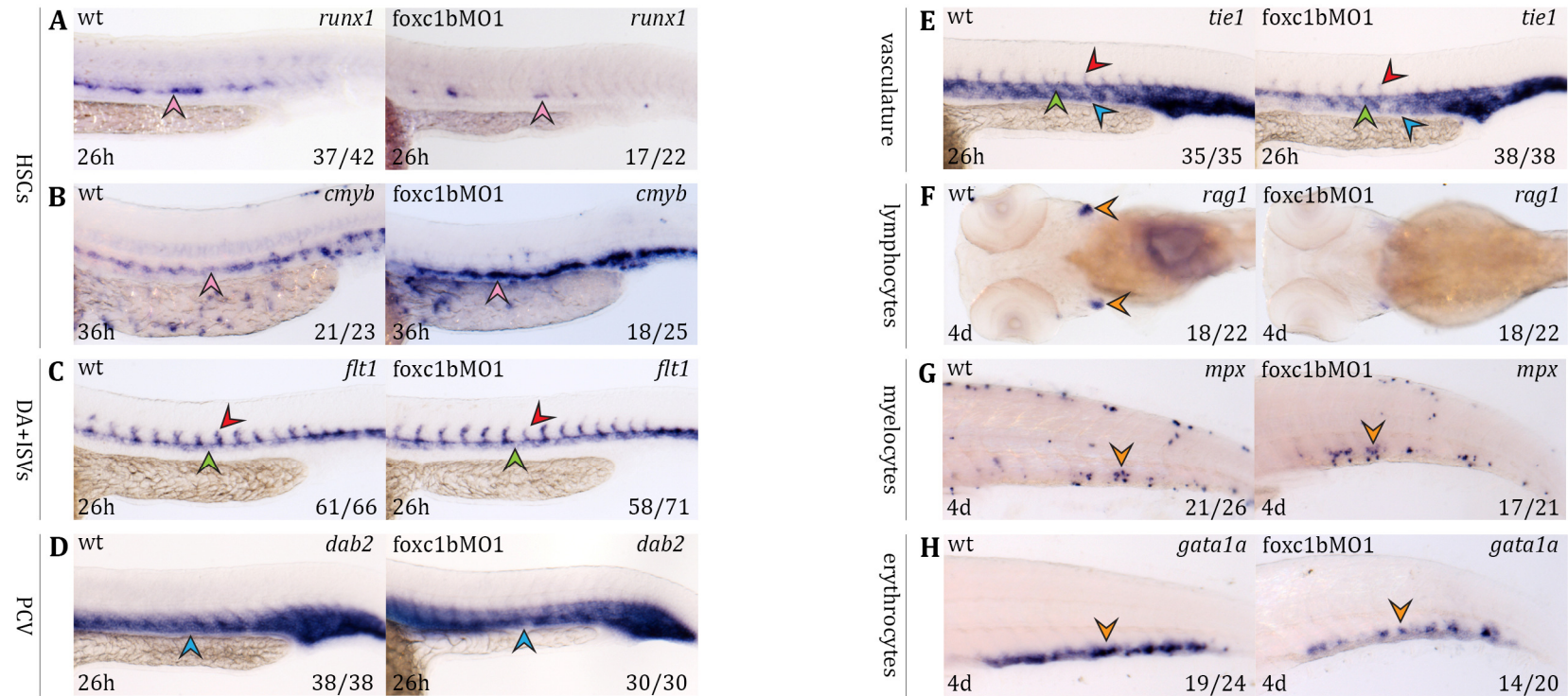


Figure 5-12. MO screen to assess the potential role of *foxc1b* in HSC development.

At 26 hpf, there are fewer *runx1* positive cells in *foxc1b*MO1 as compared to wt siblings (A), while *cmyb* positive cells are increased at (B). The arterial marker *flt1* (*vegfr1*) is not affected in *foxc1b* morphants (C). Venous expression of *dab2* is unchanged (D), as is pan-endothelial expression of *tie1* (E). *Rag1* is down-regulated in T-cells at 4 dpf (F), as is *gata1a* in erythroid cells (H) and *mpx* (*mpo*) in myeloid cells (G) of *foxc1b* morphants.. Arrows indicate: HSCs (pink), DA (green), ISVs (red), PCV (blue) and HSC derivatives (orange). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

circulation was also normal (data not shown). Furthermore, expression of venous and vascular markers assessed is normal in the absence of *foxc1b* (Figure 5-12-D&E).

Accordingly, the clear effect of *foxc1b* on initial HSC specification followed by the intriguing up-regulation of HSC markers 10 hours later in development, and the reduction in all HSC-derived lineages, make *foxc1b* a very intriguing *dll4* target with a role in HSC development. This is unlikely to be a secondary effect due to arterial mis-specification, as the arterial programme does not appear to be affected in *foxc1b* morphants, consistent with previous studies (De Val et al., 2008). Consequently, *foxc1b* was studied in further detail in Chapter 6.

5.10 Discussion

5.10.1 Overview

In Chapter 4, downstream targets of *dll4* that are likely to be upstream of *runx1* have been established by comparative RNA-seq, and best candidate genes for a regulatory role in definitive haematopoietic development have subsequently been chosen. Here, the 7 best candidate genes have been subjected to functional assessment of their potential roles in HSC development by means of a MO screen. These top 7 genes have been subjected to qPCR validation as *dll4* targets and homology across vertebrate species has been assessed (data not shown) prior to functional testing. A single MO was employed for each candidate gene, titrated for its optimal dose and vasculature, HSC programme and HSC derivatives were subsequently assessed in mostly a single biological replicate.

Two areas of argument have been highlighted throughout this Chapter that will be discussed here: firstly the choice of the most appropriate functional screening tool for this project and the design of a highly subjective "perfect" screen, and secondly the success and biological relevance of the screening outcome.

5.10.2 Choosing the best reagent for a functional screen

The zebrafish has long been known to be a very good model for forward genetic screens with a high success rate (eg. Driever et al., 1996, Haffter et al., 1996). This traditional technique is not appropriate for the project presented here, as the the objective was to target specific genes of interest, rather than generating random mutants. Previously characterized zebrafish mutants are available for *zfat* (ZMP, 2014), *hif1ab* (ZMP, 2014), and *hes6* (Schröter et al., 2012), but not for *cldn5b*, *foxd1*, *foxc1b* or *her8a*, precluding this approach for our functional analysis.

Recently, several new technologies for reverse genetics have become available in the zebrafish, including targeting induced local lesions in genomes (Tilling) (Wienholds et al., 2003, Sood et al., 2006, Moens et al., 2008), zinc-finger nucleases (ZFN) (Doyon et al., 2008, Foley et al., 2009), transcription activator-like effector nucleases (TALEN) (Bedell et al., 2012, Cade et al., 2012, Dahlem et al., 2012, Moore et al., 2012) and clustered regularly interspaced short palindromic repeats (CRISPR) (Hwang et al., 2013). However, at the time of experimentation, morpholinos were the only available technology for highly penetrant targeted disruption of gene activity in the F0 generation (Summerton, 2007, Jao et al., 2013). There are advances being made towards the generation of biallelic mutations in the F0 generation using TALEN and CRISPR technology (Nakajima et al., 2012, Blitz et al., 2013, Jao et al., 2013), however the most

promising estimate of approximately 56-81% biallelic mutation of the target gene achieved in the F0 (Jao et al., 2013), is still too low for the functional screening attempted in this chapter.

It has been proposed before that the false negative rate in MO screens that use novel MOs lies just above 10% (Huang et al., 2013, Melvin et al., 2013). This is likely to be lower here, as all MOs have been shown to work on the respective target genes before, although this has not been confirmed here. In other MO screens, 8% of genes that showed an initial phenotype could not be verified, thus classifying as false positives (Huang et al., 2013). This issue has not been generally addressed in this screen, but further analysis of the best candidate gene (*foxc1b*) includes the standard control of a second MO (Pickart et al., 2006) in Chapter 6.

A major concern with any functional screen however is non-specific off-target effects: p53-mediated neural toxicity is commonly observed at high MO concentrations and has been suggested to be a non-specific MO off-target effect (Egger, 2000, Nasevicius and Egger, 2000, Robu et al., 2007, Bedell et al., 2011) in as many as 15-20% of all MOs (Robu et al., 2007, Eisen and Smith, 2008, Bedell et al., 2011). This phenomenon can be circumvented by titration to a lower efficacy dose at which these neural phenotypes cannot be detected (Robu et al., 2007). Overall severe impediment of gross developmental morphology is a further common off-target effect, often manifesting as shortened and contorted tails, considerable body curvatures and small heads and eyes (Egger, 2000, Woods and Schier, 2008, Bedell et al., 2011).

This entire range of presumed non-specific off-target effects was observed in the titrations of the 8 MOs employed in this chapter: neural toxicity was observed in *foxc1b*MO, *hif1ab*MO and *hes6*MO, shortened and contorted tails in *foxc1b*MO, *zfat*MO1

and *zfat*MO2, considerable body curvatures in *hif1ab*MO, *zfat*MO1, *zfat*MO2 and *her8a*MO and small heads and eyes in *hes6*MO. Despite the hallmarks of off-target effects clearly present in most MOs used here, it was in all cases possible to use concentrations at which these effects were absent (Robu et al., 2007, Bedell et al., 2011), confirming the importance of MO-specific titration.

As a consequence of the screen design it is not possible to draw any firm conclusions about the phenotypes observed for any of the genes assessed, as MO specificity has not been addressed here and phenotypes have not been reproduced with an alternative MO. However, with the main objective of this chapter being the identification of the most promising *dll4* target gene for a role in HSC specification, this confirmation of MO specificity was conducted on the best candidate gene (in Chapter 6) rather than as part of the screening process.

The choice of parameters for a functional screen such as this is highly subjective, however it has been clearly successful here as three good candidate genes were discovered for a role in HSC specification, and all 7 genes screened showed interesting phenotypes in the context of haemato-vascular development.

5.10.3 All genes included in the MO screen are involved in haematopoiesis

All 7 genes screened in the MO screen presented here showed a HSC phenotype. Knockdown of *cldn5b* also caused major vascular abnormalities as indicated by severe disruption of circulation and misexpression of arterial markers. Circulation was normal in all other morphants at all timepoints assessed (26 hpf, 36 hpf, 4 dpf), indicating that the effects on vascular gene expression observed are not severe enough to cause

vascular malfunction (Schuermann et al., 2014). No effects on vascular patterning were observed in the case of *foxc1b*. *Runx1* expression was compromised in all morphants assessed, and those for *foxc1b* are presumed to be specific to a requirement for haematopoiesis, due to the absence of any arterial defects. A summary of the results of the screen is shown in Table 5-1.

This results in a phenotypic hit rate of 100% of genes assessed here, which is higher than any previously described for a published MO screen in zebrafish: a rate of 20% was described for a MO screen of a transcriptome comparison study of HUVECs and bone marrow cells (Eckfeldt et al., 2005). A rate as low as 4% was observed in a MO based sclerotome screen (Pickart et al., 2004), and a MO screen of chromatin modifiers resulted in a 9% hit rate for involvement in primitive or definitive haematopoiesis (Huang et al., 2013).

The marked success of the screen presented here is likely due to the stringent pre-selection criteria of: transcription factor or signalling activity, literature-based survey of haematopoietic activity or haematopoietic interaction, and expression in tissues related to HSC specification. This list of criteria is confirmed in its strength when considering the baseline of the screen conducted: although all genes screened here were initially considered to be *dll4* targets, only 4 could be confirmed by qPCR (Figure 5-3, p. 162). Despite this, the criteria for selection of genes to be included in a MO screen for a role in HSC specification were highly successful, and it is likely that this success could be extrapolated to a wider screen and still hold up.

Table 5-1. MO screen summary.

Legend: ~ no effect, ↑ expression up-regulated, ↓ expression down-regulated, ↔ expression expanded anterior-posteriorly, →← expression reduced anterior-posteriorly, ↓↑ expression expanded dorso-ventrally, ✕ “no”, ✓ “yes”

	validated <i>dll4</i> target	HSCs	circulation normal	vasculature	lymphocytes	myelocytes	erythrocytes
<i>cldn5b</i> MO	✕	↓	✕	↔, ↑			
<i>hif1ab</i> MO	✕	↓	✓	↓	↓	~	~
<i>zfat</i> MO	✕	↓	✓	→←	↓	~	↓
<i>foxd1</i> MO	✓	↓	✓	↓↑	↓	~	~
<i>hes6</i> MO	✓	↓/↑	✓	↓	↓	↓	↓
<i>her8a</i> MO	✓	↓	✓	↑	↓	↓	↓
<i>foxc1b</i> MO	✓	↓/↑	✓	~	↓	↓	↓

Data presented here for *cldn5b* suggests a role in vascular structure. *Cldn5b* morphants lack circulation and show pooling of blood while the arterial marker *dll4* is mis-expressed as compared to wt siblings. This indicates a crucial role for *cldn5b* in vascular development, similar to the roles described for its vertebrate orthologue *Cldn5* (Yuan et al., 2012). The extent to which *cldn5b* is required for vascular programming still remains to be determined, but here an initial observation has been made that points in this direction.

While *cldn5b* appears to have a major effect on vascular development, *hif1ab* affects levels of *dll4* only slightly, although it would be important to further assess the effect of loss of *hif1ab* activity on other arterial and vascular markers. Even if the effect on arterial development is negligible, the initial marked reduction of *runx1* staining in the

DA does not appear to cause a complete absence of functional HSCs as T cells are only slightly reduced and no effect can be discerned on the myeloid and erythroid lineages. It is possible that this phenotypic recovery over developmental time is due to a compensation by a different factor, potentially *hif1aa*, the zebrafish paralogue not present in other vertebrates.

The phenotype observed for *zfat* is perplexing as *runx1* expression is nearly absent at 26 hpf while *cmyb* expression at 36 hpf is not affected and *ikzf1* (*ikaros*) is up-regulated. This could be an indication that these HSC markers mark slightly different, potentially poised, populations of HSCs that might even be biased towards specific lineages. This makes *zfat* an intriguing gene that would need further assessment in order to shed light on this hypothesis.

The *foxd1* phenotype is remarkably similar to that of *zfat*, with the exception of a marked effect on vascular specification. It appears as if expression of arterial markers is expanded into the roof of the PCV and the expression of venous markers is expanded in to the floor of the DA, while the overall endothelium is not expanded. The WISH resolution is not good enough to prove this hypothesis, but it is likely considering that vascular endothelium per se appears not affected. Vascular endothelium is clearly separated into two lumenised strands of arterial and venous-like character as circulation is normal in these morphants at all timepoints assessed (Schuermann et al., 2014). Expansion of venous expressed genes such as *dab2* into the ventral dorsal aorta, might be responsible for the reduction of HSC markers, perhaps by direct suppression or change of the microenvironment.

The phenotypes of *hes6* and *her8a* are very similar and both genes appear to be specifically required for HSC emergence and not arterial development. These

observations were supported by additional biological replicates as well an extension of the phenotypic analysis. Further, it appears that *hes6* and *her8a* are working together in their role in haematopoiesis although there seems to be a requirement for at least another factor within this relationship as the double-morphants do not completely abrogate haematopoiesis, as would be expected in this case (Appendix Figure 8-8, p. 259). The effect of *hes6* and *her8a* on HSC development is unlikely to be through arterial mis-specification as only minor effects are seen on arterial patterning and the structural integrity of all axial vessels is intact as suggested by appropriate circulation and expression of most markers in the axial vessels. The reduction in the erythroid lineage is consistent with a previous report of *hes6* requirement in erythropoiesis (da Cunha et al., 2010).

Foxc1b stands out amongst the genes assessed for their function in HSC development here through its clear reduction in *runx1* at 26 hpf and the reduction of all HSC-derived lineages while not affecting arterial development (De Val and Black, 2009). The up-regulation of all assessed HSC markers (*runx1*, *cmyb*, *ikzf1* (*ikaros*)) at the time of initiation of EHT is intriguing and deserves further investigation. *Foxc1b* is one of the four confirmed *dll4* targets in an RNA-seq equivalent qPCR experiment, and is the only gene assessed here that showed no evidence for an effect on vascular development. Consequently, *foxc1b* is considered the best candidate gene downstream of *dll4* to regulate HSC specification through *runx1*, and this hypothesis is tested in Chapter 6.

5.10.4 Conclusions and directions

In conclusion, the MO screen conducted in this chapter was highly successful at revealing new biology for a set of 7 genes that have not previously been assessed for their role in zebrafish HSC development.

QPCR validation of these presumed *dll4* targets was attempted, and only *foxd1*, *hes6*, *her8a* and *foxc1b* could be validated. Unvalidated genes were nevertheless included in the functional screen, due to their high potential as HSC regulators, based on previous selection criteria. Indeed, a HSC phenotype was observed for all 7 genes assessed, proving the pre-screen selection criteria to be highly efficient. Here, all genes showed some effect on definitive haematopoietic development, either directly or indirectly.

The objective of the screen was achieved with *foxc1b* appearing to be required for HSC specification while not affecting arterial development, while also showing a secondary phenotype that is intriguing in its initial depletion of *runx1* positive cells, followed by an increase in non-functional HSCs at 26 hpf, resulting in a depletion of HSC derivatives. The observations made for *foxc1b* in this chapter provide the baseline for an in-depth analysis of the role of *foxc1b* downstream of *dll4* in HSC emergence in Chapter 6.

Chapter 6

Foxc1b is required for HSC
emergence

6.1 Introduction

In the zebrafish, *foxc1b* has been shown to be required for axial and angiogenic vascular development in conjunction with *foxc1a* (De Val et al., 2008, Skarie and Link, 2009, Veldman and Lin, 2012), with only a minor role on its own, if any (De Val et al., 2008). When co-injecting *foxc1a* and *foxc1b* morpholinos (*foxc1a/b*), defects in angiogenesis, disruptions in vascular basement membrane integrity, increased vascular permeability and arteriovenous deficiencies are observed (De Val et al., 2008, Skarie and Link, 2009). *Foxc1a/b* morphants exhibit abnormal blood flow due to vessel malformations, a phenotype not observed in either single morphant (Skarie and Link, 2009). Similarly, arterio-venous fusions are seen in *Foxc1/Foxc2* compound null mice and expression of many arterial markers, including those of the NOTCH pathway (*Notch1*, *Notch4*, *Dll4*, *Jag1* and *Hey2*), is absent in these mutants, while pan-endothelial markers are expressed (Kume et al., 2001, Seo et al., 2006).

Foxc1 and *Foxc2* are the only members of the vertebrate *FOXC* family; they are highly similar in developmental expression pattern, phenotype and their DNA binding domain sequences (for alignments and synteny analysis, see Appendix Figure 8-9, p. 262) and have been shown to function redundantly (reviewed in Koo and Kume, 2013). The zebrafish has a duplication of *FOXC1* (*foxc1a* and *foxc1b*) and lacks a *FOXC2* homologue (Topczewska et al., 2001b), but *foxc1a* and *foxc1b* have been suggested to fulfill a similar, if not identical, role to *FOXC1* and *FOXC2* in other vertebrates (Topczewska et al., 2001b, Skarie and Link, 2009). The role of *Foxc1* in HSC development has not previously been addressed; and in the zebrafish, no specific role for *foxc1b* has yet been described (De Val et al., 2008, Skarie and Link, 2009, Veldman and Lin, 2012).

Here, I assess the function of *foxc1b* by describing the first specific phenotype for this gene, in its requirement for HSC emergence. In Chapter 4, I identified *foxc1b*, but not *foxc1a*, as a *dll4* target in the zebrafish trunk/tail tissues at 26 hpf, and described a clear phenotype for *foxc1b* in definitive haematopoiesis in Chapter 5, indicating a *foxc1a*-independent role for *foxc1b* in HSC specification. Here, I investigate this role in further detail.

6.2 *Foxc1b* is expressed in the sclerotome and the vasculature

FOXC1 is expressed in vertebrate vascular endothelium and is believed to promote angiogenic activity (Kume et al., 2001, Topczewska et al., 2001b, De Val et al., 2008, Hayashi and Kume, 2008, Skarie and Link, 2009), consistent with the expression of zebrafish *foxc1a* and *foxc1b* during the early stages of the forming vasculature (De Val et al., 2008 and green arrow Figure 6-1-B). However, in both zebrafish and in mice, *FOXC1* is also expressed in non-vascular cells proximal to the *FOXC1* positive vasculature, including somitic mesoderm (Topczewska et al., 2001b, Hosaka et al., 2004, Seo et al., 2006, Skarie and Link, 2009, Sasman et al., 2012, Omatsu et al., 2014). In the zebrafish at the 10 ss, *foxc1b* is expressed in the forming somites (orange arrows, Figure 6-1-A) but not the posterior lateral mesoderm (PLM) that harbours the haemangioblasts. At 26 hpf, when *foxc1b* is regulated by *dll4*, it is expressed in the DA (green arrow, Figure 6-1-B&C), the sclerotome (orange arrow) and the tailbud (lilac arrow), as shown previously (Skarie and Link, 2009; Figure 6-1-C). To confirm *foxc1b* expression in the dorsal aorta at the latter stage, Tg(*dll4:gfp*) gfp-positive and gfp-negative cells were subjected to qPCR analysis, using the cardiac restricted gene *nkx2.5* as a baseline that could be

interpreted as absence of expression (Chen and Fishman, 1996; expression shown in Figure 6-1-D). This analysis clearly shows that *foxc1b* is expressed in both the arterial cell population (gfp positive) and the other tissues of the tail (gfp negative), as predicted from the WISH and previous expression analyses (De Val et al., 2008, Skarie and Link, 2009).

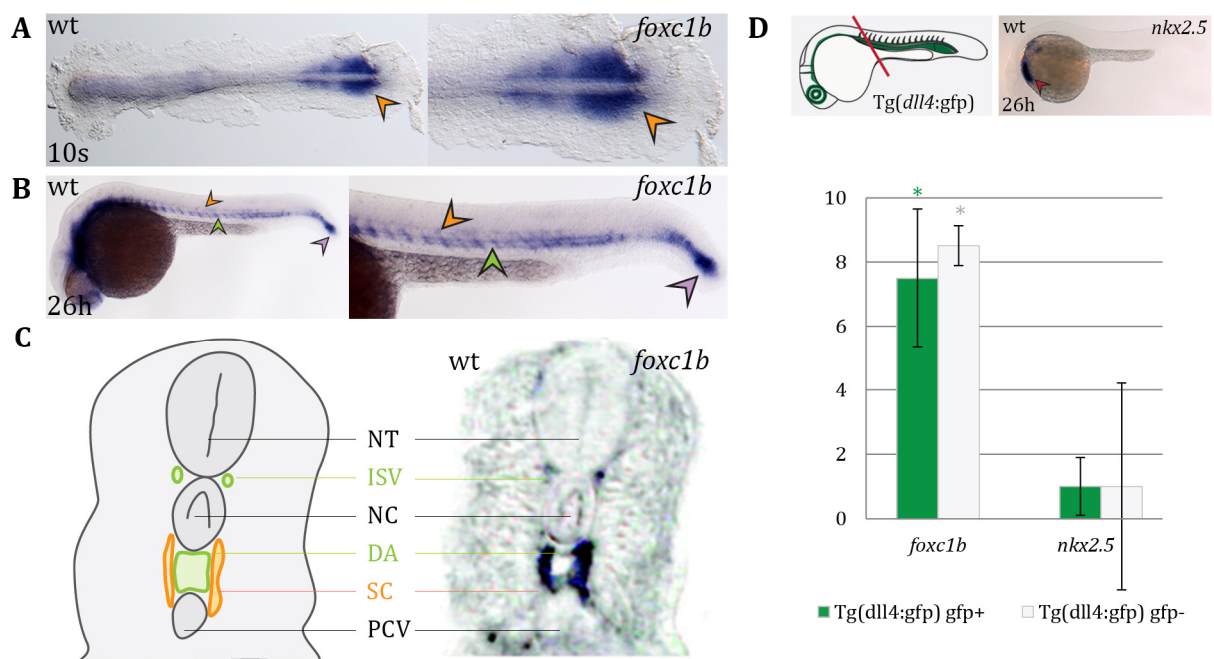


Figure 6-1. *Foxc1b* is expressed in the *dll4*-gfp positive and negative fractions of the zebrafish trunk/tail region.

A-C *Foxc1b* is expressed in the developing somites but not the PLM at the 10 somite stages (**A**, embryo kindly flat-mounted and photographed by Dr. Jana Koth) and in the DA, the sclerotome and the tailbud at 26 hpf (**B**). Cross-section shows that *foxc1b* is expressed in the dorsal aorta as well as the immediately adjacent sclerotome (**C**, from Skarie and Link, 2009). **D** qPCR analysis of FACS sorted Tg(*dll4:gfp*) cells from dissected trunk/tail tissues confirms that *foxc1b* is expressed in both the gfp+ and gfp- population. Expression of *nkx2.5* (red arrow) was used as a baseline (set to 1). Arrows indicate DA (green), somites (orange), tailbud (lilac), heart (red). Legend: NT = neural tube, ISV = intersomitic vessel, NC = notochord, DA = Dorsal aorta, SC = sclerotome, PCV = posterior cardinal vein.

In Chapter 5, I validated *foxc1b* as a *dll4* target by qPCR on trunk/tail tissues of *dll4* morphants. To further confirm this, *foxc1b* expression was assessed by WISH in *dll4* morphants. The down-regulation of *foxc1b* in the absence of *dll4* observed by RNA-seq and qPCR, is also observed here: *foxc1b* is expressed at lower levels in both arteries and sclerotome in *dll4* morphants at 26 hpf (Figure 6-2-A).

Dll4 is itself regulated by *Foxc1* in mouse endothelial cells (Seo et al., 2006, Hayashi and Kume, 2008), therefore, in order to address the possibility of a feedback loop between *foxc1b* and *dll4*, the expression of *dll4* in *foxc1b* morphants was also assessed here at 26 hpf, and found not to be under the control of *foxc1b* in the zebrafish (Figure 6-2-B). While I have shown above that both genes are required for *runx1* expression at this stage (Figure 3-5-B, p. 91 and Figure 5-12, p. 182), neither *foxc1b* nor *dll4* are differentially expressed in the dorsal aorta of *runx1* morphants (green arrows, Figure 6-2-A&B). Note the reduction of *dll4* expression in the intersomitic vessels (red arrows) in *runx1* morphants, which is likely a secondary effect of the ISV sprouting phenotype observed in these morphants (Kalev-Zylinska et al., 2002, Burns et al., 2005, Gering and Patient, 2005).

This data allows me to postulate here that at 26 hpf there is a hierarchical relationship between these three genes with *dll4* being required for *foxc1b* expression which in turn is required for *runx1* expression (Figure 6-2-C&D), in the absence of any regulatory feedback loops.

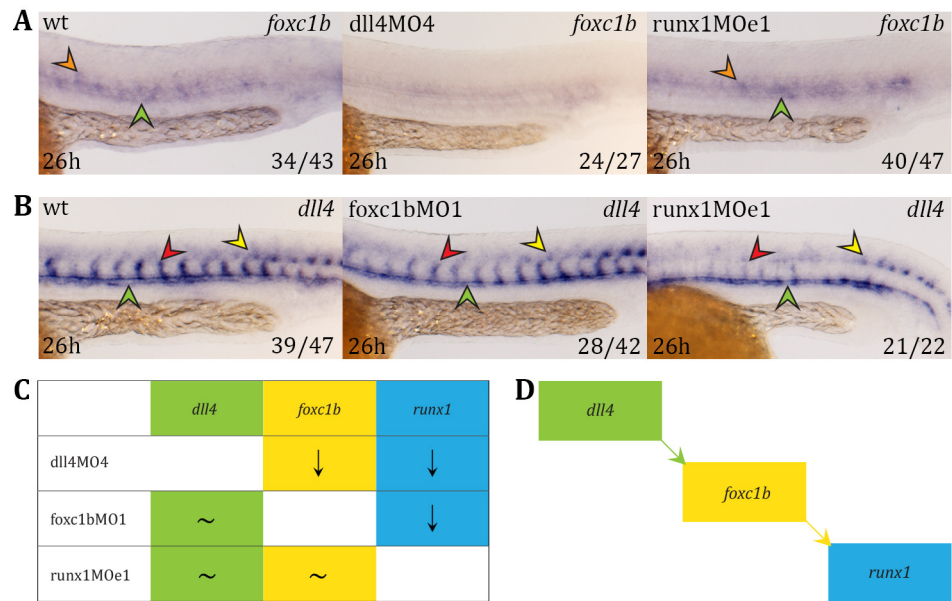


Figure 6-2. *Dll4* is required for *foxc1b* expression which is required for *runx1* expression at 26 hpf.

Expression of *foxc1b* is reduced in the sclerotome and the DA in *dll4* but not *runx1* morphants at 26 hpf (**A**). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235). *Dll4* expression is not affected in the DA of *foxc1b* and *runx1* morphants; lower *dll4* expression is observed in the ISVs of *runx1* morphants (**B**). Summary of morphant data (**C**). Proposed model for the genetic regulatory network between *dll4*, *foxc1b* and *runx1* (**D**).

Taken together with the RNA-seq and qPCR data, I have shown here that *foxc1b* is a target of *dll4* at 26 hpf in the posterior zebrafish tissues. RNA-seq and qPCR on trunk/tail tissues have shown that *foxc1b* is differentially expressed in *dll4* morphants, and ISH on whole embryos further confirms that this reduction is in both the arterial and the somatic expression domains of *foxc1b*. This makes *foxc1b* the best validated *dll4* target assessed in this thesis.

6.3 *Foxc1b* is dispensable for vascular development

Next, *foxc1b* was assessed in the context of its role downstream of *dll4* in HSC specification. The *foxc1b* morphant phenotype was assessed for the same tissues and cell populations assessed in Chapter 3 for *dll4*. To ensure the specificity of MO-induced phenotypes, two morpholinos were used (Eisen and Smith, 2008, Bill et al., 2009). Both have previously been shown to be specific for *foxc1b* and not to have off-target effects (Topczewska et al., 2001a, De Val et al., 2008). As *foxc1b* only has one exon, the morpholinos targeted the ATG start codon (MO1) and the 5' UTR region of the gene (MO2), with neither causing gross developmental abnormalities, except for a slight developmental delay, as is commonly observed in morpholino injections (Topczewska et al., 2001a, De Val et al., 2008, Skarie and Link, 2009).

Dll4 causes only minor defects in vascular development at 26 hpf in the zebrafish, as described in Chapter 3. Since I have hypothesised that *foxc1b* acts downstream of *dll4* in HSC development, a similar phenotype to *dll4* loss of function is expected in the absence of *foxc1b*. Although previous studies have shown that the absence of *foxc1b*

during zebrafish development causes no major vascular defects (De Val et al., 2008), as proper specification of vascular identity is required for HSC specification (reviewed in Adamo and García-Cardena, 2013), the extent of the vascular phenotype in *foxc1b* morphants was here addressed in more detail. Please note that all experiments presented in this Section have been run with a positive control (*runx1* at 26 hpf or *rag1* at 4 dpf, data not shown), validating that any absence of vascular phenotype is not due to inactivity of MO or failure of injection.

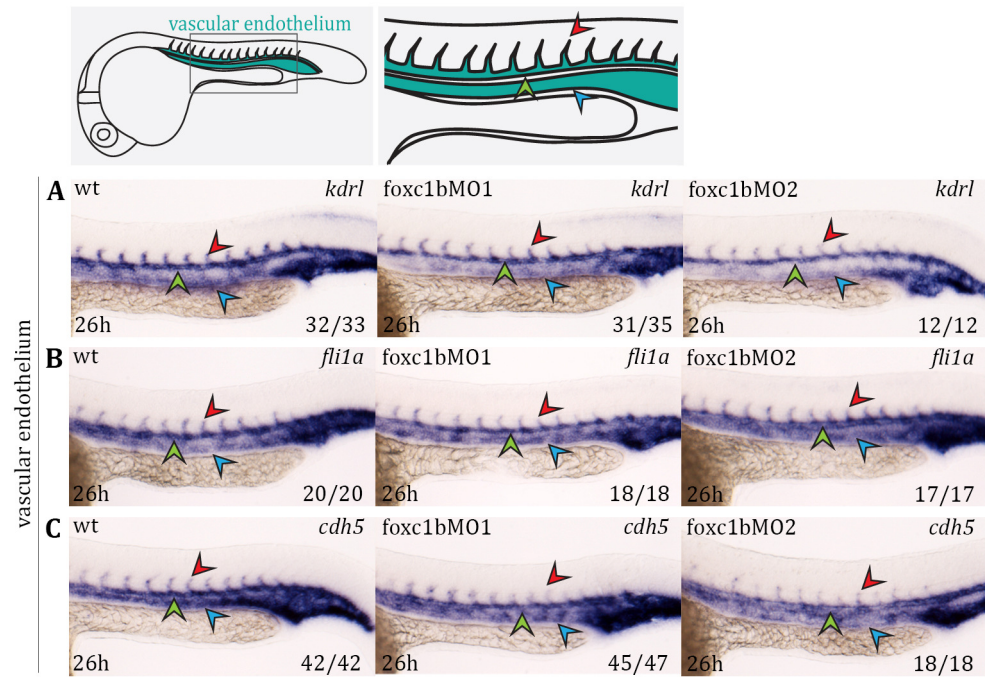


Figure 6-3. *Foxc1b* is not required for the endothelial programme at the time of HSC emergence.

Expression of the pan-endothelial marker *kdr1* (*flk1/vegfr2*) is not affected in embryos injected with *foxc1bM01* or *M02* (A). Similarly, expression of *fli1a* (B), and *cdh5* (*VE-cadherin*) (C) are also unchanged in both *foxc1b* morphants. Arrows indicate DA (green), ISVs (red), PCV (blue). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

Pan-endothelial markers are unchanged in embryos injected with *foxc1bM01* or *foxc1bM02* (Figure 6-3), consistent with the observation that *Foxc1* does not affect overall vascular development (Kume et al., 2001, Seo et al., 2006, De Val et al., 2008). *Kdr1* (*flk1/vegfr2*), *fli1a* and *cdh5* (*VE-cadherin*) are expressed at levels comparable to wt siblings in the dorsal aorta, posterior cardinal vein and intersomitic vessels (green, blue and red arrows in Figure 6-3, respectively), consistent with the observation made previously that *tie1* is not affected in embryos injected with *foxc1bM01* (Figure 5-12-E, p.180). The boundaries between the DA and the PCV can clearly be distinguished in all cases, indicating appropriate separation of arterial and venous vessels. Further,

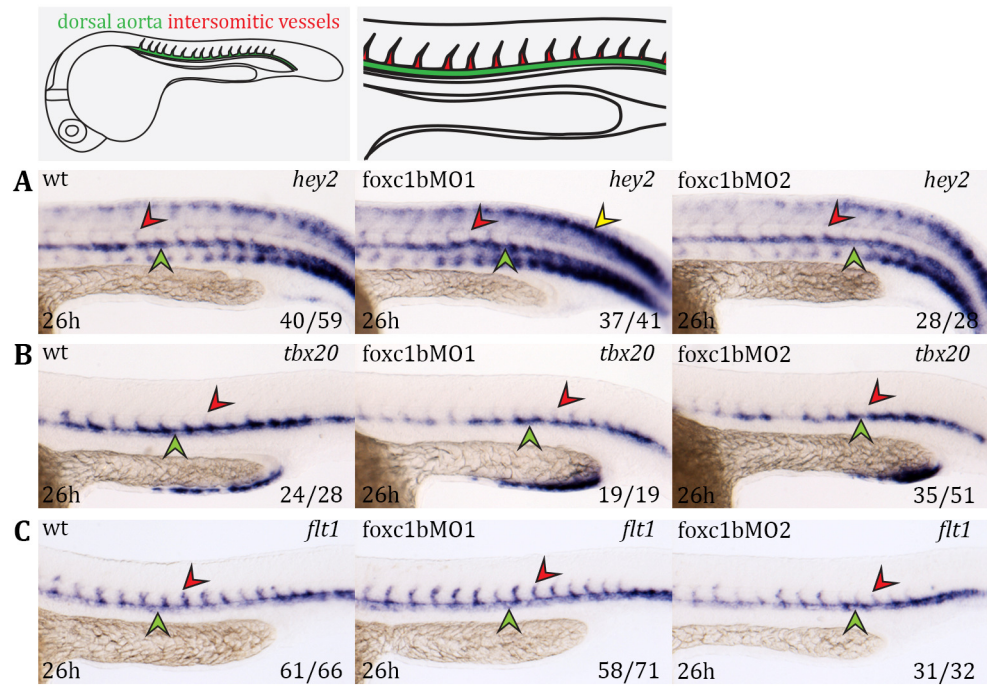


Figure 6-4. Arterial programming is normal at the time of HSC emergence in *foxc1b* morphants.

Expression of arterial markers *hey2* (*gridlock*) (A), *tbx20* (*hrt*) (B) and *flt1* (*vegfr1*) (C) is not affected in *foxc1b* morphants. Arrows indicate DA (green), ISVs (red), CNS (yellow). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

circulation is normal in morphants (data not shown), supporting the presence of a normally functioning vasculature.

Arterial programming is also not majorly affected in *foxc1b* morphants at 26 hpf, similar to *dll4* morphants. Arterial expression of *hey2* (*gridlock*) and *tbx20* (*hrt*) is not affected in either morphant (Figure 6-4-A&B). The width of the aorta and its patterning appear normal, as are spacing, progression and marker expression of the intersomitic vessels. However, neural expression of *hey2* is markedly up-regulated in *foxc1b*M01, but not M02 (yellow arrow, Figure 6-4-A) and expression levels of *flt1* (*vegfr1*) appear slightly down-regulated in the DA of *foxc1b*M02, but no effect is observed for *foxc1b*M01 (Figure 6-4-C). These two observations indicate a M0 specific effects on *hey2* and *flt1*

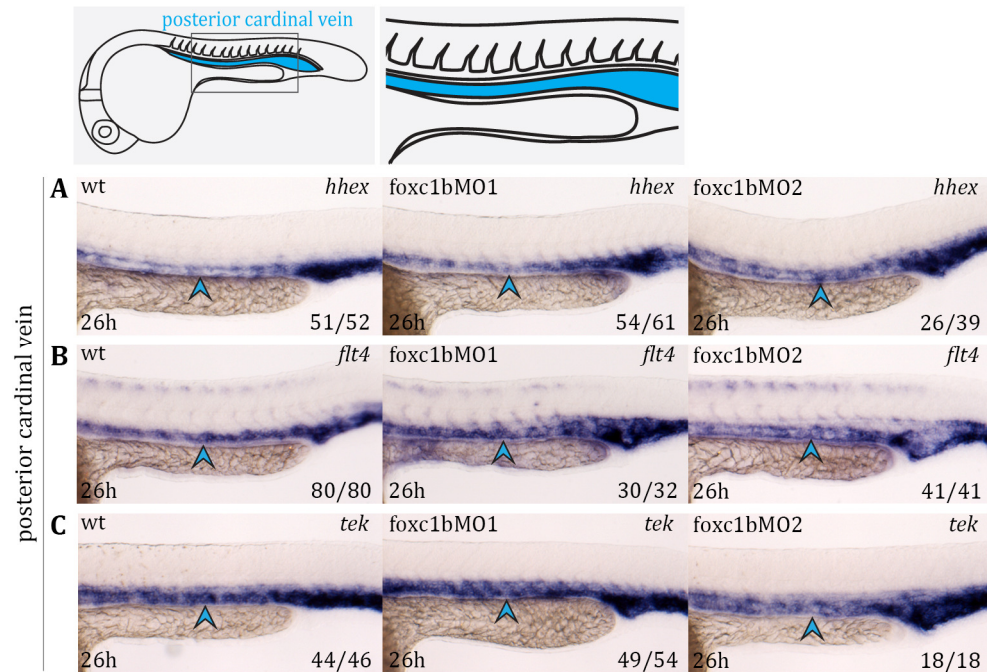


Figure 6-5. The venous programme is not majorly affected in the absence of *foxc1b*.

No major changes in markers assessed for a change in venous patterning are observed in either morphant: *hhex* (A), *flt4* (*vegfr3*) (B) and *tek* (C) are not changed. Arrows (blue) indicate PCV. Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

(*vegfr1*), respectively; likely morpholino-specific off-target effects, supporting the importance of employing two independent MOs for phenotypic analysis (reviewed in Ekker, 2000). Therefore, as neither of these effects is reproduced across both morpholinos, they are discounted here (Eisen and Smith, 2008, Bill et al., 2009).

Similar to the arterial and endothelial programme, the venous programme is not majorly affected in *foxc1b* morphants. *Hhex*, *flt4* (*vegfr3*) and *tek* (*tie2*) are preferentially expressed in venous cells of morphant and wild type embryos. Expression patterns and intensity of these genes appear unchanged (Figure 6-5-A-C), consistent with the unchanged expression of *dab2* observed previously (Figure 5-12-D, p.180). The lack of a severe effect on the venous programme is consistent with observations made in

Foxc1/Foxc2 compound homozygous mice, where despite arterio-venous fusions venous markers are expressed normally (Seo et al., 2006). *Foxc1/Foxc2* null mouse mutants have severe vascular defects (Kume et al., 2001, Seo et al., 2006), however the published zebrafish *foxc1b* phenotype indicates a fully functional vasculature (De Val et al., 2008). I here confirm and extend the previous vascular analyses of *foxc1b* in the zebrafish and show that *foxc1b* morphants have no major defects in vascular development, as previously suggested (De Val et al., 2008), similar to *dll4*.

6.4 *Foxc1b* is required for HSC specification

The MO screen suggested a requirement for *foxc1b* in HSC specification (Figure 5-12-A, p.180). Severe down-regulation of *runx1* expression at 26 hpf is also observed in *foxc1b*MO2 (Figure 6-6-A) and is consistent across multiple biological replicates, mirroring the *dll4* HSC phenotype (Figure 3-5-B, p. 91), further supporting a role for *foxc1b* in HSC specification.

The absence of *runx1* positive cells in the DA at 26 hpf likely reflects a reduction in HSCs as T-cell markers are nearly completely absent from the thymus at 4 dpf. The depletion of T-cell markers *rag1*, *rag2* and *cmyb* (Figure 6-6-B-D) indicates that T-cells are not being generated or do not mature properly in *foxc1b* morphants, while the smaller reduction in *ikzf1* (*ikaros*) expression suggests that T-cell progenitors are not affected as severely, implying that *foxc1b* has an additional role in T-cell maturation. This hypothesis is supported by the smaller reduction in *ikzf1* (*ikaros*) expression in the thymus from its initiation at 3dpf (data not shown).

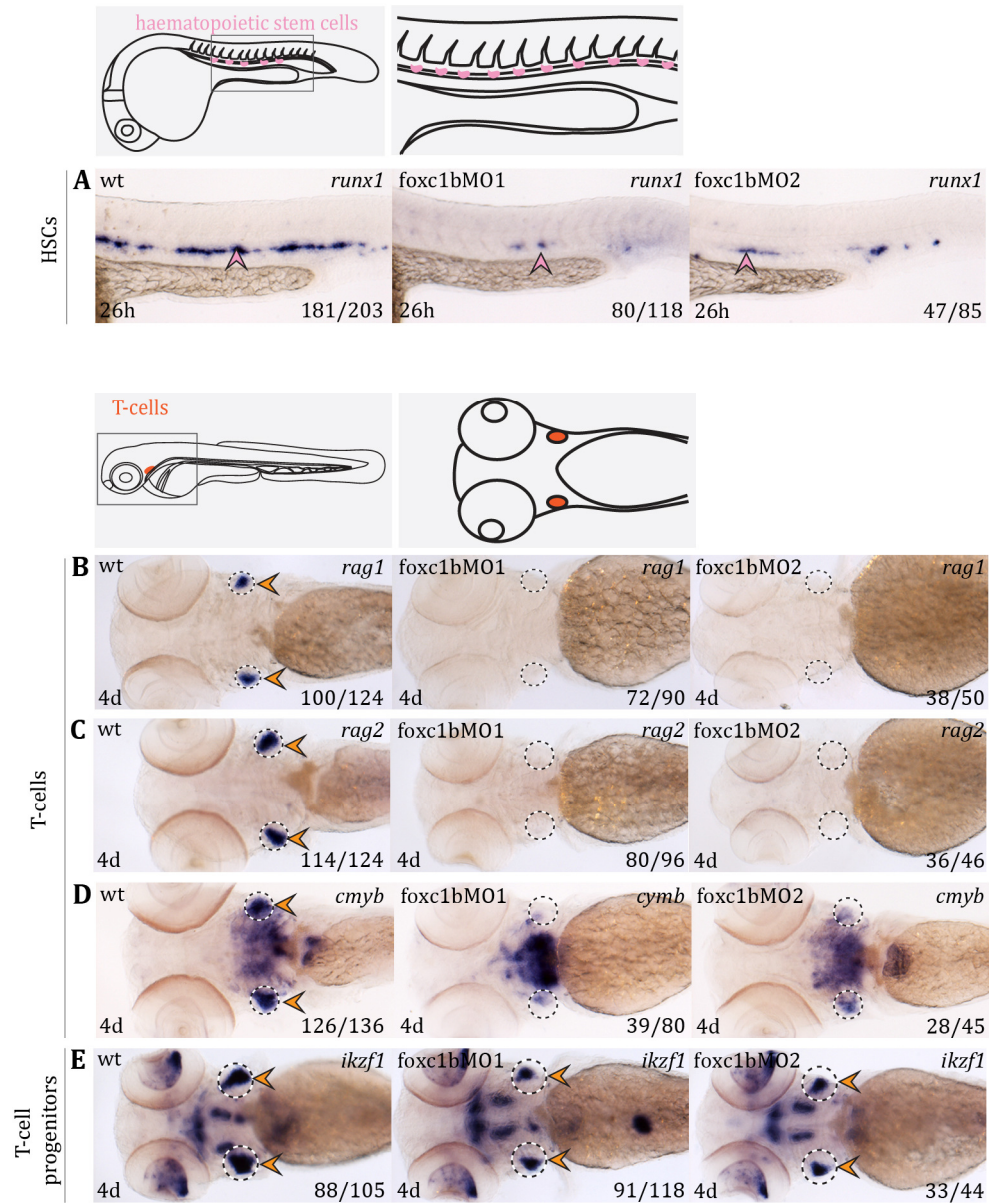


Figure 6-6. *Foxc1b* is required for HSC emergence and HSC-derived T-cell maturation.

At 26 hpf, the number of *runx1* positive cells in the DA is severely reduced in embryos injected with *foxc1bM01* or *M02*, as compared to uninjected wt siblings (A). At 4 dpf, expression of *rag1* (A), *rag2* (B) and *cmyb* (C) is absent in the thymus of embryos injected with *foxc1bM01* or *M02* (circles). Expression of *ikzf1* (*ikaros*) however is reduced in the morphants (circles, black arrows)(D) revealing an additional block on T-cell maturation. Arrows indicate: HSCs (pink) and T-cells (orange). Ventral view, anterior to the left. Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

At 4 dpf, *cmyb* and *ikzf1* (*ikaros*) are also expressed in the caudal haematopoietic tissue (CHT), where they mark multipotent progenitor cells (HS(P)Cs) and definitive myeloid cells (Bertrand et al., 2007, Paffett-Lugassy et al., 2007, Zhang and Rodaway, 2007, Bertrand et al., 2008). In *foxc1b* morphants, *cmyb* positive cells are reduced in the CHT at 4 dpf (Figure 6-7-A), as are *ikzf1* positive cells (Figure 6-7-B).

Consistent with the hypothesis that *foxc1b* is required for HSC specification, *foxc1b* morphants have less leukocytes: *lyz* is expressed in neutrophils (Liongue et al., 2009, Hall et al., 2012), and reduced in *foxc1b* morphants (orange arrows in Figure 6-7-D). *Lcp1* is expressed in macrophages, neutrophils and granulocytes (Le Guyader et al., 2008, Bolli et al., 2011, Feng et al., 2012), and its expression is likely reduced across all lineages, evident in the reduction of *lyz* staining in the CHT, circulating cells and cells that have migrated to the kidney (blue and green arrows in Figure 6-7-C, respectively).

Finally, the erythroid marker *gata1a* is reduced in the CHT and in the circulation of *foxc1b* morphants at 4 dpf (orange and red arrows in Figure 6-7-E, respectively), indicating that the definitive erythroid lineage is also compromised in *foxc1b* morphants, consistent with the functional requirement of *foxc1b* in HSC specification.

The reduction of markers for HS(P)Cs and HSC-derived lineages suggests a reduction of fully functional HSCs in the *foxc1b* morphants, in line with the observed reduction of *runx1* positive cells at the time of HSC specification. In conclusion, the *foxc1b* phenotype largely mirrors that of *dll4* at 26 hpf and 4 dpf, with minimal effects on vascular development, a severe reduction in *runx1* positive cells in the dorsal aorta at the time of HSC specification (26 hpf) and a reduction in HS(P)Cs and HSC-derived lineages at 4 dpf. These observations suggest that the requirement for *dll4* in HSC emergence is mediated through *foxc1b* at 26 hpf.

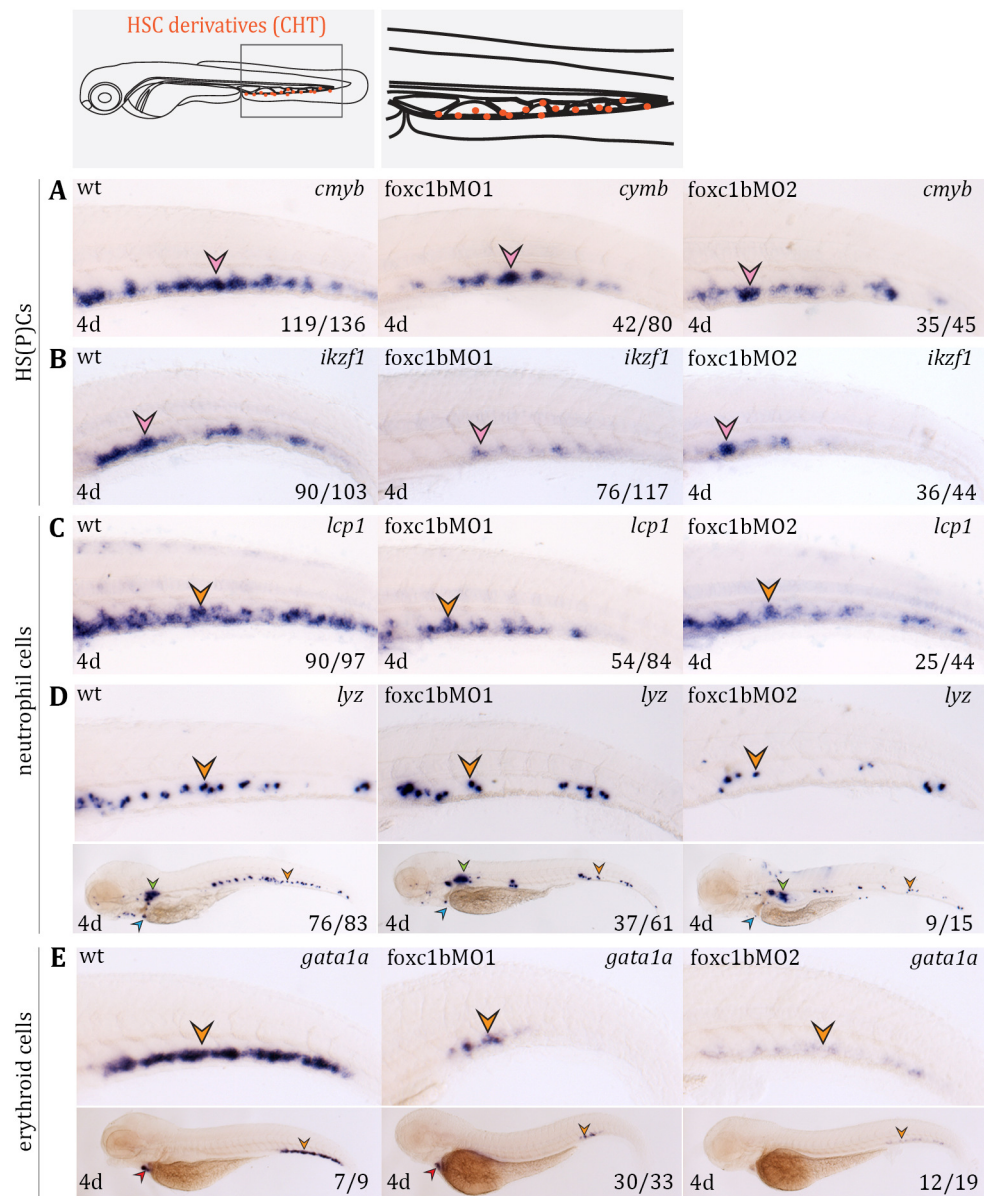


Figure 6-7. Cells of the definitive blood lineage are reduced in the CHT of *foxc1b* morphants.

Markers for HS(P)Cs and myeloid cells *cmyb* (A) and *ikzf1* (*ikaros*) (B) are reduced in the CHT of *foxc1b* morphants. All markers used to assess the neutrophil lineage are also reduced at 4 dpf: *lcp1* (*l plastin*) (C) and *lyz* (*lysozyme c*) (D). Note also the reduction of *lyz* positive cells in circulation/vasculature (blue arrows) and in the head kidney (green arrows) (D). Staining for erythroid marker *gata1a* is also reduced in the CHT of *foxc1b* morphants at 4 dpf (E). Arrows indicate HS(P)Cs (pink), HSC derivatives (orange), circulating cells (blue/red), cells in head kidney (green). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

6.5 *Foxc1b* also has a *dll4*-independent role in HSC development

Interestingly, the MO screen showed that HSC marker *cmyb* (Figure 5-12-B, p.180) is up-regulated in the DA of 36 hpf *foxc1b* morphants, a phenotype that is not observed in the *dll4* morphants. This indicates an additional, *dll4* independent, later role for *foxc1b* in HSC development, in addition to its role in T-cell maturation. In order to investigate this surprising phenotype further, additional HSC markers were assessed at this timepoint (36 hpf).

In addition to the up-regulation of *cmyb* (Figure 5-12, p. 182 and Figure 6-8-A), *ikzf1* is also over-expressed in the DA of *foxc1b* morphants at 36 hpf (Figure 6-8-B), indicating that despite the severe reduction of *runx1* in the DA earlier, HSC markers are increased at the onset of EHT in these embryos. Even *runx1* is up-regulated at this timepoint (Figure 6-8-C), despite its earlier decrease. These data suggest an accumulation of HSC-like cells in the DA at 36 hpf. This phenotypic divergence from the *dll4* phenotype, indicates that *foxc1b* is not regulated by *dll4* at this stage.

Two explanations are possible for this increase of HSC-like cells: excessive proliferation of stem cells or reduced egression/migration. In order to address if the phenotype was caused by the latter, and to investigate the dynamics of the phenotype, a detailed timecourse of *runx1* expression was conducted (Figure 6-9).

Runx1 positive cells are reduced in the DA of *foxc1b* morphants from 24 hpf (Figure 6-9-A), just after the initial onset of *runx1* expression in this tissue (Gering and Patient, 2005, Wilkinson et al., 2009). Down-regulation of *runx1* is also observed at 26 hpf and at 28

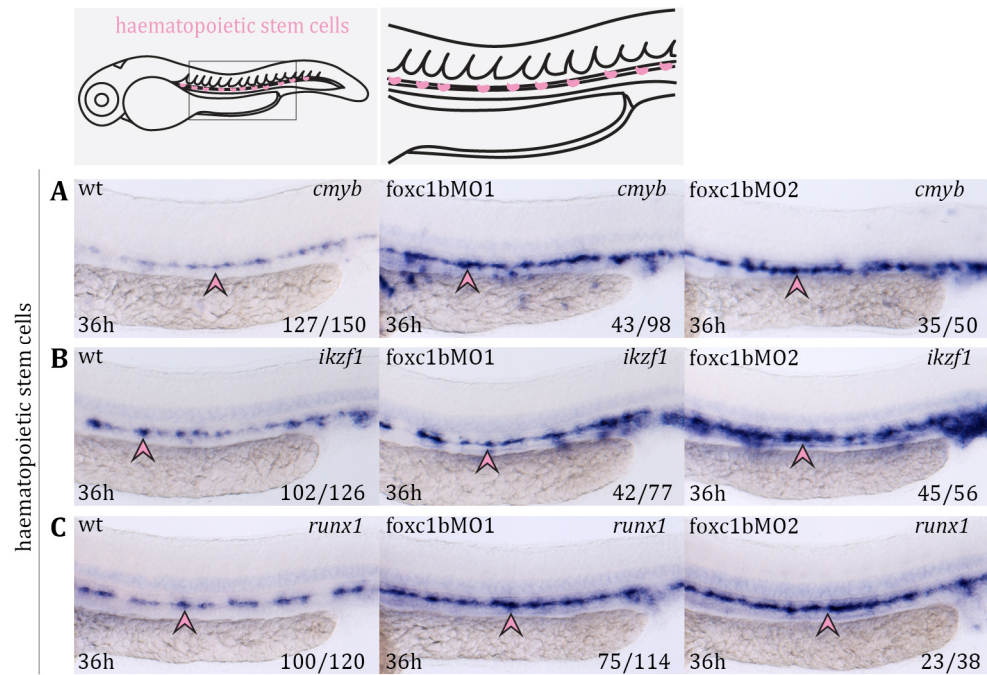


Figure 6-8. *Foxc1b* morphants show increased expression of HSC markers at 36 hpf.

At 36 hpf, HSC markers *cmyb* (A), *ikzf1* (*ikaros*) (B) and *runx1* (C) are markedly increased in the DA of *foxc1b* morphants. Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

hpf, although the phenotype is beginning to get milder (Figure 6-9-B&C). At 30 hpf, 32 hpf and 34 hpf, no marked difference in *runx1* expression to wt siblings can be observed (Figure 6-9-D-F). The up-regulation of *runx1* expressing cells is first convincingly seen at 36 hpf and is maintained until 72 hpf (Figure 6-9-G-J). At 48 hpf, in wt embryos the first *runx1* positive cells are observed in the caudal haematopoietic tissue (CHT), the equivalent tissue to the foetal liver in the mouse (dark pink arrows in Figure 6-9-H), while most *runx1* staining remains in the DA (light pink arrows). At this stage, fewer *runx1* positive cells are observed in the CHT of *foxc1b* morphant embryos as compared to wt siblings (Figure 6-9-H), similar to the phenotype observed at 56 hpf (Figure 6-9-I). By 72 hpf, *runx1* staining is not observed any more in the DA of wt embryos when most

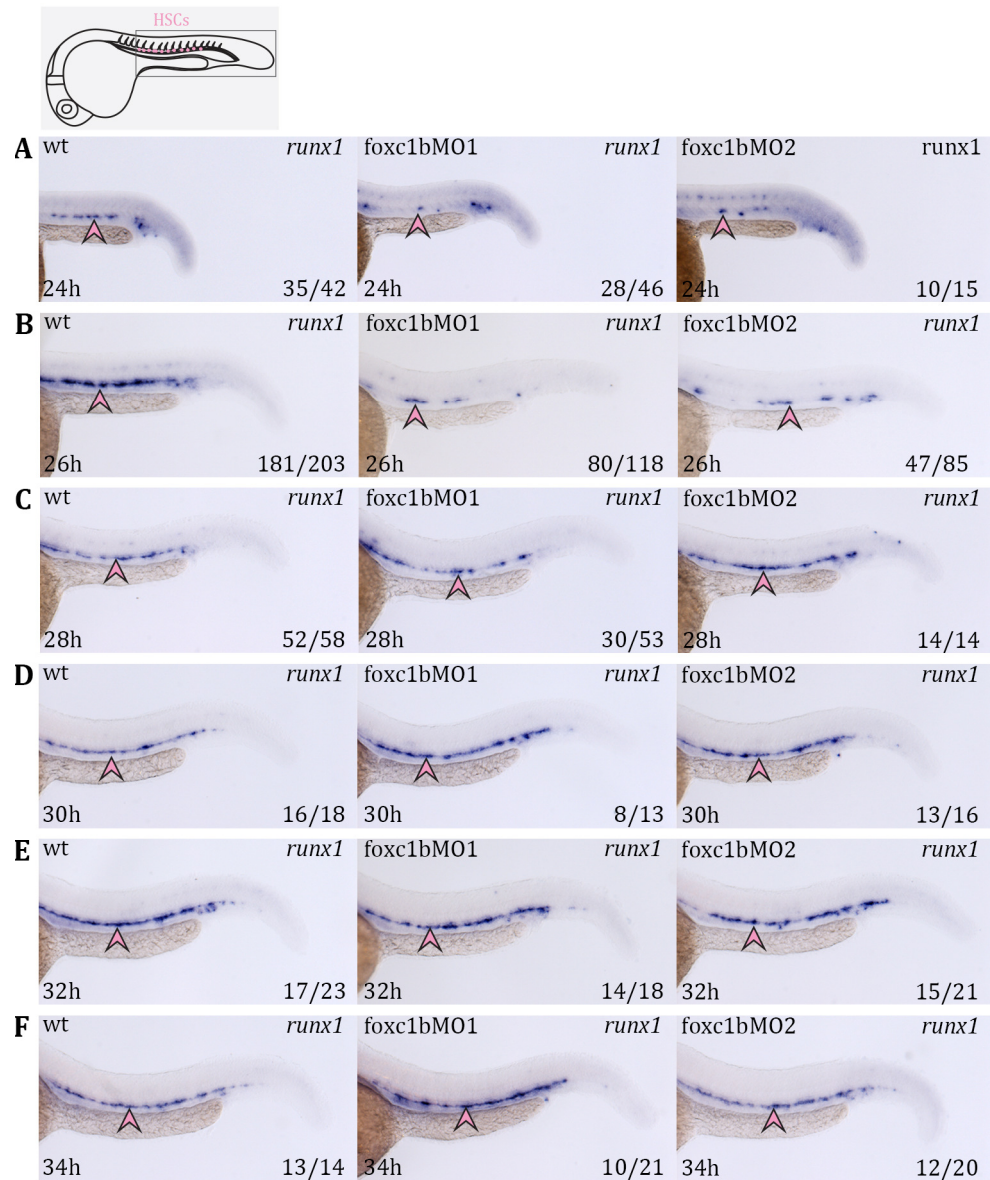
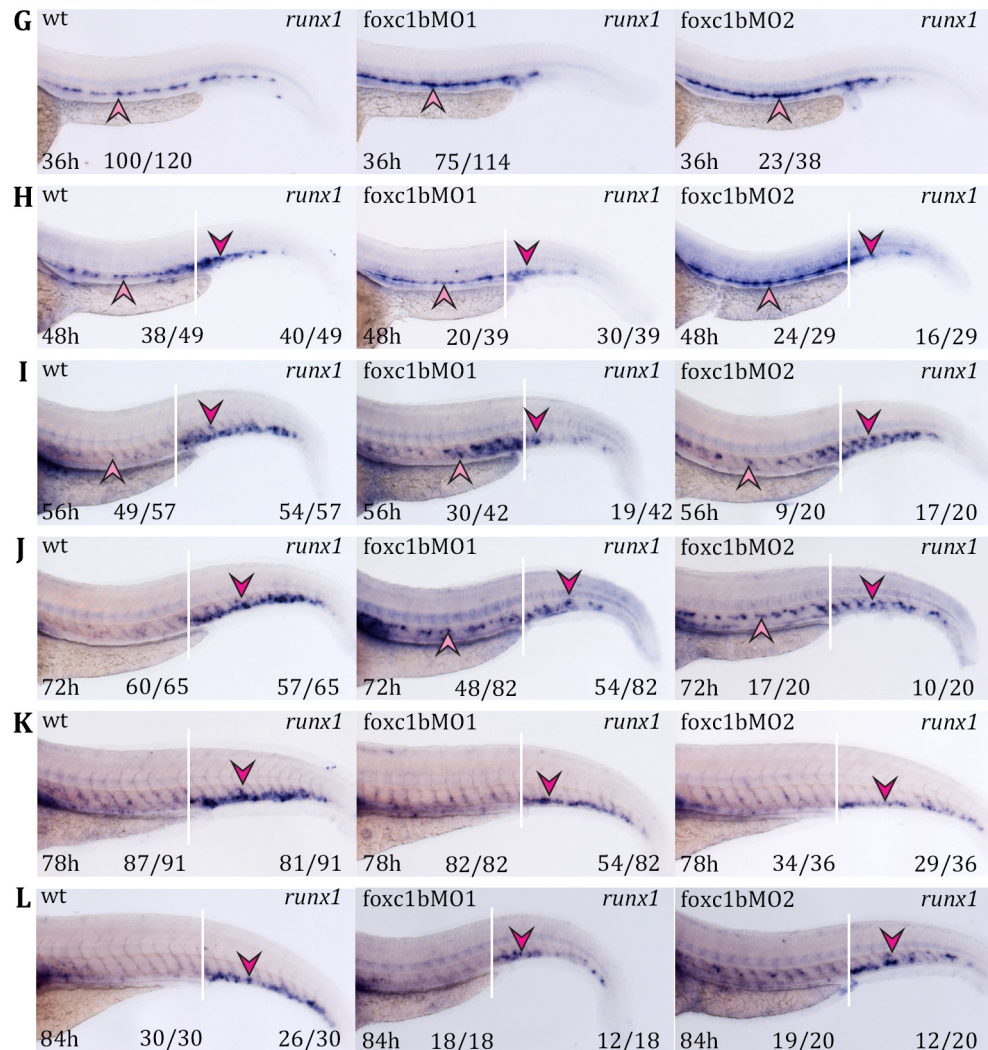
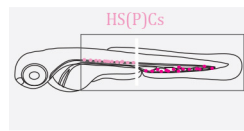


Figure 6-9. Timecourse of *runx1* expression in the DA and CHT in *foxc1b* morphants.

A-C. Early, there are fewer cells expressing *runx1* in the dorsal aorta of embryos injected with *foxc1bM01* or *M02*: at 24 hpf, numbers of *runx1* positive cells are markedly reduced in both *foxc1b* morphants (**A**). At 26 hpf, this phenotype is still clearly visible (**B**) but gets milder by 28 hpf (**C**). **D-F.** A phase of comparable numbers of *runx1* positive cells in the DA follows: at 30 hpf, no clear difference in the numbers of *runx1* expressing cells can be observed in *foxc1b* morphants as compared to wt siblings (**D**). This phenotype persists at 32 hpf (**E**) and 34 hpf (**F**). **G-H.** Later, *runx1* positive cells remain in the DA and less *runx1* positive cells are observed in the CHT. At 36 hpf, a clear up-regulation of *runx1* positive cells can be seen in the DA of embryos injected with *foxc1bM01* or *M02* (**G**). At 48 hpf, *runx1* starts to be expressed in the CHT of wt embryos;



this expression is reduced in *foxc1b* morphants (**H**). At 56 hpf, *runx1* expression reduces in the DA of wt embryos and CHT expression increases, while in *foxc1b* morphants *runx1* expression remains high in the DA at the expense of expression in the CHT (**I**). From 72 hpf, no *runx1* expression can be observed in the DA of wt embryos while it is maintained in *foxc1b* morphants; *runx1* positive cells in the CHT are reduced as compared to wt siblings (**J**). At 78 hpf, *runx1* expression in the DA is absent in wt and morphant embryos, and expression in the CHT is still reduced in the morphants (**K**). This phenotype is maintained at 84 hpf (**L**). Arrows indicate *runx1* staining in the DA (light pink) and in the CHT (dark pink), white bars separate DA region (left) from CHT region (right). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1 (p. 235).

HSCs have undergone EHT by this time (Kissa and Herbolme, 2010); in the *foxc1b* morphants however, *runx1* staining persists in the DA at high levels, while fewer *runx1* cells are observed in the CHT than in wt control embryos (Figure 6-9-J). Levels of *runx1* staining in the DA of *foxc1b* morphants plummet by 78 hpf (Figure 6-9-K) and remain low at 84 hpf (Figure 6-9-L), indicating that very few HSCs remain in the DA. This reduction of *runx1* positive cells in the DA is not accompanied by higher numbers of *runx1* in the CHT, indicating that these cells do not migrate there. No ectopic expression of *runx1* is detected in these embryos, suggesting either apoptosis or transcriptional repression of *runx1*.

To visualise the dynamics of this timecourse, data were plotted over time and by tissue: Figure 6-10 shows the largest percentage of embryos at any given timepoint that deviate in *runx1* expression from the wt average. The wt average is represented by a y-value of 0 (ie. the location of the x axis), and up-regulation is represented as positive y-values above the x axis, while down-regulation is represented as negative y-values below the x axis. Consequently, data points represented for the wt group lie close to the average, except at the initial timepoints at 22 and 24 hpf when *runx1* expression first becomes visible in the DA (Gering and Patient, 2005, Wilkinson et al., 2009) and variation is observed in initiation of *runx1* expression (Figure 6-10-A). By 26 hpf, the percentage of wt embryos that show lower-than-average expression of *runx1* is at 11% and the vast majority of embryos maintain average levels throughout the rest of the timecourse.

Three different phases of the *runx1* phenotype in *foxc1b* morphants become apparent: hardly any overexpression can be seen between 22 hpf and 28 hpf while around 70% of embryos show a down-regulation of *runx1* in the DA of both *foxc1b* morphants (light pink plot, Figure 6-10-B&C). Between 28 and 30 hpf, these dynamics change and around

25% of morphant embryos show up-regulated *runx1* expression in the DA. From 30 hpf onwards an increasing percentage of morphant embryos show increased *runx1* expression in the DA between an initial 20% at 32 hpf to above 65% at 36 hpf. Between 36 and 72 hpf, this percentage varies between approximately 50% and 70%. By 72 hpf, *runx1* is no longer expressed in the DA of wt embryos but is still maintained in *foxc1b* morphants (Figure 6-9-J). From 78 hpf, no *runx1* expression can be observed in the DA of wts or *foxc1b* morphants.

Runx1 expression in the caudal haematopoietic tissue (dark pink plot, Figure 6-10-B&C) of *foxc1b* morphants behaves differently to that in the DA (light pink plot). From 48 hpf, *runx1* expression can be detected in the CHT of wt embryos, and is also present in the CHT of *foxc1b* morphants, albeit at reduced levels, in a majority of embryos. The percentage of embryos with a down-regulation of *runx1* expression in the CHT is comparable between morphants and timepoints, and varies between 50% and 85%. No increased *runx1* expression is observed in the CHT of either *foxc1b* morphants above the level of variation observed in the wt samples. *Runx1* is reduced in a majority of *foxc1b* morphant embryos, even after *runx1* expression vanishes in the DA from 78 hpf, indicating that those cells in the DA do not successfully migrate to the CHT. Dynamics of *runx1* expression in the DA and CHT are highly similar for both *foxc1b* morpholinos.

This data indicates three distinct phases of the *foxc1b* phenotype: Phase I is characterised by the initial reduction of *runx1* expression in the DA between 22hpf and 28 hpf, highly similar to the *dll4* phenotype described in Chapter 3, and data presented here support the hypothesis that this early phenotype is regulated by *dll4*. Phase II is

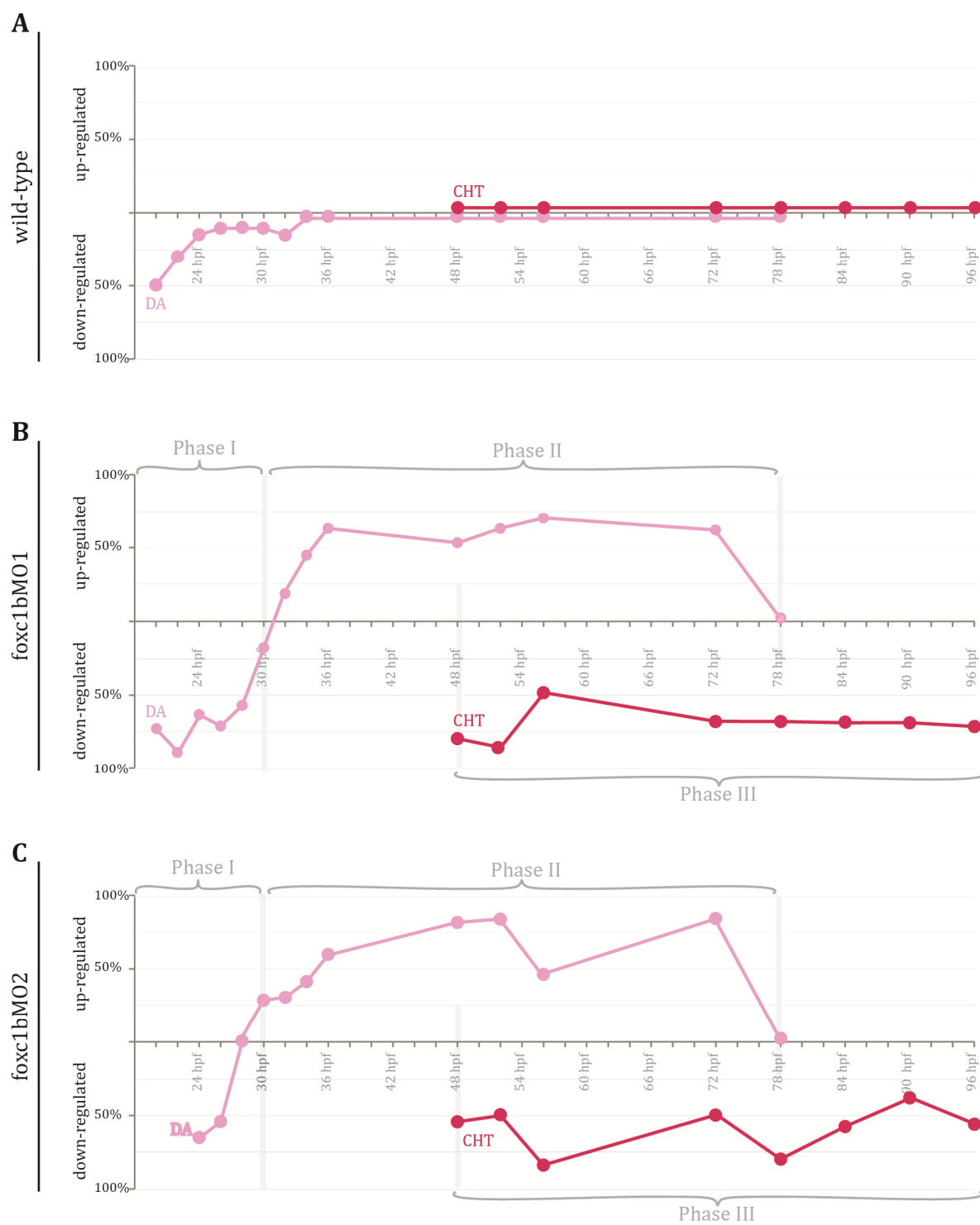


Figure 6-10. Percentage of *foxc1b*M0 embryos with changed *runx1* expression in the DA and CHT over time.

Schematic representation of results obtained for *runx1* expression timecourse in wt (A), and embryos injected with *foxc1b*M01 (B) or *foxc1b*M02 (C). y-axis represents percentage up- (blue, positive) and down-regulation (green, negative) within batches of embryos subjected to WISH. Y-values of 0 represent wt average. Intervals along the x-axis represent the developmental timecourse at 2 hour intervals, starting at 20 hpf. Solid lines represent *runx1* expression in the DA, dotted lines represent *runx1* expression in the CHT. Phase I, II and III are distinguished by the phenotype observed.

between 30 hpf and 78 hpf, when the effect of the absence of *foxc1b* becomes an up-regulation of *runx1* and other HSC markers in the DA. This phase is distinct from the *dll4* phenotype, which is characterised by a consistent down-regulation of HSC markers, and thus the Phase II phenotype is likely to be *dll4*-independent.

Phase III is observed in the CHT, from 48 hpf, overlapping Phase II in the DA. This phase is marked by the down-regulation of *runx1* expression in the CHT. The distinct phenotype for this phase is observed from 48 hpf, as soon as the first *runx1* positive cells reach the CHT. Therefore, it makes sense to classify these phases not by developmental timing, but by tissue.

This suggests that *foxc1b* is not regulated by *dll4* after 28 hpf, as the *foxc1b* HSC phenotype is opposite to that of *dll4* from this point, a hypothesis that awaits further testing. Previous studies have shown that *Foxc1* directly regulates *Dll4* in murine endothelial cells (Seo et al., 2006, Hayashi and Kume, 2008), and observations here indicate a stage-dependent repression of *dll4* in the DA by *foxc1b* in the DA at 28 hpf (Appendix Figure 8-10, p. 262).

6.6 Do *cmyb*^{hkz3} mutants show a similar phenotype to *foxc1b* morphants?

Zhang et al. (2011) have described a *cmyb* mutant zebrafish line that shows an accumulation of HSCs in the DA in the absence of HS(P)Cs in the CHT, similar to the phenotype observed for *foxc1b* morphants. Interestingly, while *cmyb* is increased in the *foxc1b* morphants (Figure 6-8, p. 207), these *cmyb*^{hkz3} mutants result in a loss of *Cmyb* protein function, indicating that levels of *cmyb* are crucial for restraining HSC numbers. *Cmyb*^{hkz3} mutants harbor a T to A splice site mutation (Appendix Figure 8-11, p. 263), resulting in a 13 nucleotide insertion in the *cmyb* transcript caused by the utilization of a cryptic splice acceptor (Zhang et al., 2011). Zhang and colleagues have shown that this mutation results in a *Cmyb* protein lacking the negative regulatory domain, with a functional DNA binding domain and a truncated transactivation domain, resulting in a loss of function phenotype.

Cmyb^{hkz3} mutants show a severe reduction in HSC-derived cells of the lymphoid, erythroid and myeloid lineages in the CHT and thymus at 4 and 5 dpf (Zhang et al., 2011 and Appendix Figure 8-11, p. 263). Vasculature is normal in these embryos, while an accumulation of HSCs is observed in the DA at 72 hpf (Zhang et al., 2011), reminiscent of the phenotype observed in the absence of *foxc1b*. Zhang et al. interpreted the absence of an effect on *runx1* staining in the DA at 30 hpf as an indication that the *cmyb* phenotype is independent or downstream of *runx1*, however, the comparison to the *foxc1b* data suggests this might be due to timing. The intriguing similarity between the published *cmyb*^{hkz3} and *foxc1b* phenotypes called for an extended analysis of the previously described *cmyb*^{hkz3} phenotype.

In order to compare the *foxc1b* and *cmyb*^{hkz3} phenotypes, a timecourse on *runx1* expression was conducted on a subset of the timepoints assessed in Figure 6-9. Four representative timepoints were chosen: 26 hpf, when a down-regulation of *runx1* is expected if *cmyb*^{hkz3} does mirror the *foxc1b* phenotype, a timepoint that was not assessed by Zhang et al. (2011). Indeed, *runx1* is severely down-regulated in 25% of embryos from a heterozygous incross, consistent with the expected percentage for homozygous embryos in the Mendelian ratio (Figure 6-11-A). Next, *runx1* expression was assessed at 30 hpf, and confirmed the previous observation that *runx1* is not differentially expressed in *cmyb*^{hkz3} at this timepoint (Zhang et al., 2011 and Figure 6-11-B). *Runx1* and HSC marker expression have not previously been assessed at 36 hpf in these mutants, at the time when an up-regulation of all HSC markers is first observed in *foxc1b* morphants (Figure 6-8, p.205). Similar to the *foxc1b* phenotype, a slight up-regulation in *runx1* staining is observed in the DA of *cmyb*^{hkz3} animals. Finally, in order to assess whether an up-regulation can be observed in the DA at the same time as a down-regulation of *runx1* positive cells in the CHT of *cmyb*^{hkz3} mutants, mutants were assessed at 52 hpf. Again, consistent with the *foxc1b* phenotype, 25% of *cmyb*^{hkz3} embryos exhibit an up-regulation of *runx1* staining in the DA, while a down-regulation of *runx1* positive cells is observed in the CHT, consistent with the previously described phenotype at 72 hpf (Zhang et al., 2011).

In conclusion, the extended analysis of the *cmyb*^{hkz3} phenotype indicates that although there is a clear accumulation of HSCs in the DA of these embryos, it appears earlier than previously described, and is preceded by a reduction of *runx1* positive cells in the DA, indicating an earlier role for *cmyb*, upstream of *runx1* which has not been described

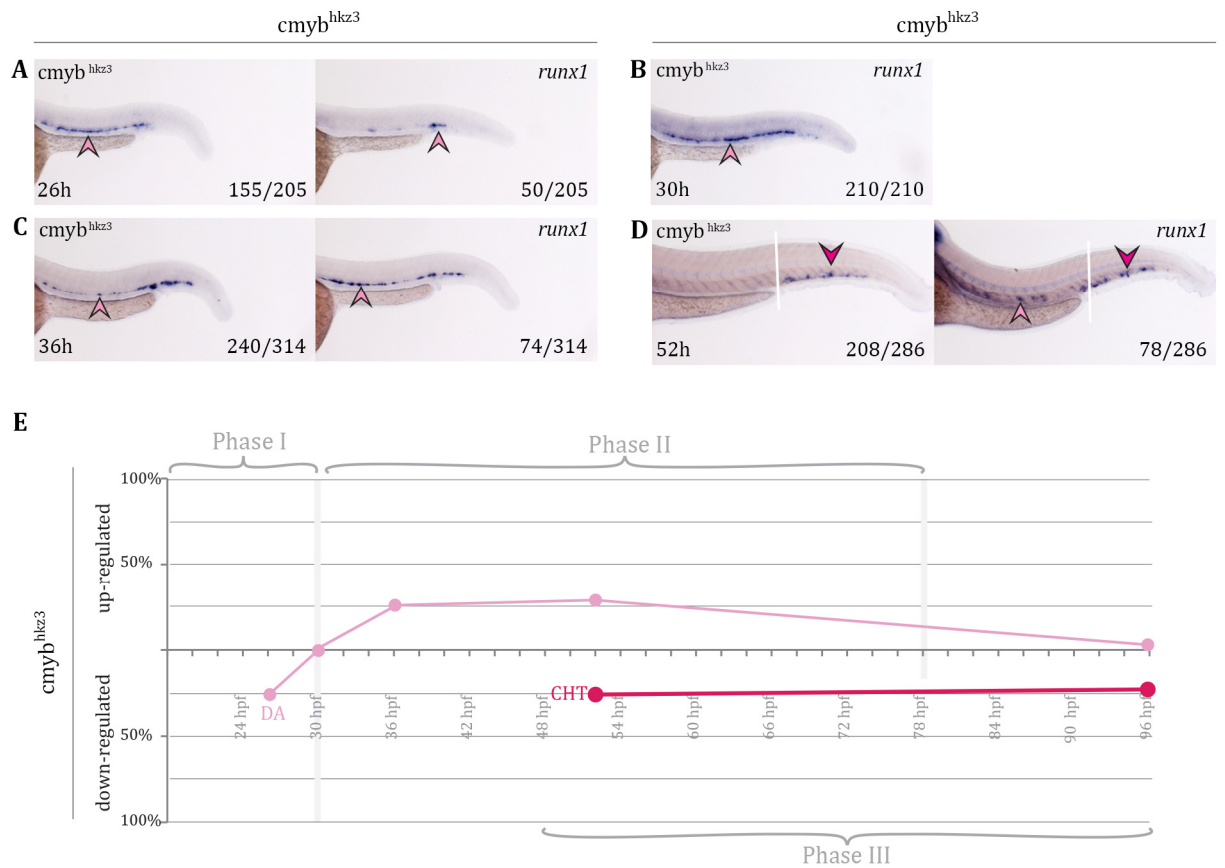


Figure 6-11. *Runx1* expression timecourse in *cmyb^{hkz3}* mutants.

At 26 hpf, *runx1* positive cells are clearly reduced in the DA in 25% of *cmyb^{hkz3}* mutants (A). At 30 hpf, 100% of *cmyb^{hkz3}* embryos show similar levels of *runx1* expression in the DA (B). At 36 hpf, 24% of *cmyb^{hkz3}* mutant embryos show a slight up-regulation of *runx1* expressing cells in the DA (C). At 52 hpf, 27% of *cmyb^{hkz3}* show residual *runx1* expression in the DA, while the other 73% have no *runx1* positive cells in the DA at this timepoint (D). Arrows indicate *runx1* staining in the DA (pink), *runx1* staining in the CHT (orange). E Schematic representation of results obtained for the *runx1* expression timecourse in *cmyb^{hkz3}* mutants: y-axis represents percentage up- (blue, positive) and down-regulation (green, negative) within batches of embryos subjected to WISH. Y-values of 0 represent wt average. Intervals along the x-axis represent the developmental timecourse at 2-hour intervals, starting at 20 hpf. Solid lines represent *runx1* expression in the DA, dotted lines represent *runx1* expression in the CHT. Phase I, II and III are distinguished by the phenotype observed.

before. This appears to be in addition to its later role downstream of *runx1*. This comparative analysis with the *foxc1b* morphants indicates that their phenotypes are highly similar, and thus that *foxc1b* and *cmyb* might act within the same *dll4*-independent mechanism.

6.7 Discussion

6.7.1 Overview

A role for *Foxc1/Foxc2* in vascular development has been described in mice, and *Foxc1* has recently been shown to be required for HSC niche formation in the murine bone marrow, while no phenotype has been ascribed to *foxc1b* alone in the fish (Seo et al., 2006, De Val et al., 2008, Skarie and Link, 2009, Veldman and Lin, 2012, Omatsu et al., 2014). I have established here, for the first time, a *foxc1a*-independent role for *foxc1b* in zebrafish development. *Foxc1b* but not *foxc1a* is regulated by *dll4* at 26 hpf in the posterior tissues of the zebrafish. *Foxc1b* is required for initial *runx1* expression in the DA: in its absence, severe reduction in HS(P)Cs and the HSC-derived definitive blood lineages is observed. I have also confirmed that this is not due to vascular mis-specification, as previously described (De Val et al., 2008, Skarie and Link, 2009). This phenotype mirrors that of *dll4*, and I propose here that *foxc1b* acts downstream of *dll4* in HSC specification through direct or indirect regulation of *runx1*.

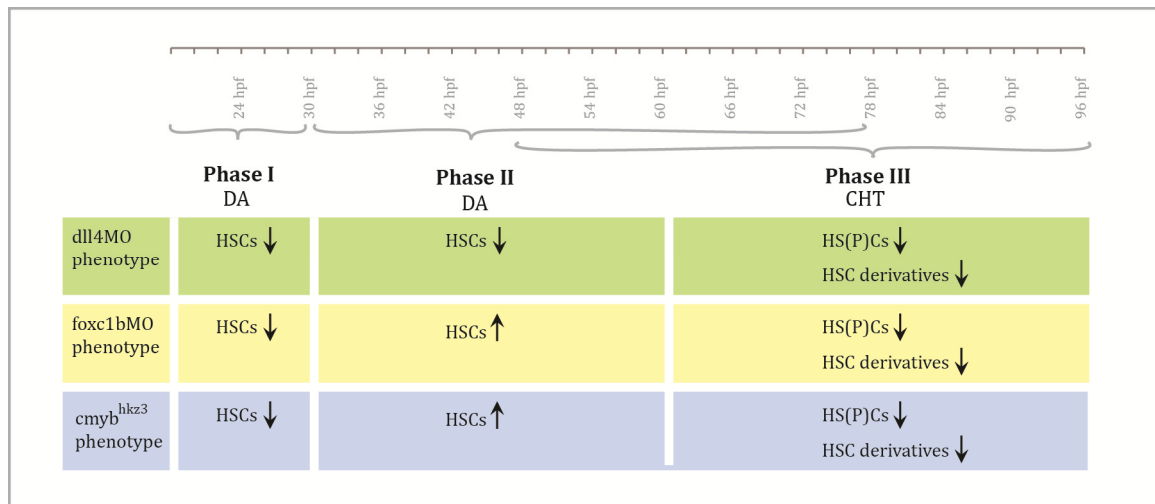


Figure 6-12. Overview of the *dll4*, *foxc1b* and *cmyb*^{hkz3} phenotypes.

Three distinct phases characterize the *dll4*, *foxc1b* and *cmyb*^{hkz3} phenotypes: Phase I shows a down-regulation of HSCs in the DA in all three cases. This is followed by Phase II, which is a maintained reduction of HSCs in the DA of *dll4*MO embryos, but an accumulation of cells expressing HSC markers in the DA of *foxc1b*MO and *cmyb*^{hkz3} embryos. Phase III is localized to the CHT where HS(P)Cs and HSC derivatives are reduced in numbers in *dll4*MO, *foxc1b*MO and *cmyb*^{hkz3} embryos.

Later, when *dll4* is no longer expressed in the DA, the absence of *foxc1b* causes an up-regulation of HSC markers, that persist longer in the DA than in wt, in a similar manner to the *cmyb* phenotype. I have identified three distinct phases of *foxc1b* action in HSC development in the zebrafish (summarised in Figure 6-12), and here their respective relevance is discussed.

6.7.2 The role of *foxc1b* downstream of *dll4* in HSC specification

Foxc1 and *Foxc2* have been suggested to be important downstream effectors of Notch signalling, but expression of the arterial Notch pathway markers (*Notch1*, *Notch4*, *Dll4*, *Jag1* and *Hey2*) are absent in *Foxc1/Foxc2* compound null mutants (Kume et al., 2001, Seo et al., 2006, Hayashi and Kume, 2008), indicating action upstream of Notch. This suggests a highly complex regulatory relationship between *Foxc* and the Notch pathway.

I have shown above that *foxc1b* is regulated by *dll4* at 26 hpf, by means of RNA-seq, qPCR and WISH. The initial reduction of *runx1* in the DA of *foxc1b* morphants at 26 hpf is comparable to that observed in *dll4* morphants, and I propose that *foxc1b* mediates the effects of *dll4*. This remains to be proven, for example by a rescue experiment in which *foxc1b* overexpression would be expected to rescue the *runx1* down-regulation in *dll4* morphants at 26 hpf.

It further remains to be established whether this regulation of *runx1* by *foxc1b* is direct or indirect. Interestingly, a crude bioinformatic analysis performed here reveals the presence of a FOX:ETS consensus site just 36 bp outside the central 235 bp of the + 23 *Runx1* enhancer in the mouse (based on data provided in Bee et al., 2009 and De Val et al., 2008). This *Runx1* enhancer is located in exon 1 of the mouse *Runx1* locus and drives haematopoietic activity of *Runx1*, independent of promoter usage, targeting reporter gene expression to the emerging HSCs in the mouse embryo (Nottingham et al., 2007, Bee et al., 2009). This crude preliminary analysis suggests that FOXC can bind in combination with ETS in this crucial regulatory part of *Runx1* in the mouse, although experimental confirmation is necessary to confirm this. If

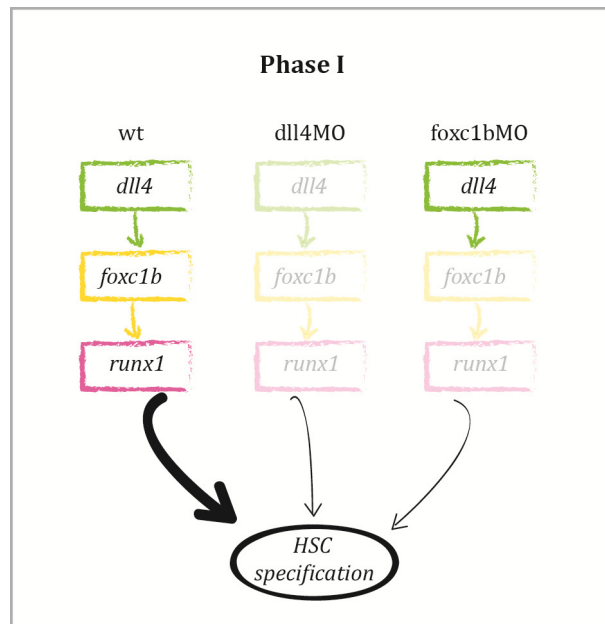


Figure 6-13. Speculative genetic regulatory model for Phase I.

During Phase I, *dll4* induces expression of *foxc1b* in wt embryos, which in turn induces *runx1* expression which is required for HSC specification. In *dll4* morphants, levels of *foxc1b* and *runx1* expression are reduced, causing a reduction in HSC specification. In *foxc1b* morphants, *dll4* expression is unaffected while expression of *runx1* is reduced, thus also resulting in reduced HSC specification.

true and conserved in zebrafish, this would suggest that the regulation of *runx1* by *foxc1b* is direct.

It also remains to be determined whether regulation of *foxc1b* by *dll4* is direct or indirect. It has been shown in HUVECs that *FOXC1* directly binds to the *DLL4* promoter to regulate *DLL4* expression (Seo et al., 2006, Hayashi and Kume, 2008). I have shown here that while *dll4* regulates *foxc1b* at 26 hpf in the zebrafish, no reciprocal regulation of *dll4* by *foxc1b* is observed at this time. Interestingly though, at 28 hpf *foxc1b* appears to repress *dll4* expression in the dorsal aorta, but not the ISVs and neural tissues (Appendix Figure 8-10, p. 262). Furthermore, this switch in regulation is concomitant with the switch observed in HSC phenotype, at the beginning of the transition between

Phase I and Phase II of *runx1* regulation (Figure 6-10, p. 213). This indicates that the regulatory interactions between *dll4* and *foxc1b* are highly dynamic in time and tissues of HSC emergence, reflecting a change in the arterial and HSC programmes around this time.

The notion of a cell non-autonomous role for *foxc1b* in HSC emergence follows from observations made in the mouse: Omatsu and colleagues employed a cross of Tie-Cre mice and *Foxc1^{f/f}* mice to ablate *Foxc1* expression in endothelial and haematopoietic cells, throughout development (Omatsu et al., 2014). Offspring showed no signs of impaired HSCPC maintenance and haematopoiesis, indicating that murine *Foxc1* is not required in endothelial and haematopoietic cells for normal haematopoiesis (Omatsu et al., 2014). Data presented in this chapter clearly indicate a requirement for *foxc1b* in HSC development in the zebrafish. One explanation is that the mouse and the zebrafish differ in their requirement for *Foxc1* in haematopoiesis, another is that the requirement in both cases is cell non-autonomous. The expression of *foxc1b* at the time of HSC emergence in the zebrafish trunk suggests a cell-autonomous role through its arterial expression or a cell non-autonomous role through its sclerotomal expression (Figure 6-1, p. 195 and De Val et al., 2008, Skarie and Link, 2009).

Omatsu and colleagues go on to show that *Foxc1* is required in the mouse for the formation of a functional niche for HS(P)C homing and maintenance in the bone marrow (Omatsu et al., 2014). There *Foxc1* initiates and maintains the expression of *CXCL12* and *SCF*, which in turn maintain identity of a specialized reticular cell type in the marrow cavities which function as a niche for hematopoiesis, the CXC chemokine ligand (CXCL) 12-abundant reticular (CAR) cells, (Omatsu et al., 2014). This requirement is cell

autonomous within the CAR cells, and not provided non-cell autonomously through the endothelial lineage (Omatsu et al., 2014).

Dll4 itself is expressed in the arterial and neural compartment in the zebrafish trunk at the time of HSC specification and several *notch* receptors are expressed in the surrounding tissues. A cell non-autonomous relationship between *dll4* and *foxc1b* through *notch* therefore needs to be considered. In the zebrafish, several Notch receptors are expressed in the somites, where *foxc1b* itself is expressed (Oates et al., 2005, Thisse et al., 2001, De Val et al., 2008, Lee et al., 2009, Skarie and Link, 2009, Kim et al., 2014). *Notch3* is expressed in the DA and the somites, amongst other tissues, but its somitic activity has recently been shown to be required for HSC specification in the zebrafish, and also to regulate *foxc1b* expression in the somites (Kim et al., 2014). While the authors have located the requirement for *notch3* in HSC specification downstream of the *wnt16* pathway and the *dlc/dld* notch ligands, they do not exclude the additional possibility that somitic *notch3* interacts with arterial *dll4*.

6.7.3 Speculative mechanisms for the action of *foxc1b* in Phase II

The perplexing switch in phenotype between Phase I and Phase II observed in *foxc1b* deserves more detailed attention. Zebrafish mutants for two genes, *cmyb* and *cbfb*, show a highly similar phenotype in the developmental time frame of Phase II.

Cmyb has multiple roles in haematopoietic development; of particular note is the absence of foetal liver haematopoiesis (or equivalent) in *cmyb* mutants across species, ascribed to impaired HSPC proliferation and differentiation (Mucenski et al., 1991, Badiani et al., 1994, Sandberg et al., 2005, Thomas et al., 2005, Lieu and Reddy, 2009,

Moriyama et al., 2010, Soza-Ried et al., 2010, Zhang et al., 2011). Normal egression and migration of HSCs from the DA to the caudal haematopoietic tissue is inhibited in *cmyb^{hkz3}* zebrafish mutants, prior to proliferation and differentiation defects observed in the CHT (Soza-Ried et al., 2010, Zhang et al., 2011), which I have confirmed here. Zhang and colleagues describe this phenotype of increased HSC numbers in the DA from 48 hpf as the earliest requirement for *cmyb* in HSC development, and based on these observations propose a role in HSC migration for *cmyb*.

Data presented here indicates a role of *foxc1b* upstream of *cmyb*, as *cmyb* is affected by the absence of *foxc1b*. However, it remains to be tested if *foxc1b* is differentially expressed in *cmyb^{hkz3}* mutants. Zhang et al. observe increased levels of the *chemokine (C-X-C motif) ligand 12a (cxcl12)*, previously known as *stromal cell-derived factor 1a (sdf1a)* in *cmyb^{hkz3}* mutants, while levels of its receptors *cxcr4a* and *cxcr4b* are not affected (Zhang et al., 2011). *Cxcl12* has been shown to act as a chemo-attractant for adult HSCs in the zebrafish and a very recent study has shown that *cxcl12b (sdf1b)* regulates HSC numbers in the zebrafish embryo (Glass et al., 2011, Nguyen et al., 2014). These, and studies in other models, clearly indicate that CXCL12 signalling can induce an expansion of HSC-like cells after initial specification, like that seen in the *foxc1b* morphants. Preliminary data suggests a down-regulation of *cxcl12* in the DA of 36 hpf but not 30 hpf *foxc1b* morphant embryos (Peterkin and Pinheiro, *personal communication*), consistent with its reported up-regulation in *cmyb^{hkz3}* mutants. Intriguingly however, Zhang and colleagues report a lack of effect on HSC development when knocking down *cxcl12 (sdf1a)*, while our preliminary data observes reduced levels of *cxcl12* in embryos with increased HSC marker expression, highly similar to the phenotype associated with increased *cxcl12* levels (Zhang et al., 2011). This requires

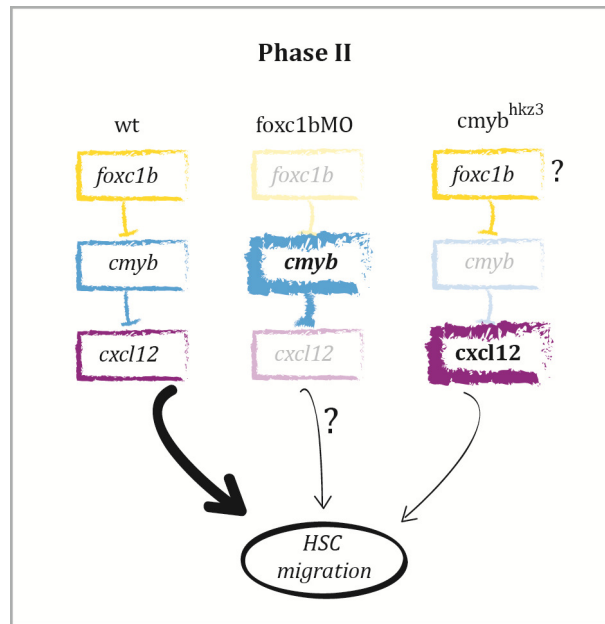


Figure 6-14. Speculative genetic regulatory mechanism for Phase II.

Foxc1b represses *cmyb* expression during Phase II in wt embryos, and *cmyb* in turn represses *cxcl12* (*sdf1a*) expression, allowing HSCs to migrate from the DA. In *foxc1b* morphants *cmyb* expression is increased, resulting in reduced levels of *cxcl12* (*sdf1a*), interfering with normal HSC migration. In *cmyb*^{hkz3} mutants *foxc1b* levels are presumed to be normal, while the absence of functional *Cmyb* protein causes increased levels of *cxcl12*, impeding on migration of HSC from the DA. Question marks represent assumptions that have not been tested.

further investigation and it would be highly informative to investigate the relative expression of *cxc4a/b* and *cxcl12b* in *foxc1b* and *dll4* morphants to address the hypothesis that *foxc1b* regulates part of this signalling pathway, similarly to or through *cmyb*, and independently of *dll4*.

While Zhang and colleagues give evidence of a requirement for *cmyb* in HSC migration from the DA, data obtained here suggests an additional earlier and migration-independent requirement of *cmyb* in HSC development, similar to that of *foxc1b*: while *runx1* is not adversely affected in *cmyb*^{hkz3} mutants at 30 hpf (Zhang et al., 2011), I observe reduction in *runx1* expression at 26 hpf. This suggests that the absence of *cmyb* results in fewer *runx1* positive cells initially, at the time of HSC specification.

Immediately afterwards however, absence of *cmyb* appears to drive proliferation or differentiation, as a clear increase in cells expressing HSC markers is present, indicated by the compensation of the earlier phenotype by 30 hpf and a clear up-regulation of HSCs by 36 hpf. This suggests that the few HSCs present at 26 hpf have proliferated to compensate for the early defect; at a time when EHT is just initiating and no substantial numbers of HSCs are leaving the DA in the wt embryos (Kissa and Herbomel, 2010), signifying that the accumulation of HSCs at this timepoint cannot be solely ascribed to a defect in migration from the DA. *Cmyb* is known to regulate HSC proliferation and differentiation in the foetal liver (Mucenski et al., 1991; Sandberg et al., 2005), both of which could be relevant in this context.

Interestingly, *foxc1b* morphants phenocopy the *cmyb* loss of function phenotype. This is counter-intuitive as *cmyb*^{hkz3} represents a *cmyb* loss of function (Zhang et al., 2011), while *foxc1b* morphants cause an up-regulation of *cmyb* staining (Figure 6-8-A, p. 207), here interpreted as an increase in *cmyb* activity and/or *cmyb* positive cells. This observation indicates that appropriate levels of *cmyb* are required for normal HSC proliferation and migration, and that aberrations in *cmyb* levels have a similar, if not identical, effect on HSC numbers, early and late.

A recent study has described another remarkably similar phenotype in zebrafish *cbfb* mutants: Two independent *cbfb* null mutants showed an increase in cells expressing *runx1* in the DA from 36 hpf onwards, and an absence of all HSC derived lineages in the CHT and the thymus (Bresciani et al., 2014). Interestingly, the authors did not investigate the expression of *runx1* earlier in the *cbfb* null mutants, so it remains to be determined whether the absence of *cbfb* in the zebrafish only phenocopies Phase II of the *foxc1b* phenotype or also the earlier reduction of *runx1* observed in Phase I at 26 hpf.

The authors also confirm that *cbfb* is regulated by the Notch pathway, although they do not attempt to identify the Notch ligand responsible (Bresciani et al., 2014).

One important finding of the study conducted by Bresciani et al. (2014) is that the role of *cbfb* can be uncoupled from that of *runx1* in the zebrafish, as evidenced by their distinct phenotypes. This is surprising considering that the two proteins form a heterodimeric complex (Ogawa et al., 1993, Wang et al., 1993, Jin et al., 2009, Sood et al., 2010). While *runx1* mutants show no evidence of definitive haematopoiesis, *cbfb* mutants initiate definitive haematopoiesis (as indicated by *runx1* and *cmyb* expression in the DA at 36 hpf), but these cells fail to migrate to the subsequent sites of HSC development (Jin et al., 2009, Sood et al., 2010, Bresciani et al., 2014). Taken together with the *foxc1b* phenotype, this raises the possibility that *foxc1b* regulates *runx1* during Phase I, and switches to *cbfb* in Phase II, which remains to be shown experimentally. The authors further show that *cbfb* is regulated by Notch (Bresciani et al., 2014), which in turn suggests that *foxc1b* might remain under the control of the Notch pathway, but through a different ligand, a possibility which is explored in more detail below.

6.7.4 Speculative non-cell autonomous models of *dll4*-independent regulation of *foxc1b*

The clear division of the *foxc1b* phenotype into three discernible phases suggests that *foxc1b* is subject to different regulatory contexts. While I have shown that *foxc1b* is regulated by *dll4* in Phase I, the *dll4*-independent regulatory pathway employed in Phase II remains to be elucidated. In the zebrafish trunk, *foxc1b* is expressed in the DA and the immediately adjacent part of the somites, the sclerotome (De Val et al., 2008, Skarie and Link, 2009). The sclerotome is required for appropriate HSC specification in the

zebrafish through *wnt/notch* signalling (Clements et al., 2011, Kim et al., 2014), and thus it is possible that *foxc1b* acts in a cell non-autonomous manner in this context.

One possible cell non-autonomous *dll4*-independent regulator of *foxc1b* is Wnt signalling. The Wnt/ β -catenin pathway has previously been implicated in HSC specification and maintenance (Goessling et al., 2009, Staal and Luis, 2010) and non-canonical *wnt16* signalling from the somites has been placed upstream of the Notch pathway during zebrafish early somitogenesis (10-16 ss) (Clements et al., 2011). Clements and colleagues show that this early Notch signalling is mediated through the activity of the *Dll1* paralogues *dlc* and *dld* in the sclerotomal compartment of the somites, and Kobayshi and colleagues (2014) place it downstream of *jam1a*. Both *foxc1a* and *foxc1b* are reduced in somites in the absence of *dlc*, *dld* and *wnt16*, but not in the artery (Clements et al., 2011). *Dll4* expression is not affected in *wnt16* morphants (Clements et al., 2011), indicating that this reduction in *foxc1b* expression is not due to reduced expression of *dll4*.

This *wnt16*-mediated regulation of *foxc1b* however is unlikely to be responsible for the later observed increase in HSC markers in the DA of *foxc1b* morphants, as loss of *wnt16* and the *dll1* paralogues results in a loss of *cmyb* at 36 hpf (Clements et al., 2011), rather than a gain. Somitic expression of *foxc1b* requires *dlc* and *dld* activity, as single knockdowns only result in a partial reduction of *foxc1b* expression (Clements et al., 2011), providing an explanation for the lack of regulation of *foxc1b* by *dlc* and *dld* in the DA, as *dld* is not expressed in this tissue (Figure 3-2-G&H, p.84). Regulation of *foxc1b* by the other *Dll1* paralogues *dla* and *dlb* is unlikely as their expression is restricted to the neural tissues at the time of HSC emergence (Figure 3-2-A-D, p.84) and throughout

development (Appel and Eisen, 1998, Haddon et al., 1998, Kim et al., 2008, Qiu et al., 2009).

Another potential *dll4*-independent mechanism of regulation of *foxc1b* is through the *jagged* family of Notch ligands. Despite none of the *jagged* genes being clearly expressed in the DA of the zebrafish around the time of HSC specification, all are expressed in the somites if at low levels (Figure 3-1, p.82), *Jagged1* and *Jagged2* have been reported to be required for HSC emergence in chick and mouse (Robert-Moreno et al., 2008, Richard et al., 2013). No functional analysis of *jag1* has been published in the context of HSC development in the zebrafish, and preliminary results obtained here fail to show an effect of *jag1a* or *jag1b* MOs on *runx1* expression at 36 hpf (data not shown). However, their compensatory action has not been assessed in a double-knockout, an experiment necessary to exclude the possibility of a *jag1*-mediated action. So although it appears that *jag1a* and *jag1b* alone are not sufficient to regulate *foxc1b*, it cannot be excluded that the *jagged* family is required for this later *dll4*-independent regulation of *foxc1b*, in a likely cell non-autonomous fashion.

Vegf was considered a further good candidate regulator of *foxc1b* during Phase II: loss of *veg*f can be rescued by Notch, indicating that Notch acts downstream of *veg*f in a common genetic pathway, required for HSC development (Lawson et al., 2002, Gering and Patient, 2005, Ciau-Uitz et al., 2010, Leung et al., 2013). In compound homozygous *Foxc1/Foxc2* mouse mutants, *Vegfa* expression is upregulated in the paraxial mesoderm, neural tube and endoderm (Seo et al., 2006). A similar up-regulation of *vegfaa* expression is observed the somites of *foxc1b* zebrafish morphants at 36 hpf, but not 26 hpf (Appendix Figure 8-12, p. 263). While this indicates a repression of *vegfaa* by *foxc1b* in the somites during Phase II but not Phase I, a similar pattern is observed in *dll4*

morphants (Peterkin and Pinheiro, *personal communication*), suggesting that *vegfaa* is not the *dll4*-independent mechanism through which *foxc1b* regulates later HSC development in Phase II. Nevertheless, this observation indicates that at least part of the pathway acts cell-non-autonomously.

6.7.5 Conclusions and directions

It has previously been suggested that FOXC1 could act in distinctively opposite (ie. pro and anti-angiogenic) manners, depending on the context of the tissue of expression (Koo and Kume, 2013), and I suggest that this concept can be extended to developmental timing of HSC development. Initially, *foxc1b* appears to act positively on initial *runx1* expression in the dorsal aorta, while later it appears to assume a negative role.

The reduction of *runx1* in the DA at 26 hpf is the earliest described phenotype for *foxc1b* to date, and it is likely regulated through *dll4*. The repressive action of *foxc1b* on *runx1* and other HSC markers observed just afterwards is indicative of a *dll4*-independent phase of HSC development, mirroring that of *cmyb* loss of function. While the regulatory mechanism upstream of this Phase (II) of *foxc1b* involvement in HSC development still remains to be elucidated, data presented here provides an indication that *foxc1b* might act through *cmyb* in its second, *dll4*-independent function.

Consequently it is likely that the observed phenotype of Phase III is a consequence of the down-regulation of *runx1* at 26 hpf, as in the *dll4* phenotype. Alternatively, if the increased HSC-like cells are functional, the reduction of HS(P)Cs and HSC-derived cells observed could be a result of inhibited migration from the DA, as in *cmyb*^{hkz3}. Or, most likely a combination of both.

Chapter 7

Discussion

In this thesis, I have established the earliest yet known role for *dll4* in the zebrafish: *dll4* is required for the initiation of *runx1* expression and consequently is essential for HSC specification. This role is earlier than any observed and described arterial phenotype (Hogan et al., 2009, Bridge et al., 2012), and therefore likely independent of the role of *dll4* in arterial endothelium. This is further supported by the scarcity of arterially expressed *dll4* targets detected in the expression screen conducted.

It appears likely that *dll4* acts primarily through *runx1* at this early time point (26 hpf): not only are 79% of all *dll4* targets also targets of *runx1*, but *runx1* is sufficient to rescue the *dll4* HSC phenotype. This suggests that the early HSC phenotype observed in the absence of *dll4* can be explained by its regulation of *runx1*. This finding provides the first direct evidence for a requirement of *dll4* in HSC development. A zebrafish mutant line, predicted to be null for *dll4*, has recently become available through the zebrafish mutation project (ZMP, 2014), and would enable further confirmation of the phenotypes described here in a knockout rather than knockdown model.

Although the loss of *runx1* expression has been reported before in E9.5 *dll4* mutant mice, this is preceded by severe arterial defects and has thus not been studied in more detail (Robert-Moreno et al., 2008). Consequently, it appears likely that this role for *dll4* in HSC specification is conserved across species, while there appears to be an evolutionary divergence of the arterial role of *dll4* between mice and zebrafish. It is this very loss of the requirement for *dll4* in arterial specification that has here allowed the examination of the specific role of *dll4* in HSC emergence *in vivo*, which has not been possible in the mouse. *Dll4*-Notch signalling is also essential for T-cell progenitor development, and its ectopic expression in developing T-cells, causes T-cell acute lymphoblastic leukemia (T-ALL) (Calderón and Boehm, 2012, Xiong et al., 2013, Hagenbeek et al., 2014).

Furthermore, a bias towards a myeloid fate of human embryonic stem cell-derived (hESC) haematopoietic progenitor cells (HPCs) is enabled by the expression of *Dll4* in hESC-derived vascular feeder cells (Rafii et al., 2013), supporting the haematopoietic potential of *Dll4* and further suggesting a role for *dll4* in haematopoiesis, independent of its contribution to arterial specification.

I have further shown that the mechanism through which *dll4* regulates *runx1* expression is at least partially through *foxc1b*. I have shown that *foxc1b* is regulated by *dll4* at the time of HSC specification, and although a formal rescue of the loss of *runx1* expression in *dll4* morphants through *foxc1b* is still outstanding, it appears likely this experiment would be successful as the phenotypes of *dll4* and *foxc1b* morphants are highly similar at this time point. Interestingly, this similarity in phenotypes is only temporally, followed by an apparently *dll4*-independent *foxc1b* requirement in definitive haematopoiesis. I have shown that *foxc1b*'s role is to repress expression of definitive blood markers, including *cmyb* at this later time point, indicating that *foxc1b* regulates *cmyb* in a direct or indirect fashion. This is of interest as the *cmyb*^{hkz3} mutants show a highly similar phenotype to the *foxc1b* morphants in this phase of HSC development, characterized by an increase in HSC-like cells in the DA, that appear to be unable to emigrate (Zhang et al., 2011).

Cmyb^{hkz3} mutants exhibit an up-regulation of *cxcl12* (*sdf1a*) in the DA, disturbing the migratory microenvironment for HSCs (Zhang et al., 2011). The RNA-seq experiment revealed that *dll4* is required for expression of its receptor *cxcr4a* at 26 hpf but not *cxcl12* itself. Cxc signalling might therefore provide a mechanism through which Phase I and Phase II could interact through *dll4* dependent (*cxcr4*) and independent (*cxcl12*)

signalling. This theory awaits further testing through assessment of the regulation of *cxc4* and *cxc12* by *dll4*, *foxc1b* and *cmyb*.

In this *Cxc* driven hypothesis, it would be predicted that HSCs in the DA of *foxc1b* morphants and *cmyb*^{hkz3} mutants undergo EHT but fail to migrate afterwards. Assessment of the process of EHT could therefore help to shed light on the nature of the observed defect and the relation between the two phenotypes, and to help unravel whether *foxc1b* is required for migration of cells or for EHT itself.

Chapter 8

Appendices

Appendix Table 8-1. Breakdown of MO phenotypes represented in Figures.

Summary of observed phenotypes in this thesis. Morphant numbers are in relation to wt averages: ↑ increased expression, ~ average expression (wt-like), ↓ decreased expression. Numbers represent the number of embryos assessed.

Figure 3-5

Figure 3-5

		wt				dll4MO1				dll4MO4				
		↑	~	↓	total	↑	~	↓	total	↑	~	↓	total	
A	<i>runx1</i>	24 hpf	0	43	5	48	0	16	35	51	0	11	46	57
B	<i>runx1</i>	26 hpf	1	173	30	204	0	43	163	206	1	40	150	191
C	<i>runx1</i>	28 hpf	0	103	11	114	0	28	94	122	0	23	107	130
D	<i>runx1</i>	30 hpf	0	78	7	85	0	26	34	60	0	20	26	46
E	<i>runx1</i>	32 hpf	0	52	0	52	0	13	39	52	0	18	27	45

Figure 3-6

Figure 3-6

		wt				dll4MO1				dll4MO4				
		↑	~	↓	total	↑	~	↓	total	↑	~	↓	total	
A	<i>runx1</i>	36 hpf	0	82	10	92	0	15	68	83	0	29	52	81
B	<i>cmyb</i>	36 hpf	3	176	10	189	0	35	52	87	2	61	112	175
C	<i>ikzf1</i>	36 hpf	0	97	0	97	0	52	51	103	0	46	28	74

Figure 3-7-B

		wt				dll4MO4				runx1DNA + tol2RNA + Dll4MO4				runx1DNA + tol2RNA			
		↑	~	↓	total	↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
<i>cmyb</i>	36 hpf	3	71	10	84	2	20	87	109	0	62	16	78	11	59	4	74
<i>runx1</i>	36 hpf	0	115	0	115	x	x	x	x	x	x	x	x	0	115	0	115

Figure 3-8

Figure 3-8

			wt				dll4MO1				dll4MO4			
			↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
A	<i>hey2</i>	26 hpf	0	62	4	66	2	52	3	57	0	55	1	56
B	<i>efnb2a</i>	26 hpf	0	22	0	22	0	29	0	29	0	23	0	23
C	<i>flt1</i>	26 hpf	0	60	5	65	0	23	1	24	0	33	1	34

Figure 3-9

Figure 3-9

			wt				dll4MO1				dll4MO4			
			↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
A	<i>flt4</i>	26 hpf	0	47	0	47	6	54	0	60	4	46	0	50
B	<i>dab2</i>	26 hpf	0	60	0	60	4	51	0	55	4	36	0	40
C	<i>tek</i>	26 hpf	0	25	0	25	0	27	0	27	0	27	0	27
D	<i>hhex</i>	26 hpf	0	51	0	51	0	54	1	55	0	72	0	72

Figure 3-10

Figure 3-10

		wt				dll4MO1				dll4MO4				
		↑	~	↓	total	↑	~	↓	total	↑	~	↓	total	
A	<i>kdr1</i>	26 hpf	0	68	1	69	1	68	8	77	1	86	8	95
B	<i>fli1a</i>	26 hpf	0	92	0	92	0	31	3	34	0	90	1	91
C	<i>cdh5</i>	26 hpf	0	29	3	32	1	17	0	18	0	23	1	24

Figure 3-11

Figure 3-11

			wt				dll4MO1				dll4MO4			
			↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
A	Tg(itga2b:gfp)	4 dpf	1	60	0	61	n/a	n/a	n/a	n/a	0	3	22	25
B	<i>runx1</i>	4 dpf	0	126	7	133	5	18	80	103	0	25	80	105
C	<i>cmyb</i>	4 dpf	0	127	1	128	0	12	45	57	0	54	69	123
D	<i>ikzf1</i>	4 dpf	0	117	0	117	0	48	62	110	0	40	81	121

Figure 3-12

Figure 3-12

			wt				dll4MO1				dll4MO4			
			↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
A	<i>hbbe1.1</i>	4 dpf	0	73	16	89	0	13	39	52	3	32	88	123
B	<i>mpx</i>	4 dpf	0	96	3	99	0	64	51	115	0	42	46	88
C	<i>lcp1</i>	4 dpf	1	76	5	82	0	19	51	70	0	73	18	91

Figure 3-13

A *rag1* 4 dpf
B *rag2* 4 dpf
C *lck* 4 dpf
D *foxn1* 4 dpf

wt				dll4MO1				dll4MO4			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
0	85	3	88	0	15	63	78	0	8	87	95
0	129	8	137	0	9	54	63	0	20	124	144
3	54	6	63	0	12	48	60	0	3	35	38
1	20	2	23	0	5	19	24	0	6	22	28

Figure 5-4

A *runx1* 26 hpf
B *dll4* 26 hpf

wt				cldn5bMO			
↑	~	↓	total	↑	~	↓	total
1	12	1	14	0	1	13	14
2	26	0	28	16	2	0	18

Figure 5-5

A *runx1* 26 hpf
B *cmyb* 36 hpf
C *dll4* 26 hpf
D *rag1* 4 dpf
E *mpx* 4 dpf
F *hbbe1.1* 4 dpf

wt				hif1abMO			
↑	~	↓	total	↑	~	↓	total
2	18	4	24	0	11	13	24
0	19	1	20	0	5	8	13
0	19	1	20	0	15	8	23
0	25	2	27	0	8	19	27
1	34	6	41	1	20	4	25
0	21	4	25	0	11	3	14

Figure 5-6

A *runx1* 26 hpf
B *cmyb* 36 hpf
C *ikzf1* 36 hpf
D *dll4* 26 hpf
E *rag1* 4 dpf
F *mpx* 4 dpf
G *hbbe1.1* 4 dpf

wt				zfatMO1			
↑	~	↓	total	↑	~	↓	total
0	11	6	17	0	6	12	18
0	18	3	21	0	25	2	27
0	24	10	34	0	19	3	22
0	23	1	24	0	0	19	19
0	26	7	33	0	5	17	22
0	14	3	17	0	10	4	14
4	18	2	24	0	3	16	19

Figure 5-7

A *runx1* 26 hpf
B *cmyb* 36 hpf
C *ikzf1* 36 hpf
D *dll4* 26 hpf
E *dlc* 26 hpf
F *dab2* 26 hpf
G *kdr1* 26 hpf
H *rag1* 4 dpf
I *mpx* 4 dpf
J *hbbe1.1* 4 dpf

wt				foxd1MO			
↑	~	↓	total	↑	~	↓	total
3	42	2	47	2	27	43	72
0	13	0	13	0	16	1	17
1	12	2	15	0	4	14	18
2	39	4	35	31	9	0	41
0	23	3	26	28	1	0	29
0	23	0	23	15	1	0	16
0	12	0	12	0	17	0	17
0	8	1	9	0	4	4	8
0	11	0	11	0	14	9	23
0	16	3	19	0	16	2	18

Figure 5-8

A *runx1* 26 hpf
B *cmyb* 36 hpf
C *dlc* 26 hpf
D *dab2* 26 hpf
E *kdr1* 26 hpf
F *rag1* 4 dpf
G *mpx* 4 dpf
H *hbbe1.1* 4 dpf

wt				hes6MO			
↑	~	↓	total	↑	~	↓	total
1	87	4	92	0	23	106	129
3	83	5	91	1	34	52	87
0	78	8	86	20	54	1	75
0	22	0	2	0	15	0	15
0	51	0	51	1	49	3	53
1	92	10	103	0	14	33	47
0	77	8	85	6	53	21	80
2	81	5	88	0	18	96	114

Figure 5-9

A	<i>runx1</i>	36 hpf
B	<i>gfi1aa</i>	36 hpf
C	<i>ikzf1</i>	36 hpf
D	<i>dll4</i>	26 hpf
E	<i>hey2</i>	26 hpf
F	<i>tbx20</i>	26 hpf
G	<i>fli1a</i>	26 hpf
H	<i>lck</i>	4 dpf
I	<i>cmyb</i>	4 dpf
J	<i>ikzf1</i>	4 dpf
K	<i>lyz</i>	4 dpf
L	<i>lcp1</i>	4 dpf

wt				hes6MO			
↑	~	↓	total	↑	~	↓	total
0	63	4	67	0	36	19	55
0	19	3	22	0	4	13	17
2	42	2	48	28	14	1	43
1	22	1	24	0	32	1	33
0	19	3	22	0	18	5	23
0	17	1	18	0	40	3	43
0	36	0	36	0	27	3	30
0	17	1	18	0	10	12	22
0	22	3	25	0	7	22	29
0	18	3	21	0	6	23	29
0	18	3	21	0	15	15	30
1	14	2	17	0	12	18	30

Figure 5-10

A	<i>runx1</i>	26 hpf
B	<i>cmyb</i>	36 hpf
C	<i>flt1</i>	26 hpf
D	<i>dab2</i>	26 hpf
G	<i>rag1</i>	4 dpf
E	<i>hbbe1.1</i>	4 dpf
F	<i>mpx</i>	4 dpf

wt				her8aMO			
↑	~	↓	total	↑	~	↓	total
1	78	12	91	0	16	82	98
0	39	1	40	0	19	23	42
0	12	4	16	0	29	2	31
0	25	0	25	0	19	0	19
0	78	2	80	0	17	92	109
4	72	3	79	3	32	48	83
0	12	0	12	0	17	19	36

Figure 5-11

A	<i>runx1</i>	36 hpf
B	<i>ikzf1</i>	36 hpf
C	<i>dlc</i>	26 hpf
D	<i>dll4</i>	26 hpf
E	<i>hey2</i>	26 hpf
F	<i>tbx20</i>	26 hpf
G	<i>kdr1</i>	26 hpf
H	<i>foxn1</i>	4 dpf

wt				her8aMO			
↑	~	↓	total	↑	~	↓	total
0	8	0	8	0	4	10	15
0	39	1	40	0	3	22	25
0	47	7	54	0	83	7	90
0	17	8	25	4	29	6	39
0	11	2	13	0	31	0	31
0	16	1	17	0	24	3	27
0	29	0	29	0	46	2	48
0	14	0	14	0	32	0	32

Figure 5-12

A	<i>runx1</i>	26 hpf
B	<i>cmyb</i>	36 hpf
C	<i>flt1</i>	26 hpf
D	<i>dab2</i>	26 hpf
E	<i>tie1</i>	26 hpf
F	<i>rag1</i>	4 dpf
G	<i>mpx</i>	4 dpf
H	<i>gata1a</i>	4 dpf

wt				foxc1bMO1			
↑	~	↓	total	↑	~	↓	total
0	37	5	42	0	5	17	22
0	21	2	23	18	5	2	25
1	61	4	66	0	58	13	71
0	38	0	38	0	30	0	30
0	35	0	35	0	38	0	38
0	18	4	22	0	4	18	22
1	21	4	26	0	17	4	21
3	19	2	24	0	6	14	20

Figure 6-2-A

<i>foxc1b</i>	26 hpf
<i>dll4</i>	26 hpf

wt				dll4MO4				foxc1bMO1				runx1MOe1			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
2	34	7	43	0	3	24	27	x	x	x	x	0	40	7	47
2	39	6	47	x	x	x	x	6	28	8	42	0	21	1	22

Figure 6-3

A	<i>kdr1</i>	26 hpf
B	<i>fli1a</i>	26 hpf
C	<i>cdh5</i>	26 hpf

wt				foxc1bMO1				foxc1bMO2			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
0	32	1	33	0	31	4	35	0	12	0	12
0	20	0	20	0	18	0	18	0	17	0	17
0	42	0	42	0	45	2	47	0	18	0	18

Figure 6-4

A	<i>hey2</i>	26 hpf
B	<i>tbx20</i>	26 hpf
C	<i>flt1</i>	26 hpf

wt				foxc1bMO1				foxc1bMO2			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
2	40	17	59	4	37	0	41	0	28	0	28
1	24	3	28	0	19	0	19	16	35	0	51
1	61	4	66	0	58	13	71	0	31	1	32

Figure 6-5

A	<i>hhex</i>	26 hpf
B	<i>flt4</i>	26 hpf
C	<i>tek</i>	26 hpf

wt				foxc1bM01				foxc1bM02			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
0	51	1	52	0	54	7	61	26	13	0	39
0	80	0	80	2	30	0	32	0	41	0	41
1	44	1	46	1	49	4	54	0	18	0	18

Figure 6-6

A	<i>runx1</i>	26 hpf
B	<i>rag1</i>	4 dpf
C	<i>rag2</i>	4 dpf
D	<i>cmyb</i>	4 dpf
E	<i>ikzf1</i>	4 dpf

wt				foxc1bM01				foxc1bM02			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
3	181	19	203	0	38	80	118	0	38	47	85
0	100	24	124	0	18	72	90	0	12	38	50
0	114	10	124	0	16	80	96	0	10	36	46
1	126	9	136	0	41	39	80	0	17	28	45
7	88	10	105	1	26	91	118	0	11	33	44

Figure 6-7

A	<i>cmyb</i>	4 dpf
B	<i>ikzf1</i>	4 dpf
C	<i>lcp1</i>	4 dpf
D	<i>lyz</i>	4 dpf
E	<i>gata1a</i>	4 dpf

wt				foxc1bM01				foxc1bM02			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
4	119	13	136	1	37	42	80	0	10	35	45
1	90	12	103	1	40	76	117	0	8	36	44
0	90	7	97	0	54	30	84	1	18	25	44
0	76	7	83	7	17	37	61	0	9	6	15
1	7	1	9	0	3	30	33	0	7	12	19

Figure 6-8

A	<i>cmyb</i>	36 hpf
B	<i>ikzf1</i>	36 hpf
C	<i>runx1</i>	36 hpf

wt				foxc1bM01				foxc1bM02			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
8	127	15	150	43	41	14	98	35	13	2	50
12	102	12	126	42	19	16	77	45	11	0	56
11	100	9	120	75	38	1	114	23	14	1	38

Figure 6-9

A	<i>runx1</i>	24 hpf
B	<i>runx1</i>	26 hpf
C	<i>runx1</i>	28 hpf
D	<i>runx1</i>	30 hpf
E	<i>runx1</i>	32 hpf
F	<i>runx1</i>	34 hpf
G	<i>runx1</i>	36 hpf
H	<i>runx1</i>	48 hpf
I	<i>runx1</i>	56 hpf
J	<i>runx1</i>	72 hpf
H	<i>runx1</i>	48 hpf
I	<i>runx1</i>	56 hpf
J	<i>runx1</i>	72 hpf
K	<i>runx1</i>	78 hpf
L	<i>runx1</i>	84 hpf

wt (DA)				foxc1bM01 (DA)				foxc1bM02 (DA)			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
0	35	7	42	6	12	28	46	0	5	10	15
3	181	19	203	0	38	80	118	0	38	47	85
0	52	6	58	2	21	30	53	0	14	0	14
0	16	2	18	3	8	2	13	2	13	1	16
3	17	3	23	3	14	1	18	6	15	0	21
1	13	0	14	9	10	2	21	7	12	1	20
11	100	9	120	75	38	1	114	23	14	1	38
0	38	7	45	20	15	4	39	24	5	0	29
0	49	8	57	10	30	2	42	9	11	0	20
2	60	3	65	48	30	4	82	17	2	1	20
wt (CHT)				foxc1bM01 (CHT)				foxc1bM02 (CHT)			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
0	40	9	49	0	9	30	39	0	13	16	29
0	54	3	57	0	23	19	42	0	3	17	20
3	57	5	65	0	28	54	82	1	9	10	20
2	81	8	91	0	28	54	82	0	7	29	36
1	26	3	30	0	6	12	18	0	12	8	20

Appendix Figure 8-8

A	<i>runx1</i>	26 hpf
B	<i>runx1</i>	36 hpf
C	<i>cmyb</i>	36 hpf
D	<i>rag1</i>	4 dpf

wt				hes6M0				her8aM0				hesM0+her8aM0			
↑	~	↓	total	↑	~	↓	total	↑	~	↓	total	↑	~	↓	total
1	28	0	29	0	7	21	28	0	6	24	30	0	2	42	44
0	8	0	8	0	0	10	10	1	4	10	15	0	0	22	22
1	35	3	39	0	8	15	23	0	5	19	24	2	7	27	36
0	67	1	68	0	4	35	39	1	14	57	72	0	14	50	64

Appendix Figure 8-10

A	<i>dll4</i>	26 hpf
B	<i>dll4</i>	28 hpf

wt				foxc1bM01			
↑	~	↓	total	↑	~	↓	total
2	39	6	47	7	28	7	42
4	34	4	42	53	29	2	84

Appendix Figure 8-12

A	<i>vegfaa</i>	26 hpf
B	<i>vegfaa</i>	36 hpf

wt				foxc1bM01			
↑	~	↓	total	↑	~	↓	total
8	89	1	98	2	66	5	73
3	84	0	87	40	6	0	46

8.1 Appendix to Chapter 3 - The role of the Notch ligand *dll4* in definitive haematopoiesis

Appendix Table 8-2. Schematic representation of the expression of Notch receptors and ligands during zebrafish embryonic development.

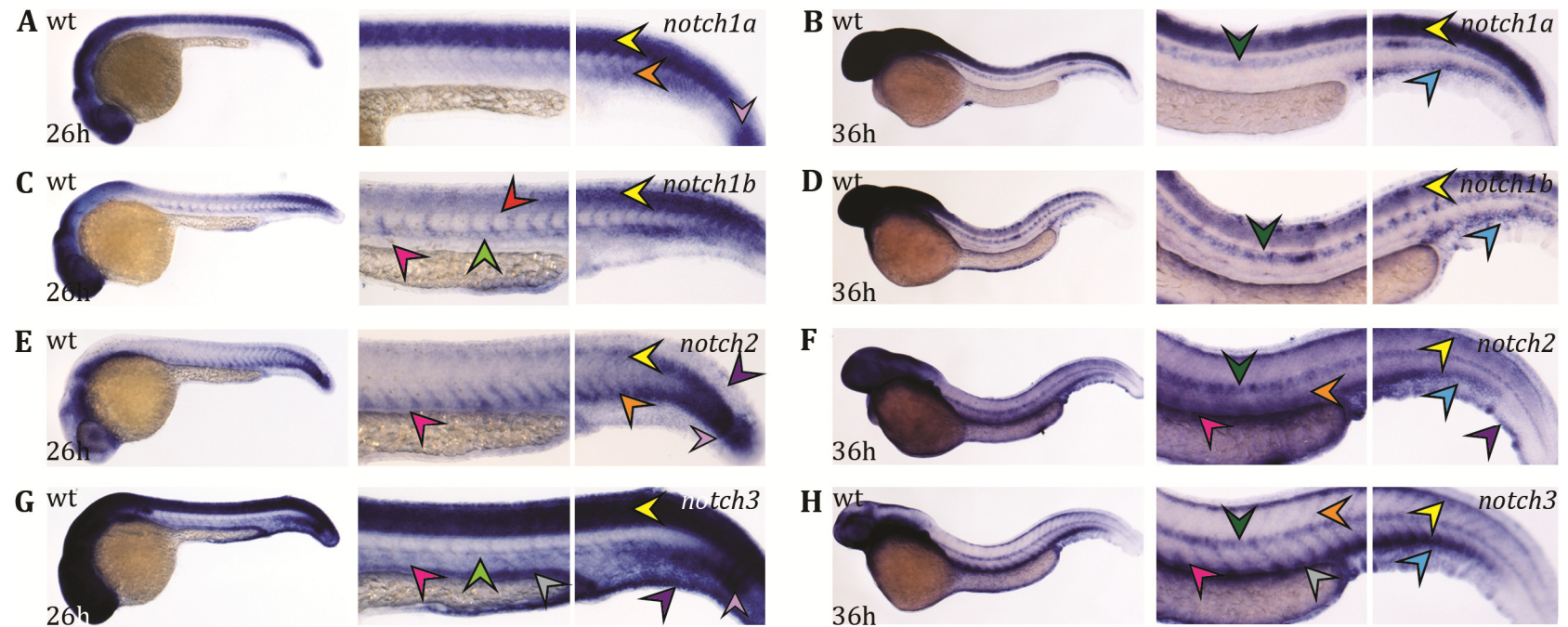
Based on literature review and expression patterns obtained in this thesis.

Legend: dorsal aorta (DA), intersomitic vessels (ISVs), posterior blood island (PBI), somites (SOM), central nervous system (CNS), notochord (NC), liver (LIV), pronephric duct (PND), gut (GUT), ectoderm (ECT), tailbud (TB), lateral line (LL), anus (ANS). Coloured fields indicate gene expression in tissue, fields without reference are presumed expression, lighter coloured fields indicate low level broad expression.

Appendix Table 8-2-A. Schematic representation of the expression of Notch receptors during zebrafish embryonic development.						
	tissue	segmentation period	26 hpf	27 hpf - 35 hpf	36 hpf	37 hpf - 5 dpf
notch1a	PBI				Appendix Figure 8-1-B	
	SOM	Oates, 2005	Appendix Figure 8-1-A			
	CNS	Niikura, 2006	Appendix Figure 8-1-A	////////////////////////////////////	Appendix Figure 8-1-B	Kim, 2008
	NC				Appendix Figure 8-1-B	
	LIV					Lorent, 2004
	PND	Ma, 2007				
	ECT	Hsiao, 2007				
	TB	Haddon, 1998; Oates, 2005	Appendix Figure 8-1-A			
notch1b	DA	Thisse, 2005; Sacilotto, 2013	Appendix Figure 8-1-C	Geudens, 2010		
	ISVs		Appendix Figure 8-1-C	Geudens, 2010		
	PBI				Appendix Figure 8-1-D	
	CNS	Thisse, 2005	Appendix Figure 8-1-C	Thisse, 2005; Geudens, 2010	Appendix Figure 8-1-D	Thisse, 2005
	NC				Appendix Figure 8-1-D	
	LIV					Lorent, 2004
	GUT		Appendix Figure 8-1-C			
	ECT	Hsiao, 2007				
notch2	PBI				Appendix Figure 8-1-F	
	SOM	Thisse, 2001; Oates, 2005	Appendix Figure 8-1-E	Thisse, 2001; Oates, 2005	Appendix Figure 8-1-F	
	CNS		Appendix Figure 8-1-E	////////////////////////////////////	Appendix Figure 8-1-F	
	NC	Thisse, 2001			Appendix Figure 8-1-F	
	LIV					Lorent, 2004
	GUT		Appendix Figure 8-1-E	Thisse, 2001	Appendix Figure 8-1-F	
	ECT	Hsiao, 2007	Appendix Figure 8-1-E	////////////////////////////////////	Appendix Figure 8-1-F	
	TB	Thisse, 2001	Appendix Figure 8-1-E			
notch3	DA	Patterson, 2005; Patterson, 2007	Appendix Figure 8-1-G	Burns, 2005; Busmann, 2007		
	ISVs		Appendix Figure 8-1-G	Hogan, 2008		
	PBI			Busmann, 2007	Appendix Figure 8-1-H	
	SOM	Oates, 2005; Lee, 2009		Kim, 2014		
	CNS	Oates, 2005; Warga, 2007	Appendix Figure 8-1-G	Nikolaou, 2009	Appendix Figure 8-1-H	Zaucker, 2013
	NC	Oates, 2005			Appendix Figure 8-1-H	
	LIV					Lorent, 2004
	PND	Ma, 2007	Appendix Figure 8-1-G	Liu, 2007	Appendix Figure 8-1-H	
	ECT	Hsiao, 2007	Appendix Figure 8-1-G			
	TB	Oates, 2005	Appendix Figure 8-1-G			
	LL			Chong, 2008; Nechiporuk, 2008	Appendix Figure 8-1-H	Chong, 2008

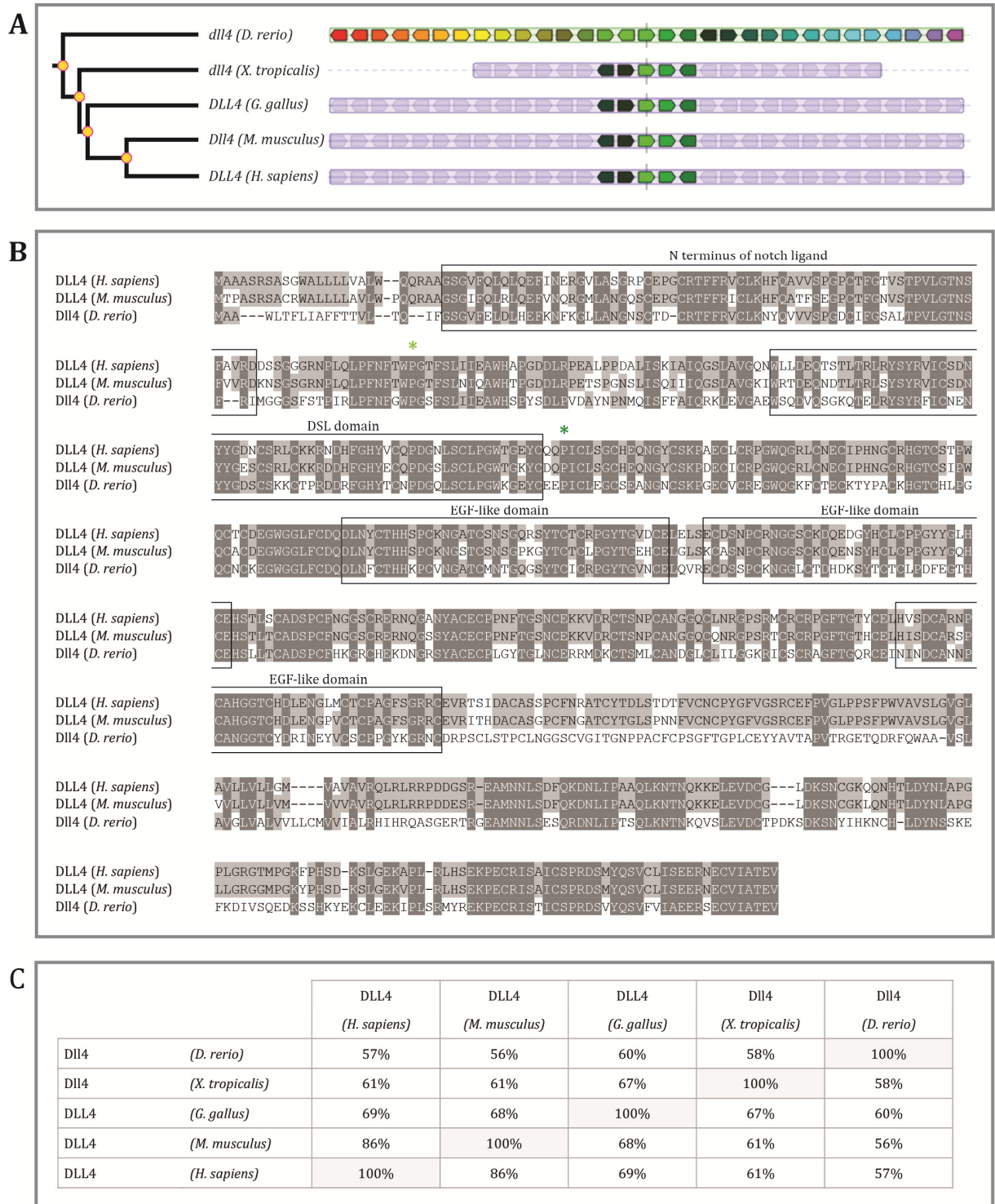
Appendix Table 8-2-B. Schematic representation of the expression of Notch ligands of the jagged family during zebrafish embryonic development.						
	tissue	segmentation period	26 hpf	27 hpf - 35 hpf	36 hpf	37 hpf - 5 dpf
<i>jag1a</i>	DA					
	SOM					
	CNS	Thisse, 2001; Zecchin, 2005		Thisse, 2001; Zecchin, 2005	Figure 4 2-B	Thisse, 2001; Zecchin, 2005
	NC	Thisse, 2001; Zecchin, 2005				
	LIV					Lorent, 2004
	GUT				Figure 4 2-B	
	LL				Figure 4 2-B	
<i>jag1b</i>	DA	Clements, 2011				
	SOM		Figure 4 2-C			
	CNS		Figure 4 2-C			
	NC	Zecchin, 2005			Figure 4 2-D	
	LIV					Lorent, 2004
	PND	Zecchin, 2005	Figure 4 2-C		Figure 4 2-D	
<i>jag2a</i>	DA					
	SOM					
	CNS					
	NC					
			Figure 4 2-E			
	ECT		Figure 4 2-E	////////////////////////////////////	Figure 4 2-F	
	LL				Figure 4 2-F	
<i>jag2b</i>	SOM	Thisse, 2005; Zecchin, 2005	Figure 4 2-G	Tian, 2010	Figure 4 2-G	
	CNS	Thisse, 2005; Zecchin, 2005; Yeo, 2007	Figure 4 2-G	Thisse, 2005; Zecchin, 2005	Figure 4 2-G	
	NC				Figure 4 2-G	
	LIV					Lorent, 2004
	PND	Thisse, 2005; Zecchin, 2005 ;Ma, 2007	Figure 4 2-G	Thisse, 2005; Zecchin, 2005; Liu, 2007	Figure 4 2-G	Liu, 2007; Ma, 2007
	ECT	Zecchin, 2005; Ma, 2007	Figure 4 2-G	Zecchin, 2005	Figure 4 2-G	Zecchin, 2005
	LL			Thisse, 2005		

Appendix Table 8-2-C. Schematic representation of the expression of Notch ligands of the delta family during zebrafish embryonic development.						
	tissue	segmentation period	26 hpf	27 hpf - 35 hpf	36 hpf	37 hpf - 5 dpf
<i>dlia</i>	CNS	Appel, 1998; Haddon, 1998; Thisse, 2001; Qiu, 2009	Figure 4 3-A	Appel, 1998	Figure 4 3-B	Kim, 2008
	CNS	Haddon, 1998; Thisse, 2008	Figure 4 3-C	Thisse, 2008	Figure 4 3-D	Thisse, 2008
<i>dlb</i>	DA	Smithers, 2000; Lawson, 2001	Figure 4 3-E	Smithers, 2000; Burns, 2005; Patterson, 2007; Qian, 2007		
	ISVs	Smithers, 2000; Lawson, 2001	Figure 4 3-E	Smithers, 2000; Patterson, 2007		
	SOM	Haddon, 1998; Smithers, 2000; Kawamura, 2005; Thisse, 2005; Giudicelli, 2007; Thisse, 2008; Sacilotto, 2013	Figure 4 3-E	Thisse, 2008, Smithers, 2000	Figure 4 3-F	
	CNS	Smithers, 2000; Thisse, 2005	Figure 4 3-E	Smithers, 2000; Thisse, 2005; Thisse, 2008	Figure 4 3-F	Thisse, 2005; Thisse, 2008
	NC	Smithers, 2000; Thisse, 2005			Figure 4 3-F	
	TB	Haddon, 1998; Smithers, 2000	Figure 4 3-E	Smithers, 2000; Tian, 2010		
	SOM	Haddon, 1998; Childs, 2002; Kawamura, 2005; Oates, 2005	Figure 4 3-G	//////////////////////////////////// ////////////////////////////////////	Figure 4 3-H	
<i>dlc</i>	CNS	Haddon, 1998	Figure 4 3-G	Cau, 2008	Figure 4 3-H	
	NC				Figure 4 3-H	
	TB	Oates, 2005; Clements, 2011	Figure 4 3-G			
	LL					Jiang, 2014
	ANS		Figure 4 3-G	////////////////////////////////////	Figure 4 3-H	
	DA	Leslie, 2007; Wang, 2011	Figure 4 3-I	Hogan, 2008; Pendeville, 2008; Hogan, 2009; Geudens, 2010; Krueger, 2011	Wang, 2011	
<i>dli4</i>	ISVs	Leslie, 2007; Wang, 2011	Figure 4 3-I	Hogan, 2008; Pendeville, 2008; Hogan, 2009; Geudens, 2010; Krueger, 2011	Wang, 2011	
	CNS	Leslie, 2007	Figure 4 3-I	Leslie, 2007		
	NC				Figure 4 3-J	
	PND			Geudens, 2010		



Appendix Figure 8-1. Expression of Notch receptors in the zebrafish embryo around early stages of HSC development.

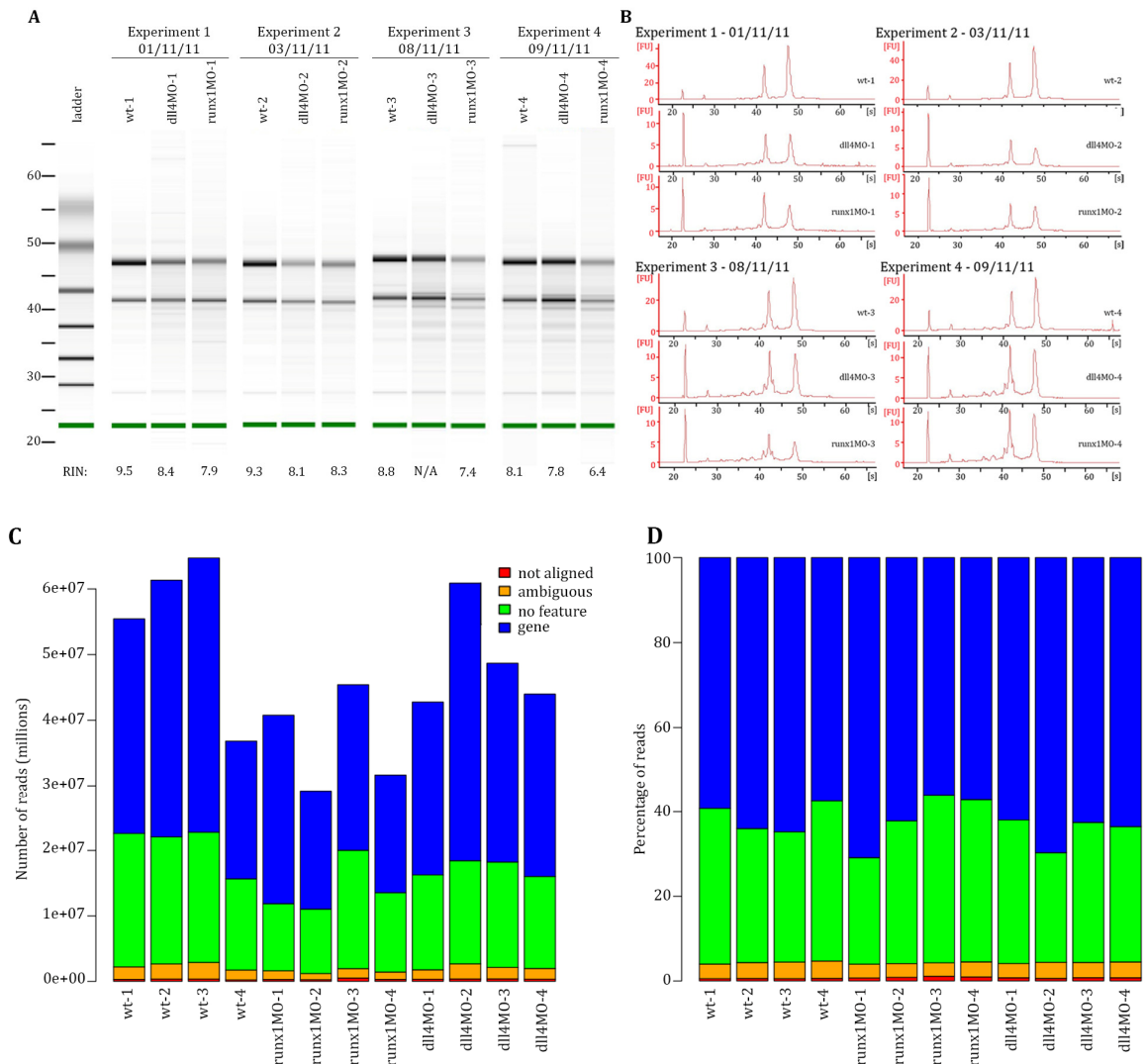
notch1a is expressed in the CNS, the somites and the TB at 26 hpf (A), at 36 hpf *notch1a* remains expressed in the CNS and is expressed in the NC and PBI (B). *notch1b* is expressed in the heart, DA and the ISVs, the CNS and the gut at 26 hpf (C); at 36 hpf, *notch1b* is expressed in the CNS, NC and PBI (D). *notch2* (*notch6*) is expressed in the ectoderm, the gut, the somites, the TB and parts of the CNS at 26 hpf (E). At 36 hpf, additional expression is gained in the NC and PBI (F). At 26 hpf *notch3* (*notch5*) is expressed in the DA and ISVs, the ectoderm, the pronephric duct, the TB and the CNS (G). At 36 hpf, expression in the DA and ISVs is lost and expression in the NC, PBI and lateral line is gained (H). Arrows indicate posterior tissues: DA (light green), ISVs (red), PBI (light blue), CNS (NT, yellow), somites (orange), NC (dark green), ectoderm (purple), pronephric duct (grey), TB (lilac), lateral line (brown) and gut (pink).



Appendix Figure 8-2. Homology analyses for vertebrate *Dll4* genes.

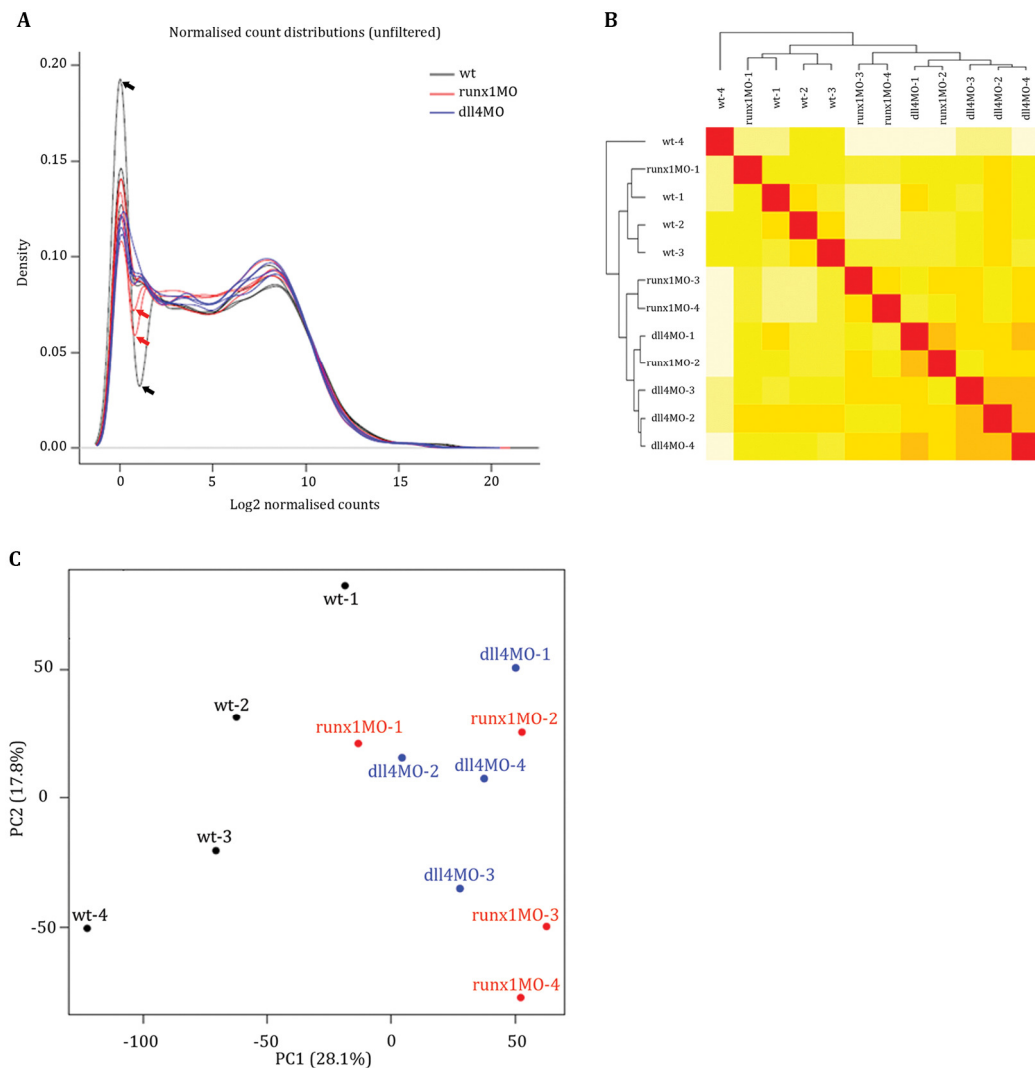
A. Gene tree and synteny analysis of *dll4* across selected vertebrate species: The gene tree for *dll4* mirrors the species phylogeny and does not include gene duplications; all nodes are speciation nodes (yellow circles). Neighboring genes are conserved resulting in a syntenic region, indicating that the zebrafish *dll4* is the true orthologue to the mouse, human chicken and xenopus *Dll4* gene. The gene tree (re-drawn) and synteny alignment were obtained from the Genomicus browser using *D. rerio* as the reference species (Osada et al., 1997), nodes were annotated by evaluation. The *dll4* locus is in the centre of the alignment. **B-C.** *Dll4* cross-species protein sequence comparisons: Alignment of the protein sequences of the *Dll4* proteins of human, mouse and zebrafish (Owen, 1846), showing conservation hotspots in the domains and the regions between them, as well as the C terminus of the *dll4* gene. Highlights in dark grey indicate amino acids conserved across all three species while lighter grey highlights indicate conserved residues across two species. Boxes show conserved domains as predicted by CDD (Paffett-Lugassy et al., 2007), asterisks indicate *dll4*MO1 (light green) and *dll4* MO4 (dark green) binding consensus (**B**). The percentage sequence identity table (values obtained from ClustalO (Palmblad et al., 2013)) shows conservation of protein sequences between any two selected species. Overall, the zebrafish *Dll4* protein is approximately 60% identical to all its orthologues. The highest sequence identity is observed between the human and mouse proteins (86%) (**C**).

8.2 Appendix to Chapter 4 - Establishing *dll4* targets



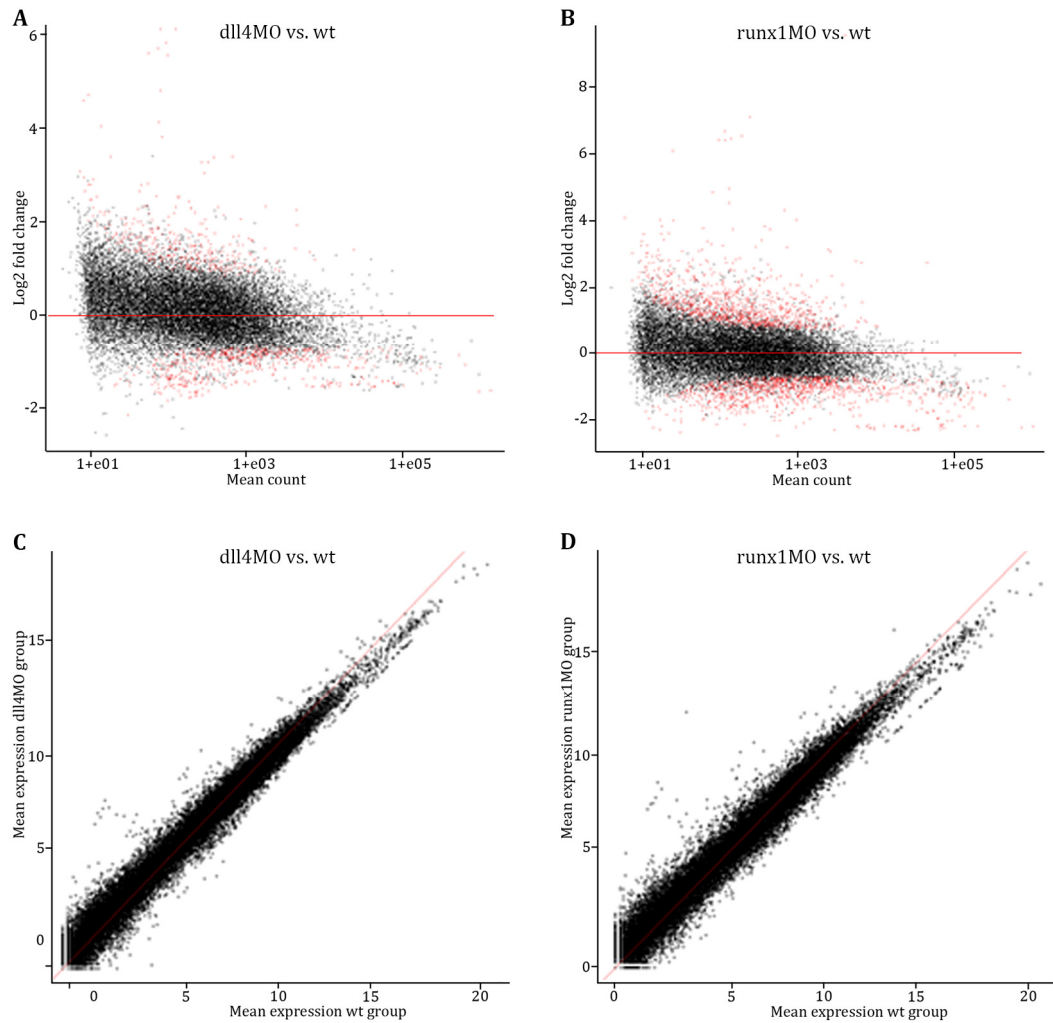
Appendix Figure 8-3. Quality of RNA submitted for sequencing and read assignment by category.

RNA quality was assessed using a bioanalyzer on samples submitted for RNA sequencing. **A.** Gel analysis of RNA samples shows minimal degradation. RIN values range between 6.4 and 9.5. **B.** Clear peaks are observed for all samples, indicating good RNA quality. Note the RIN value (N/A) for dll4MO sample 3: judging from the gel and peaks, RNA quality was good enough for sequencing and the absence of RIN value was likely due to mis-assignment of peaks by the software. **C.** Read assignment by category by number of reads: the majority of reads fall into genes, the second most common read category is “no feature” (reads mapping to the genome but outside of annotated genes) across all samples. **D.** When considering the same data as a percentage of overall reads for each sample, it becomes apparent that the assignment of reads to a given category is relatively uniform across samples.



Appendix Figure 8-4. Gene count and heatmap analyses on RNA-seq samples.

A. Normalised gene count distributions (unfiltered): individual lines represent the distribution of counts for all genes in a particular sample. Black arrows indicate sample wt-4 which follows a considerably different distribution to the other samples. Red arrows indicate samples *runx1MO-2* and *runx1MO-4* which diverge from the overall pattern to a lesser extent. This feature may be explained by their smaller library size. **B.** Heatmap analysis: the heatmap compares the similarity between samples in a pair-wise fashion. Samples clustering most closely are *dll4MO-1*, *dll4MO-2* and *dll4MO-3* while wt-4 seems to be distinct in its pattern as compared to all other samples. **C.** Principal Component Analysis (PCA) plots: PCA plots represent the variation between samples and the sources of variation in the data. Wt samples (black) cluster away from *runx1* and *dll4* morphants. Morphants do not fall into separate clusters.



Appendix Figure 8-5. Expression vs fold change plots.

A,/B Expression vs fold change plots where X axis shows expression (mean count) and y axis shows log2 fold change of *dll4MO* vs wt comparison (**A**) and *runx1MO* vs wt (**B**). Significantly differentially expressed genes are highlighted in red. In both comparisons, the vast majority is unchanged and most differentially expressed genes have a log2 fold change around 2. There are few extreme up-regulated genes in both comparisons, but no extreme down-regulated ones. The most extremely up-regulated genes is *U8 small nucleolar RNA* (ENSDARG00000082850) in *runx1MO*. **C/D** Mean expression in each group is shown by expression concordance. Data is not symmetrical around the red line indicating a systematic down-regulation compared to wt for highly expressed genes and a systematic up-regulation for weakly expressed genes as indicated by the deviation from the red line.

Appendix Table 8-3. Nanostring analysis.

Table of log2 fold-changes (log2 FC) and p-values of the nanostring probe set (probe) samples submitted for RNA-sequencing (4 biological replicates). ✓ in column “*DE in RNA-seq*” indicates that a gene was considered significantly differentially expressed in the RNA-seq DE analysis. Asterisks indicate genes considered significant ($p < 0.05$), and those with a $-1 < \log_2 \text{FC} > 1$ were considered likely biological changes (**bold**).

probe	dll4MO-vs-wt log2 FC	p-value	DE in RNA-seq	runx1MO-vs-wt log2 FC	p-value	DE in RNA-seq
actb1 (βactin1)	-0,35	0,554	✓	-0,01	0,805	✓
actb2 (βactin2)	-0,05	0,975	✓	0,17	0,556	✓
acvr11	-0,04	0,934		0,10	0,610	
alas2	0,26	0,506		0,44	0,186	
angpt1	-0,55	0,105	✓	-0,16	0,485	
angpt2	0,57	0,778		1,51	0,212	
aplnra	-0,35	0,492		-0,29	0,590	
baxa	1,05*	<0,001*	✓	1,92*	<0,001*	✓
bcl2	0,05	0,927		0,20	0,288	
bcl2l1	0,25	0,354		0,70	0,002*	
bmp2b	0,17	0,364		0,68	0,013*	
bmp4	0,15	0,462		0,83	0,015*	✓
bmpr2a	-0,13	0,555		0,11	0,841	
bmpr2b	0,11	0,847		0,20	0,410	
cbfa2t3	0,16	0,730		0,66	0,142	
cbfbeta	0,18	0,328		0,37	0,013*	
cdh17	-0,01	0,927		0,18	0,240	
cdh5	-0,06	0,682		-0,19	0,226	
cdkn1a (p21)	2,45*	<0,001*		3,88*	<0,001*	✓
cdkn1b (p27)	-0,14	0,347		0,38	0,031*	✓
cdkn1c (p57 kip2)	0,13	0,322		0,21	0,251	✓
cebp1	-0,32	0,521		-0,17	0,780	
chordin	-0,24	0,395		0,05	0,803	
cldn5a	0,02	0,918		0,06	0,661	
cldn5b	-0,50	0,043*	✓	-0,42	0,126	
cmyb	-0,19	0,538		0,23	0,406	
csf1ra	-0,34	0,100		-0,85	0,028*	
ccnd1 (cyclinD1)	0,64	0,014*		1,20*	0,000*	
dab2	-0,14	0,695		0,09	0,596	
dlc	0,48	0,077		0,41	0,012	
dld	0,09	0,876		-0,04	0,712	
dll4	-0,59	0,032*	✓	-0,07	0,585	✓
dnmt4	0,20	0,554		0,43	0,013*	
eef1a1a	0,25	0,278	✓	0,10	0,625	✓
efnb2a	-0,20	0,347		0,19	0,414	
eGFP	-1,54	0,486		-1,28	0,682	
eng	-0,48	0,665		-0,40	0,578	
ephb4a	-0,17	0,630		0,18	0,623	
etv2	0,04	0,850		0,15	0,433	
fli1a	-0,25	0,459		-0,30	0,353	
fli1b	-0,33	0,401		0,03	0,790	
flt4	0,06	0,831		0,13	0,468	
fzd2	-0,14	0,489	✓	0,02	0,859	✓
fzd7b	0,03	0,907		0,09	0,450	
gapdhs	-0,02	0,983		-0,14	0,508	
gata1	0,17	0,717		0,88	0,085	

probe	dll4MO-vs-wt log2 FC	p-value	DE in RNA-seq	runx1MO-vs-wt log2 FC	p-value	DE in RNA-seq
gata2a	0,01	0,991		0,49	0,244	
gata2b	-0,87	0,030		-0,19	0,697	
gfi1	0,05	0,744		0,17	0,478	
gfi1. 1	0,04	0,843		0,60	0,172	
gfi1b	0,10	0,896		0,95	0,102	
gli1	0,20	0,987		0,70	0,305	
gli2a	0,39	0,481		0,60	0,075	
hbbe1.1 (βe1 globin)	0,31	0,290		0,54	0,089	
hey1	0,27	0,242		0,74	<0,001*	
hey2 (grl)	-0,56	0,822		-0,67	0,741	
hhex	0,04	0,876		0,38	0,218	
id1	-0,13	0,524		0,25	0,255	
id2a	0,11	0,535		0,48	0,035*	
id2b	0,10	0,496		0,38	0,300	
id3	-0,03	0,821		0,22	0,641	
ikzf1 (ikaros)	-0,07	0,971		0,47	0,287	
itga2b (CD41)	-0,62	0,339		-0,23	0,867	
jag1a	-0,18	0,479		-0,66	0,083	✓
jag1b	0,16	0,460		0,51	0,037*	
jag2b	0,22	0,651		0,42	0,169	
kdr	-0,11	0,990		-0,06	0,962	
kdr1 (flk1)	-0,10	0,775		0,09	0,546	
klf2	-0,25	0,592		-0,21	0,677	✓
klf4	0,13	0,761		0,92	0,062*	
lcp1	-0,34	0,722		-0,16	0,933	
ldb2a	-0,32	0,075		0,00	0,995	
lmo2	0,01	0,885		0,45	0,230	
lmo4a	-0,03	0,836		0,24	0,051	
lmo4b	-0,18	0,252		0,02	0,839	
mflt1	-0,27	0,250		-0,11	0,285	
mpeg1	-0,44	0,282		-0,55	0,095	
mpx	-0,61	0,008*	✓	-1,06*	0,007*	✓
notch1a	-0,16	0,731		-0,14	0,780	
notch1b	0,27	0,811		0,33	0,537	
notch2	-0,09	0,837		0,22	0,666	
notch3	-0,32	0,657		-0,34	0,653	
nr2f2	0,46	0,166		0,80	0,004*	
pax2a	0,28	0,346		0,55	0,090	
pcna	-0,13	0,217	✓	-0,02	0,821	✓
perp	0,20	0,027*		0,43	0,004*	
plcg1	-0,21	0,638		0,06	0,806	
ptch2	0,17	0,849		0,44	0,365	
ptprc	0,17	0,441		0,43	0,023*	
rspo1	2,22*	0,004*	✓	3,74*	0,000*	✓
runx1	0,49	0,340		0,90	0,033	
tal1 (scl)	0,01	0,923		0,47	0,137	
sdha	0,06	0,769		0,19	0,401	
sflt1	-0,30	0,331		-0,29	0,272	
shha	0,31	0,142		0,54	0,109	
shhb	0,62	0,114		0,94	0,007*	
smad1	0,21	0,722		0,65	0,082	
smad2	-0,23	0,428		-0,10	0,674	
smad3a	0,02	0,914		-0,11	0,603	
smad3b	0,01	0,964		0,06	0,963	✓
smad4	-0,12	0,726	✓	0,41	0,255	✓

probe	dll4MO-vs-wt			runx1MO-vs-wt		
	log2 FC	p-value	DE in RNA-seq	log2 FC	p-value	DE in RNA-seq
smad5	-0,07	0,891	✓	0,08	0,728	✓
snai1a (snail)	0,17	0,600		0,74	0,030*	
snai1b (slug)	0,10	0,930		1,00	0,086	
spi1b (pu1)	0,13	0,513	✓	0,12	0,524	✓
taz	0,34	0,027*	✓	0,92	0,004*	✓
tbx20	0,02	0,896		0,17	0,681	
tel1	-0,12	0,530		0,37	0,086	
tgfb1a	0,16	0,478		0,20	0,263	
tgfb1b	0,19	0,246		0,13	0,402	
tgfb2	0,15	0,305		0,56	0,028*	
tgfb3	-0,02	0,802		0,24	0,247	
tgfbr1a	-0,11	0,692		-0,06	0,837	
tgfbr1b	-0,01	0,911		-0,02	0,911	
tgfbr2	0,01	0,979		0,11	0,142	
tgfbr3	0,28	0,557		0,35	0,384	
tie1	-0,17	0,328		-0,13	0,359	
tie2	-0,20	0,526		-0,18	0,554	
tif1gamma	-0,05	0,831		0,29	0,399	
tp53	2,35*	<0,001*	✓	3,38*	<0,001*	✓
twist1a	-0,05	0,774		0,07	0,737	✓
twist1b	0,01	0,884		0,15	0,474	
twist2	0,00	0,851		-0,09	0,268	
ubc	-0,28	0,261	✓	-0,04	0,886	✓
urod	0,30	0,330		0,72	0,011*	
vegfaa	0,03	0,850		0,45	0,105	
vegfab	0,11	0,508	✓	0,28	0,071	✓
vegfc	0,00	0,969		0,29	0,029*	
wnt16	0,08	0,710		0,10	0,630	
wnt2bb	-0,06	0,681		0,03	0,940	
zeb2a	0,56	0,068		0,66	0,037	
zeb2b	0,52	0,321		0,69	0,286	

Appendix Table 8-4. Significantly differentially expressed genes in dll4MO-vs-wt but not runx1MO-vs-wt (*dll4+*/*runx1-*).

This table reflects the differential gene expression (DE) analysis carried out between the *dll4*MO and wt RNA-seq datasets; genes shown were not differentially expressed in the *runx1*MO-vs-wt dataset. Only significant genes (*p*_{adj}<0.05) are listed with their respective log₂ fold changes in alphabetical order. Column “screen class” indicates if a gene has been included in the expression screen and whether it was classified as a potential transcription factor/DNA binding (TF), signalling molecule (SIG) or as a previously uncharacterised gene (UG). Some genes classed as UG have since become annotated with a gene name which has been used here; however original names are given in parentheses, where applicable. Results of the expression profiling are represented by tissue of expression in Figure 4-10, (p. 144). The column “CAGE” indicates the CAGE fold change values for those genes for which comparable values could be found. Values underlined indicate a fold change in the opposite direction as compared to the RNA-seq dataset obtained here; several CAGE values per gene represent different splice isoforms (CAGE values kindly provided by Prof. Adrian Harris). Genes were coloured according to the following priorities: TF (blue), SIG (orange), UG (pink), OTHER (purple), CAGE (green) leaving all other genes (grey).

Appendix Table 8-4. Significantly differentially expressed genes in <i>dll4+</i>/<i>runx1-</i>						
gene symbol	log ₂ Fold Change	P _{adj}	screen class	CAGE	gene name	ENSEMBL ID
<i>aar2</i> (<i>zgc:162293</i>)	-0,87	0,0461	UG(20)	<u>0.74 /</u> <u>0.74</u>	AAR2 splicing factor homolog (<i>S. cerevisiae</i>)	ENSDARG00000018658
<i>AARSD1</i> (2 of 2)	0,91	0,0223			alanyl-tRNA synthetase domain containing 1	ENSDARG00000079462
<i>abdh17aa</i> (<i>zgc:162293</i>)	-1,09	0,0383	UG(29)		abhydrolase domain containing 17 Aa	ENSDARG000000091433
<i>ACTC1</i>	-0,93	0,0208			<i>zgc:86709</i>	ENSDARG00000057911
<i>actc1a</i>	-0,95	0,0260			actin, alpha, cardiac muscle 1a	ENSDARG00000042535
<i>adams17</i>	-1,00	0,0208			ADAMTS-like 7	ENSDARG00000055439
<i>ADARB2</i>	2,09	0,0000	OTHER		adenosine deaminase, RNA-specific, B2	ENSDARG00000071823
<i>adrm1b</i>	-0,97	0,0138		0,6	adhesion regulating molecule 1b	ENSDARG00000058560
<i>agr2</i>	-1,36	0,0019			anterior gradient homolog 2 (<i>Xenopus laevis</i>)	ENSDARG00000070480
<i>AL935186.4</i>	-1,06	0,0219			novel gene (ncRNA)	ENSDARG000000085168
<i>and4</i>	-1,29	0,0449			actinodin4	ENSDARG000000090729
<i>angpt1</i>	-1,05	0,0139	SIG		angiopoietin 1	ENSDARG00000030364
<i>ankrd1b</i>	1,83	0,0397		<u>0.53 /</u> <u>1.66</u>	ankyrin repeat domain 1b (cardiac muscle)	ENSDARG000000076192
<i>apex2</i>	1,01	0,0495	TF	<u>0.15 /</u> <u>1.94</u>	APEX nuclease (apurinic/aprimidinic endonuclease) 2	ENSDARG00000008472
<i>arf1l</i>	-1,11	0,0375			ADP-ribosylation factor 1 like	ENSDARG00000016393
<i>arhgap11a</i>	0,92	0,0451			Rho GTPase activating protein 11A	ENSDARG00000015403
<i>arhgap22</i>	0,93	0,0458		<u>0.02 /</u> <u>0.15</u>	Rho GTPase activating protein 22	ENSDARG00000021255
<i>ARL9</i>	-1,05	0,0461			ADP-ribosylation factor-like 9	ENSDARG00000071881
<i>ASPSCR1</i> (2 of 2)	-1,01	0,0296			alveolar soft part sarcoma chromosome region, candidate 1	ENSDARG000000089727
<i>atf4b1</i>	-1,28	0,0074	TF	0,34	activating transcription factor 4b1 (tax-responsive enhancer element B67)	ENSDARG00000039515
<i>ATF6B</i>	-0,82	0,0425	TF		activating transcription factor 6 beta	ENSDARG00000069883
<i>atpif1b</i> (<i>zgc:153321</i>)	0,86	0,0414	UG(17)		ATPase inhibitory factor 1b	ENSDARG00000092357
<i>bag6</i>	-0,88	0,0287			BCL2-associated athanogene 6	ENSDARG00000075892
<i>blmh</i>	-1,15	0,0189			bleomycin hydrolase	ENSDARG00000025977
<i>bmp1a</i>	-0,90	0,0230	SIG (n. d.)		bone morphogenetic protein 1a	ENSDARG00000028071
<i>bop1</i>	-0,92	0,0213		0,82	block of proliferation 1	ENSDARG00000063627
<i>btbd10b</i>	-0,82	0,0492	TF	0,71	BTB (POZ) domain containing 10b	ENSDARG00000020252
<i>btg2</i>	-0,93	0,0196	SIG	0,19	B-cell translocation gene 2	ENSDARG00000020298
<i>BX936343.1</i>	0,76	0,0429	UG(18)		Uncharacterised protein	ENSDARG00000054645

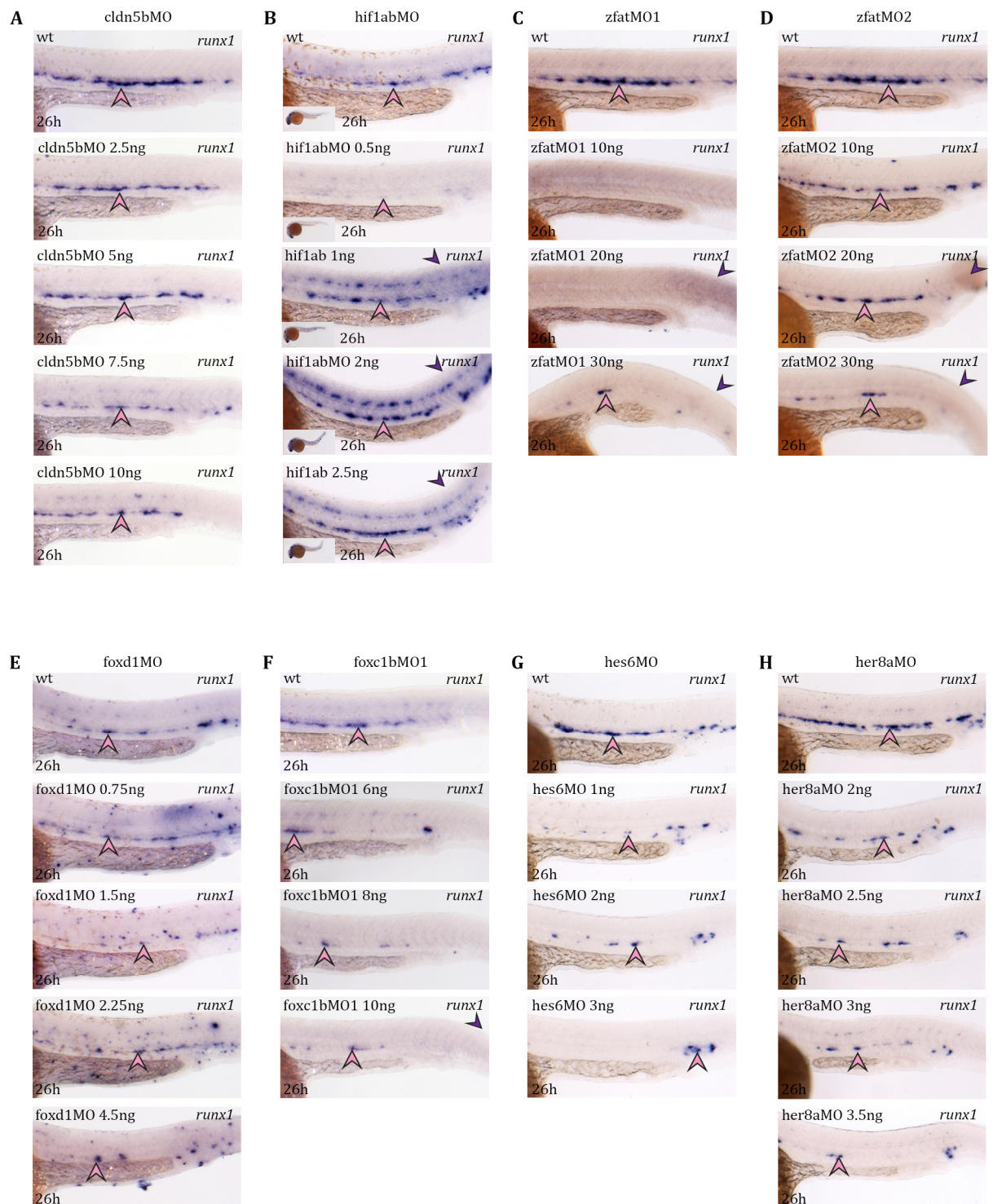
Appendix Table 8-4. Significantly differentially expressed genes in dll4+/runx1-						
gene symbol	log2Fold Change	Padj	screen class	CAGE	gene name	ENSEMBL ID
<i>BX957362. 1</i>	-1,20	0,0022	UG(32)	0,53	Uncharacterised protein	ENSDARG00000052074
<i>c1qtnf9</i>	-1,23	0,0349			<i>C1q and tumor necrosis factor related protein 9</i>	ENSDARG00000058318
<i>CABZ01069239. 1</i>	-0,85	0,0447	UG(19)		novel gene	ENSDARG00000090031
<i>caprin1a</i>	-0,99	0,0141		1,44	cell cycle associated protein 1a	ENSDARG00000009346
<i>ccdc85ca</i>	0,97	0,0413	TF		coiled-coil domain containing 85C, a	ENSDARG00000005232
<i>ccnk</i>	-0,96	0,0287		1,52	cyclin K	ENSDARG00000034146
<i>cct4</i>	-1,06	0,0342	SIG	0,59	chaperonin containing TCP1, subunit 4 (delta)	ENSDARG00000013475
<i>CDC37</i>	-0,90	0,0267			cell division cycle 37 homolog (<i>S. cerevisiae</i>)	ENSDARG00000040126
<i>cdkal1</i>	-0,90	0,0357		0,74	CDK5 regulatory subunit associated protein 1-like 1	ENSDARG00000022122
<i>CHCHD4</i>	1,34	0,0259	TF	0,07	coiled-coil-helix-coiled-coil-helix domain containing 4	ENSDARG00000040707
<i>cherp</i>	-1,24	0,0191		2,56	calcium homeostasis endoplasmic reticulum protein	ENSDARG00000021812
<i>chia. 6</i>	1,52	0,0413			chitinase, acidic. 6	ENSDARG00000093193
<i>cldn10b (zgc:110625)</i>	-0,99	0,0413	UG(26)		claudin 10b	ENSDARG00000005377
<i>cldn5b</i>	-0,89	0,0378	SIG		claudin 5b	ENSDARG00000014059
<i>CLPX (3 of 3)</i>	0,98	0,0163		1,69	<i>ClpX</i> caseinolytic peptidase X homolog (<i>E. coli</i>)	ENSDARG00000067796
<i>CREBZF</i>	1,34	0,0045	TF	0,74	CREB/ATF bZIP transcription factor	ENSDARG00000089787
<i>crygn2</i>	-1,98	0,0218	OTHER		crystallin, gamma N2	ENSDARG00000030411
<i>CU457819. 2</i>	-1,19	0,0303			Histone H2A	ENSDARG00000086552
<i>CU457819. 3</i>	-1,23	0,0266	UG(33)	0,39	Uncharacterised protein	ENSDARG00000090426
<i>CU469531. 2</i>	-1,15	0,0139		0,52	ras-related protein Rab-11B	ENSDARG00000090086
<i>cyr61l1</i>	1,14	0,0379		0,75	cysteine-rich, angiogenic inducer 61 like 1	ENSDARG00000035422
<i>dctn4</i>	1,12	0,0210			dynactin 4	ENSDARG00000023988
<i>dhrs3b</i>	-0,87	0,0381	SIG		dehydrogenase/reductase (SDR family) member 3b	ENSDARG00000044803
<i>dhx15</i>	-0,90	0,0302	SIG	0,29	DEAH (Asp-Glu-Ala-His) box polypeptide 15	ENSDARG00000015392
<i>DSCR3</i>	-0,87	0,0463		0. 57 / 8. 20 / 0. 34	Down syndrome critical region gene 3	ENSDARG00000075650
<i>echdc1</i>	-1,11	0,0303		0,22	enoyl Coenzyme A hydratase domain containing 1	ENSDARG00000005959
<i>eif3s6ip</i>	-0,95	0,0229			eukaryotic translation initiation factor 3, subunit 6 interacting protein	ENSDARG00000006729
<i>eif4a3</i>	-0,85	0,0459	SIG		eukaryotic translation initiation factor 4A, isoform 3	ENSDARG00000014472
<i>ELMO3</i>	0,84	0,0401			engulfment and cell motility 3	ENSDARG00000042126
<i>ENOSF1</i>	1,30	0,0305			enolase superfamily member 1	ENSDARG00000038359
<i>entpd4</i>	-0,94	0,0287			ectonucleoside triphosphate diphosphohydrolase 4	ENSDARG00000038422
<i>FAM129A (2 of 2)</i>	0,99	0,0284			family with sequence similarity 129, member A	ENSDARG00000042055
<i>FAM161B</i>	1,64	0,0417			family with sequence similarity 161, member B	ENSDARG00000089467
<i>fam45a</i>	1,23	0,0303			family with sequence similarity 45, member A	ENSDARG00000062965
<i>FBN3 (1 of 2)</i>	-1,20	0,0485			fibrillin 3	ENSDARG00000088660
<i>fhad (si:dkey-24f17. 5)</i>	2,07	0,0295	UG(03)		forkhead-associated (FHA) phosphopeptide binding domain 1	ENSDARG00000056723
<i>fip1l1</i>	-0,84	0,0391	SIG	1,43	<i>FIP1</i> like 1 (<i>S. cerevisiae</i>)	ENSDARG00000070431
<i>foxc1b</i>	-1,05	0,0130	TF		forkhead box C1b	ENSDARG00000055398
<i>foxd1</i>	-1,36	0,0022	TF		forkhead box D1	ENSDARG00000029179
<i>foxd1 (2)</i>	-1,53	0,0349	UG(43)		forkhead box D1 (2)	ENSDARG00000079699
<i>FP015790. 3</i>	1,57	0,0356	UG(08)		Uncharacterised protein	ENSDARG00000089541
<i>galnt10</i>	-1,05	0,0184			UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10 (GalNAc-T10)	ENSDARG00000042778
<i>gorasp2</i>	-1,05	0,0126		0,94	golgi reassembly stacking protein 2	ENSDARG00000015126
<i>grb2</i>	-0,88	0,0486	SIG	0,45	growth factor receptor-bound protein 2	ENSDARG00000038059
<i>h2afy-13 (zgc:114037)</i>	-1,42	0,0383	UG(38)		H2A histone family, member Y	ENSDARG00000087235
<i>h2afy-8 (zgc:173652)</i>	-1,67	0,0180	UG(46)		H2A histone family, member Y	ENSDARG00000070280
<i>hif1ab</i>	-0,81	0,0396	TF	0. 20 / 2. 24 / 1. 74	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) b	ENSDARG00000034293
<i>hist1h4a (wu:fe37d09)</i>	-1,17	0,0413	UG(31)		histone cluster1, H4a	ENSDARG000000091446
<i>HIST2H4B (5 of 7)</i>	-1,32	0,0022			zgc:165555	ENSDARG00000090893
<i>HIST2H4B (6 of 7)</i>	-1,22	0,0499			zgc:165555	ENSDARG00000087472
<i>HIVEP2 (1 of 2)</i>	0,97	0,0316			human immunodeficiency virus type 1 enhancer binding protein 2	ENSDARG00000039987
<i>hlcs</i>	-1,05	0,0412	SIG	3,47	holocarboxylase synthetase (biotin-(propionyl-Coenzyme A-carboxylase (ATP-hydrolysing) ligase)	ENSDARG00000039934
<i>hm:zewp0073</i>	-0,89	0,0368	UG(21)		hm:zewp0073	ENSDARG00000076126
<i>hnrnpa0l</i>	-0,90	0,0343			heterogeneous nuclear ribonucleoprotein A0, like	ENSDARG00000036161

Appendix Table 8-4. Significantly differentially expressed genes in <i>dll4+ /runx1-</i>						
gene symbol	log2Fold Change	Padj	screen class	CAGE	gene name	ENSEMBL ID
<i>hnnpd</i>	0,84	0,0316	SIG		heterogeneous nuclear ribonucleoprotein D	ENSARG00000059246
<i>jam3b</i>	0,83	0,0442	SIG	0,06	junctional adhesion molecule 3b	ENSARG00000061794
<i>kpn1</i>	-1,11	0,0040		0,25	karyopherin (importin) beta 1	ENSARG00000008680
<i>lig4</i>	1,27	0,0303	TF		ligase IV, DNA, ATP-dependent	ENSARG000000060620
<i>LRIG3</i>	-0,85	0,0452	TF		leucine-rich repeats and immunoglobulin-like domains 3	ENSARG00000079363
<i>lrp6</i>	-0,88	0,0259	SIG	0,84	low-density lipoprotein receptor-related protein 6	ENSARG00000076053
<i>lrrc8c</i>	0,95	0,0260	TF	0.15 / 0.88	leucine rich repeat containing 8 family, member C	ENSARG00000059718
<i>mafbb</i>	-1,01	0,0166	TF		v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B, duplicate b	ENSARG00000070542
<i>maff</i>	1,18	0,0114	TF	1.24 / 0.75	v-maf musculoaponeurotic fibrosarcoma oncogene homolog f (avian)	ENSARG00000028957
<i>mbip</i>	-0,97	0,0448			MAP3K12 binding inhibitory protein 1	ENSARG00000019364
<i>med14</i>	-0,82	0,0452			mediator complex subunit 14	ENSARG00000009953
<i>med24</i>	-1,11	0,0041			mediator complex subunit 24	ENSARG00000032459
<i>MEPCE (2 of 2)</i>	-0,97	0,0200			methylphosphate capping enzyme	ENSARG00000035771
<i>metrn</i>	0,92	0,0362			meteorin, glial cell differentiation regulator	ENSARG00000030367
<i>mll5</i>	-0,84	0,0438		12.76 / 1.53 / 0.49 / 1.02 / 0.75	myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog, Drosophila)	ENSARG00000007523
<i>mplkip (C2H7orf11)</i>	-0,93	0,0412	UG(23)	1,05	M-phase specificPLK1 interacting protein	ENSARG00000078986
<i>mrpl36</i>	-1,18	0,0237		0,75	mitochondrial ribosomal protein L36	ENSARG00000070607
<i>mrpl39</i>	-0,94	0,0195			mitochondrial ribosomal protein L39	ENSARG00000041570
<i>mta3</i>	-1,09	0,0056	TF	2.73 / 0.20 / 0.15 / 0.15 / 0.74	metastasis associated family, member 3	ENSARG000000054903
<i>mtmr12</i>	1,28	0,0172		0.18 / 0.05 / 2.24 / 0.74	myotubularin related protein 12	ENSARG00000059817
<i>muc5ac (CR854881.2)</i>	-0,96	0,0296	UG(24)		mucin 5AC, oligomeric mucus/gel-forming	ENSARG00000070331
<i>MUSTN1 (2 of 2)</i>	1,18	0,0006			musculoskeletal, embryonic nuclear protein 1	ENSARG000000089086
<i>mxra8a</i>	1,07	0,0280			matrix-remodelling associated 8a	ENSARG00000062222
<i>MYO5C</i>	0,89	0,0333			myosin VC	ENSARG00000013782
<i>NAPA (2 of 2)</i>	1,06	0,0403		1,02	N-ethylmaleimide-sensitive factor attachment protein, alpha	ENSARG000000067605
<i>necap2</i>	-0,86	0,0485			zgc:110468	ENSARG00000046002
<i>nkx3.2</i>	-1,09	0,0333	TF		NK3 homeobox 2	ENSARG00000037639
<i>novel gene</i>	1,03	0,0029	UG(16)	2,53	novel gene	ENSARG00000044092
<i>nox1b</i>	1,43	0,0018			NADPH oxidase organizer 1b	ENSARG00000056374
<i>nr1d2b</i>	0,96	0,0302	TF		nuclear receptor subfamily 1, group D, member 2b	ENSARG00000009594
<i>nsfl1c</i>	-0,90	0,0459			NSFL1 (p97) cofactor (p47)	ENSARG00000087640
<i>NSFL1C (1 of 2)</i>	-0,85	0,0409			NSFL1 (p97) cofactor (p47)	ENSARG00000006375
<i>nsmaf</i>	1,03	0,0208	SIG	8.19 / 0.43 / 0.14 / 0.72 / 0.31	neutral sphingomyelinase (N-SMase) activation associated factor	ENSARG00000051920
<i>ntpcr</i>	0,89	0,0362			nucleoside-triphosphatase, cancer-related	ENSARG00000021853
<i>nup50</i>	-0,93	0,0305		2,24	nucleoporin 50	ENSARG00000017454
<i>oaz2a</i>	-0,92	0,0323		0,68	ornithine decarboxylase antizyme 2a	ENSARG00000016476
<i>osr2</i>	-1,07	0,0114			odd-skipped related 2 (Drosophila)	ENSARG00000038006
<i>pde8a</i>	-0,98	0,0112	SIG		phosphodiesterase 8A	ENSARG000000088905
<i>PIEZ02 (1 of 5)</i>	-0,88	0,0476			piezo-type mechanosensitive ion channel component 2	ENSARG000000088302
<i>pl10</i>	-0,82	0,0459	SIG	0,25	pl10	ENSARG00000005774
<i>plcd4b</i>	0,95	0,0393	SIG		phospholipase C, delta 4b	ENSARG00000040684
<i>plod3</i>	-0,88	0,0421	SIG	0.97 / 0.71	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	ENSARG00000076317
<i>pom121</i>	-0,96	0,0158		0,11	POM121 membrane glycoprotein (rat)	ENSARG00000055113
<i>ppp1caa</i>	-0,95	0,0451		0.16 / 0.63	protein phosphatase 1, catalytic subunit, alpha isoform a	ENSARG00000003486
<i>ppp1r3cb</i>	0,85	0,0450	SIG	5,71	protein phosphatase 1, regulatory (inhibitor) subunit 3Cb	ENSARG00000014554
<i>ppp4cb</i>	-0,95	0,0289		0.25 / 0.75	protein phosphatase 4 (formerly X), catalytic subunit b	ENSARG00000076439
<i>prpf39</i>	-0,87	0,0421		1,03	PRP39 pre-mRNA processing factor 39 homolog (yeast)	ENSARG00000042080
<i>PRRC2B</i>	-1,03	0,0412	TF		proline-rich coiled-coil 2B	ENSARG00000079639
<i>psmb2</i>	-0,93	0,0440		0,63	proteasome (prosome, macropain) subunit, beta type, 2	ENSARG00000031511

Appendix Table 8-4. Significantly differentially expressed genes in dll4+/runx1-						
gene symbol	log2Fold Change	Padj	screen class	CAGE	gene name	ENSEMBL ID
PSMD5	-1,00	0,0211	SIG	8,2	proteasome (prosome, macropain) 26S subunit, non-ATPase, 5	ENSXDARG00000059046
psmg1	-0,96	0,0372			proteasome (prosome, macropain) assembly chaperone 1	ENSXDARG00000040620
pth1b	2,13	0,0059	SIG		parathyroid hormone 1b	ENSXDARG00000091961
PTPLA	0,86	0,0279			protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	ENSXDARG00000022633
rab24	1,15	0,0189			RAB24, member RAS oncogene family	ENSXDARG00000054032
rab3ip	-0,88	0,0467		2,73	RAB3A interacting protein (rabin3)	ENSXDARG00000010073
RALGPS1 (1 of 2)	-1,05	0,0169	TF		Ral GEF with PH domain and SH3 binding motif 1	ENSXDARG00000089443
rassf8 (si:ch211-261n11.3)	3,97	0,0001	UG(01)	0.04 / 0.25	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	ENSXDARG00000090577
rgs6	1,00	0,0176	SIG		regulator of G-protein signalling 6	ENSXDARG00000015627
rnps1	-0,88	0,0380	SIG		RNA binding protein S1, serine-rich domain	ENSXDARG00000015853
rrad	1,39	0,0173			Ras-related associated with diabetes	ENSXDARG00000052011
rragc	0,87	0,0387			Ras-related GTP binding C	ENSXDARG00000069829
rufy3	1,02	0,0287		3,73	RUN and FYVE domain containing 3	ENSXDARG00000029692
scdb	1,14	0,0333		0,25	stearoyl-CoA desaturase b	ENSXDARG00000030265
sdf4	0,87	0,0438			stromal cell derived factor 4	ENSXDARG00000070682
sels	0,78	0,0391			selenoprotein S	ENSXDARG00000070097
SEMA4B (1 of 2)	1,02	0,0229	SIG	5.21 / 1.72	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4B	ENSXDARG00000074414
SF3B2	-0,88	0,0350	SIG	0,45	splicing factor 3b, subunit 2, 145kDa	ENSXDARG00000018049
sft2d3	-1,28	0,0014			SFT2 domain containing 3	ENSXDARG00000070284
sgk2b	1,06	0,0147			serum/glucocorticoid regulated kinase 2b	ENSXDARG00000006598
SH3BP5L (1 of 2)	1,04	0,0303			SH3-binding domain protein 5-like	ENSXDARG00000007136
si:ch211-117m20.5	1,42	0,0002	UG(12)		si:ch211-117m20.5	ENSXDARG00000009196
si:ch211-125e6.8	1,12	0,0473	UG(15)		si:ch211-125e6.8	ENSXDARG000000094518
si:ch211-278f9.2	1,28	0,0201	UG(14)		si:ch211-278f9.2	ENSXDARG000000093624
si:dkey-15h8.1	1,69	0,0164	UG(06)		si:dkey-15h8.1	ENSXDARG000000095009
si:dkey-15i15.2	2,12	0,0465	UG(02)		si:dkey-15i15.2	ENSXDARG000000095084
si:dkey-238m4.1	1,56	0,0115			si:dkey-238m4.1	ENSXDARG000000092797
si:dkey-6f10.4	1,98	0,0004	UG(04)		si:dkey-6f10.4	ENSXDARG000000092390
si:dkey-7f3.9	1,43	0,0355			si:dkey-7f3.9	ENSXDARG000000079008
si:dkeyp-13d11.2	1,49	0,0299	UG(10)		si:dkeyp-13d11.2	ENSXDARG000000077519
si:dkeyp-51b9.3	1,78	0,0485	UG(05) n. d.	0,05	si:dkeyp-51b9.3	ENSXDARG000000092379
slc27a2 (zgc:158482)	-0,92	0,0292	UG(22)		solute carrier family 27 (fatty acid transporter), member 2	ENSXDARG000000067520
slc30a7	-1,08	0,0265		0.72 / 1.04	solute carrier family 30 (zinc transporter), member 7	ENSXDARG00000019998
SLC5A7 (2 of 2)	1,47	0,0143			solute carrier family 5 (choline transporter), member 7	ENSXDARG000000074860
smarcb1b	-0,96	0,0147	SIG	0,32	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1b	ENSXDARG00000011594
sncgb	0,91	0,0404		12,67	synuclein, gamma b (breast cancer-specific protein 1)	ENSXDARG000000076081
socs9	-1,12	0,0126	SIG	0.08 / 0.68	suppressor of cytokine signalling 9	ENSXDARG000000074493
soul3	-0,97	0,0167		0,15	zgc:153411	ENSXDARG000000063452
sp9	-0,92	0,0337	TF		sp9 transcription factor	ENSXDARG00000010462
SPATA7	1,12	0,0351			spermatogenesis associated 7	ENSXDARG000000075898
SPATS2 (1 of 2)	-1,09	0,0317		0.39 / 1.34	spermatogenesis associated, serine-rich 2	ENSXDARG000000067969
SRP_euk_arch	-1,25	0,0070			Eukaryotic type signal recognition particle RNA	ENSXDARG000000087732
SRP_euk_arch	-1,64	0,0107			Eukaryotic type signal recognition particle RNA	ENSXDARG000000087015
SRP_euk_arch	-1,65	0,0425			Eukaryotic type signal recognition particle RNA	ENSXDARG000000088785
SRP_euk_arch	-1,69	0,0293			Eukaryotic type signal recognition particle RNA	ENSXDARG000000091337
SRP_euk_arch	-1,71	0,0499			Eukaryotic type signal recognition particle RNA	ENSXDARG000000089620
SRP_euk_arch	-1,26	0,0280			Eukaryotic type signal recognition particle RNA	ENSXDARG000000088445
SRP_euk_arch	-1,51	0,0308			Eukaryotic type signal recognition particle RNA	ENSXDARG000000087484
SRP_euk_arch	-2,29	0,0136			Eukaryotic type signal recognition particle RNA	ENSXDARG000000087891
SRP_euk_arch	-1,43	0,0468			Eukaryotic type signal recognition particle RNA	ENSXDARG000000087774
srr (CH73-26304.4)	1,63	0,0470	UG(07)	1,34	serine racemase	ENSXDARG000000096045
SRSF2 (2 of 2)	-1,02	0,0323	SIG		serine/arginine-rich splicing factor 2	ENSXDARG00000010906
ssu72	-1,16	0,0245		3.73 / 0.25	SSU72 RNA polymerase II CTD phosphatase homolog (S. cerevisiae)	ENSXDARG00000031216
stau1	-0,84	0,0438			stau1, RNA binding protein, homolog 1 (Drosophila)	ENSXDARG000000044182
stk25b	-1,03	0,0147		0,75	serine/threonine kinase 25b	ENSXDARG00000039022
stmn1b	0,90	0,0233	SIG	0,67	stathmin 1b	ENSXDARG00000033655

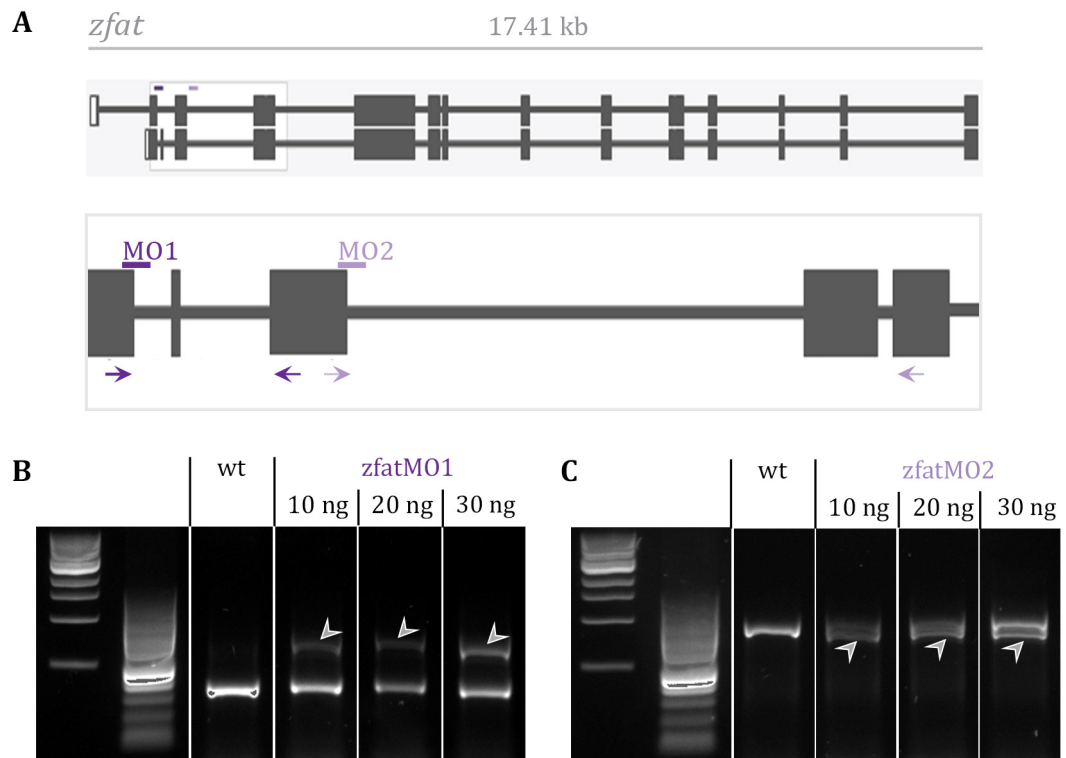
Appendix Table 8-4. Significantly differentially expressed genes in <i>dll4+</i> / <i>runx1</i> -						
gene symbol	log2Fold Change	Padj	screen class	CAGE	gene name	ENSEMBL ID
<i>stx11b.1</i>	0,93	0,0300		0,75	<i>syntaxin 11b.1</i>	ENSDARG00000029290
<i>SYNGR2 (2 of 3)</i>	-0,99	0,0337		1.74 / 0.75	<i>synaptogyrin 2</i>	ENSDARG00000030752
<i>syngr2b</i>	-0,89	0,0431		1.75 / 0.75	<i>synaptogyrin 2b</i>	ENSDARG000000086225
<i>syt13</i>	1,02	0,0340			<i>synaptotagmin XIII</i>	ENSDARG00000012306
<i>tbck</i>	-1,12	0,0073			<i>TBC1 domain containing kinase</i>	ENSDARG00000013667
<i>tecrb</i>	-0,93	0,0402			<i>trans-2,3-enoyl-CoA reductase b</i>	ENSDARG00000025904
<i>tfdp1b</i>	-0,90	0,0404	TF	0,55	<i>transcription factor Dp-1, b</i>	ENSDARG00000016304
<i>tfg</i>	-0,87	0,0382		3.13 / 0.75	<i>trk-fused gene</i>	ENSDARG00000003641
<i>thoc4</i>	-0,89	0,0303			<i>THO complex 4</i>	ENSDARG00000077732
<i>THRAP3 (2 of 3)</i>	0,88	0,0299		0,92	<i>thyroid hormone receptor associated protein 3</i>	ENSDARG00000040095
<i>tiprl</i>	-0,85	0,0492			<i>TIP41, TOR signalling pathway regulator-like (S. cerevisiae)</i>	ENSDARG00000032353
<i>tjp2b</i>	-0,89	0,0435	SIG		<i>tight junction protein 2b (zona occludens 2)</i>	ENSDARG00000023443
<i>tmco7</i>	-1,02	0,0198			<i>transmembrane and coiled-coil domains 7</i>	ENSDARG00000034001
<i>tnni1d (CU137645.1)</i>	1,29	0,0172	UG(13)		<i>troponin I skeletal, slow d</i>	ENSDARG00000016728
<i>TPRA1</i>	-0,96	0,0413			<i>transmembrane protein, adipocyte associated 1</i>	ENSDARG00000054930
<i>ttc22</i>	1,17	0,0485			<i>tetratricopeptide repeat domain 22</i>	ENSDARG00000077836
<i>txlnbb</i>	1,09	0,0017			<i>taxilin beta b</i>	ENSDARG00000076241
<i>ubl7</i>	-1,08	0,0349		1,29	<i>ubiquitin-like 7</i>	ENSDARG00000040286
<i>UFL1</i>	-0,90	0,0383			<i>UFM1-specific ligase 1</i>	ENSDARG00000007496
<i>vat1</i>	-0,77	0,0334		0,59	<i>vesicle amine transport protein 1 homolog (T. californica)</i>	ENSDARG00000056481
<i>yipf1</i>	0,94	0,0417	TF	0,15	<i>Yip1 domain family, member 1</i>	ENSDARG00000014474
<i>zc3h18</i>	-0,82	0,0450	TF		<i>zinc finger CCCH-type containing 18</i>	ENSDARG00000062506
<i>ZFAND3</i>	-0,86	0,0323	TF	0.28 / 0.15	<i>zinc finger, AN1-type domain 3</i>	ENSDARG00000052833
<i>ZFAT</i>	1,11	0,0267	TF		<i>zinc finger and AT hook domain containing</i>	ENSDARG00000077987
<i>ZFP36</i>	-0,88	0,0323	TF	0.06 / 0.51	<i>zinc finger protein 36, C3H type, homolog (mouse)</i>	ENSDARG00000044395
<i>zgc:110340(C5H8orf4)</i>	-1,09	0,0431	UG(28)		<i>chromosome 8 open reading frame 4</i>	ENSDARG00000079497
<i>zgc:110425</i>	-1,50	0,0076	UG(41)	0,35	<i>zgc:110425</i>	ENSDARG00000058479
<i>zgc:162816</i>	-1,04	0,0150	UG(27)		<i>zgc:162816</i>	ENSDARG00000070416
<i>zgc:162816 (C9H3orf70)</i>	-0,97	0,0449	UG(25)		<i>chromosome 3 open reading frame 70</i>	ENSDARG000000061664
<i>zgc:163061</i>	-1,46	0,0032	UG(40)	0,36	<i>zgc:163061</i>	ENSDARG00000075538
<i>zgc:165409</i>	-1,27	0,0449	UG(36)		<i>zgc:165409</i>	ENSDARG000000091385
<i>zgc:171818 (BX548000.3)</i>	-1,12	0,0459	UG(30)	3,43	<i>zgc:171818</i>	ENSDARG000000088772
<i>zgc:195633</i>	-1,32	0,0055	UG(37)		<i>zgc:195633</i>	ENSDARG000000088291
<i>znf503</i>	-0,81	0,0451	TF	9.69 / 0.75	<i>zinc finger protein 503</i>	ENSDARG00000018492

8.3 Appendix to Chapter 5 - Functional assessment of candidate *dll4* targets



Appendix Figure 8-6. Titration of MOs included in the expression screen.

A. Titration of *cldn5b*MO using *runx1* expression: At 2.5ng *cldn5b*MO has a negligible effect on *runx1* staining as compared to wt siblings. At higher concentrations of *cldn5b*MO reduced numbers of *runx1* positive cells are observed in the DA. No overall adverse effects on morphology are observed at the concentrations used. **B. Titration of *hif1ab*MO on *runx1* expression:** When injecting 0.5ng *hif1ab*MO, *runx1* expression is severely reduced if not absent in the DA. Higher concentrations (1ng) of *hif1ab*MO bring *runx1* expression in the DA back to wt levels while up-regulating expression in the neural cells. This phenotype is enhanced for 2ng *hif1ab*MO and levels of *runx1* expression in the neural tube drop again at 2.5ng. At concentrations of 1ng and above, axial defects are observed. **C. Titration of *zfat*MO1 for *runx1* expression:** A reduction in *runx1* positive cells is observed in the DA at 26 hpf at all concentrations tested. At 10ng MO, embryos show no overall gross developmental abnormalities while showing a clear reduction in *runx1* staining. At 20 and 30ng, necrosis in the head and tail is observed as well as defects in AP patterning as indicated by curvature of the tail. **note that the pictures for wt and *zfat*MO1 (10ng) are also shown in Figure 5-6 (p. 168)* **D. Titration of *zfat*MO2 for *runx1* expression:** *zfat*MO2 causes reduction in *runx1* expression in the DA at 26 hpf at all concentrations tested. Embryos injected with 10ng *zfat*MO2, show no overall gross abnormalities while showing a clear reduction in *runx1* staining. At 20 and 30ng, necrosis in the head and tail is observed as well as defects in AP patterning as indicated by curvature of the tail. **E. Titration of *foxd1*MO for *runx1* expression:** When injecting 1.5ng to 4.5ng *foxd1*MO, *runx1* expression in the DA is reduced, no developmental abnormalities are observed at these concentrations. **F. Titration of *foxc1b*MO1 for *runx1* expression:** *runx1* expression in the DA is reduced at 6, 8 and 10ng *foxc1b*MO1. At 10ng necrosis is observed in the tail and in the head (not shown). **G. Titration of *hes6*MO at 26 hpf using 1ng, 2ng and 3ng as compared to wt siblings:** *runx1* expression is increasingly severely down-regulated in embryos injected with *hes6*MO. **H. Titration of *her8a* for *runx1* expression:** *Her8a*MO was titrated with 2ng, 2.5ng, 3ng and 3.5ng, assessing the expression of *runx1*. Increasing amounts of morpholino result in an inverse correlation with the amount of cells expressing *runx1*. **A-H.** Arrows indicate HSCs (pink) and gross morphological defects (purple).

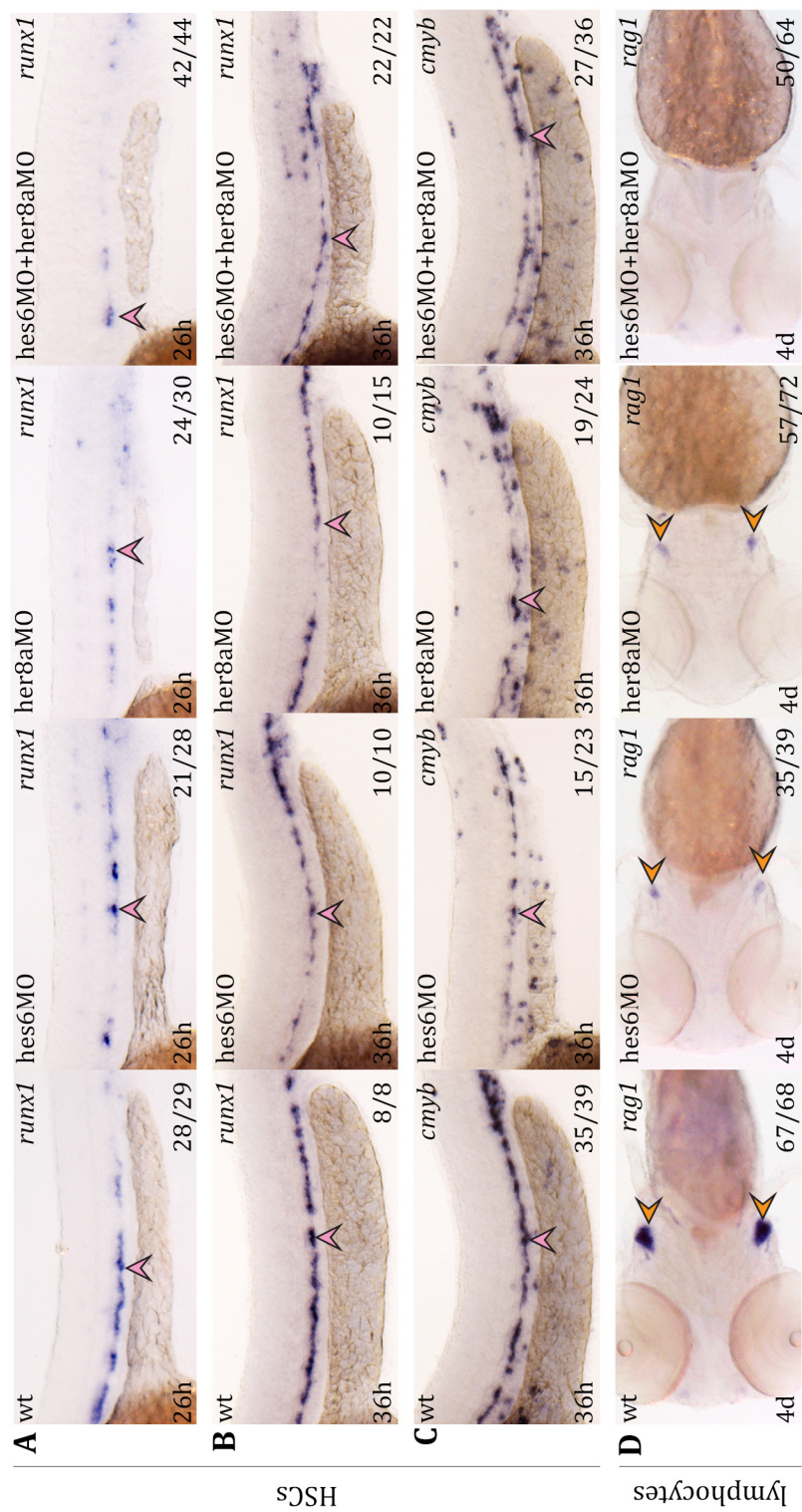


Appendix Figure 8-7. *Zfat* MO design and testing MO efficacy.

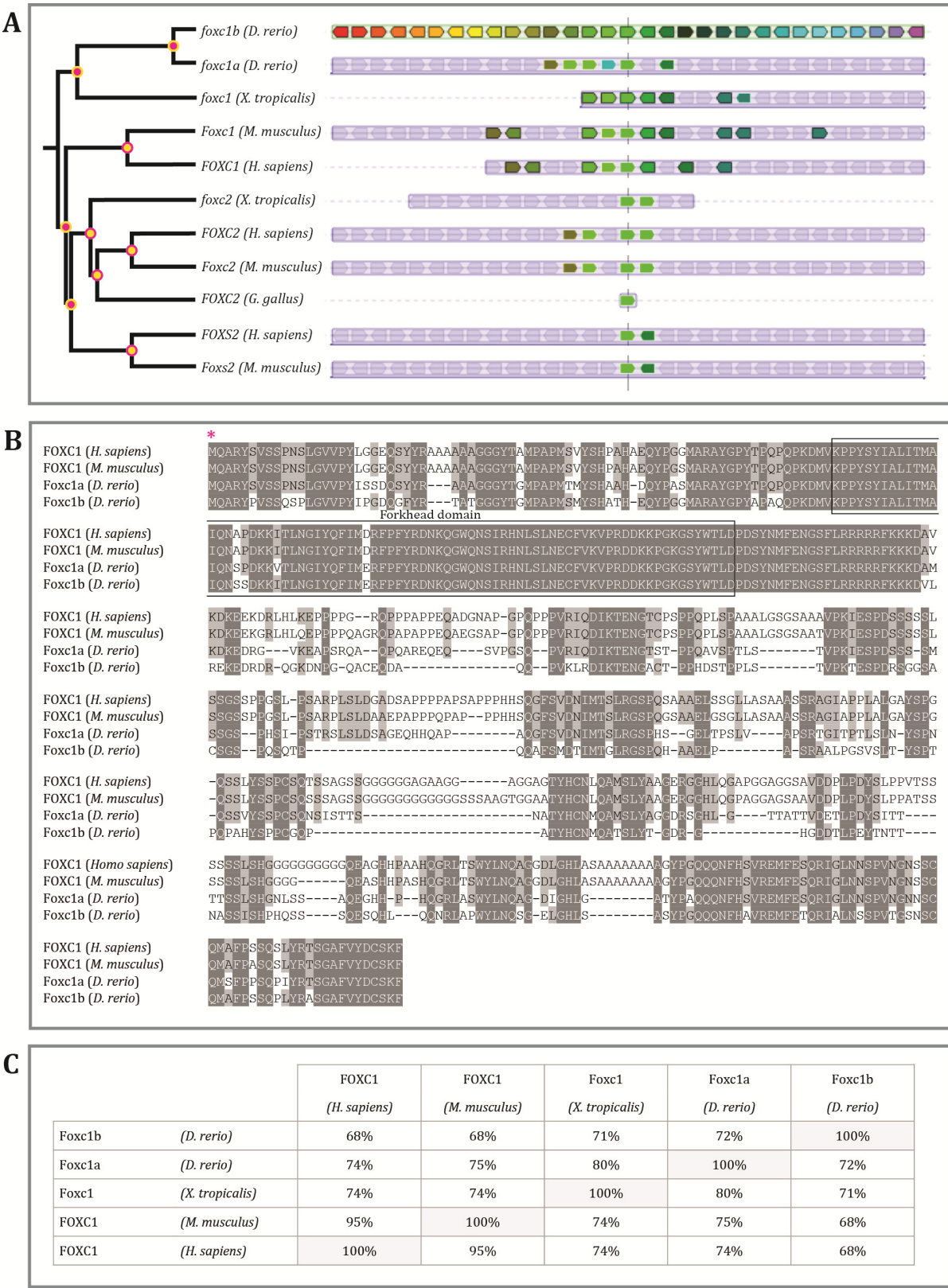
A. Schematic representation of *zfat* gene structure, MO and primer design: Both MO1 and MO2 are splice blocking MOs and designed to block both splice isoforms of the *zfat* gene. Primers were designed to identify mis-splicing. **B** PCR products for all *zfat*MO1 concentrations assessed show an additional larger band (arrows), consistent with a retained intron. **C** *zfat*MO2 PCR products show an additional shorter band (arrows), consistent with a cryptic splice site used in the morphant embryos.

Appendix Figure 8-8. *hes6* and *her8a* morphants show an additive effect on HSCs and their derivatives.

(Figure on following page) When injecting *hes6*MO and *her8a*MO alone, fewer *runx1* positive cells in the DA are observed in the DA of 26 hpf embryos as compared to uninjected wt siblings; a phenotype which is enhanced when co-injecting both MOs (**A**). A similar through weaker phenotype is observed for *runx1* (**B**) and *cmyb* (**C**) at 36 hpf. *Rag1* expressing T-cells are severely reduced in *hes6* and *her8a* morphants but absent in the double morphants (**D**). Arrows indicate: HSCs (pink) and T-cells (orange). * Note that data shown for wt and *hes6*MO in panel (**B**) is also represented in Figure 5-10 (p. 178). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1, (p. 235).

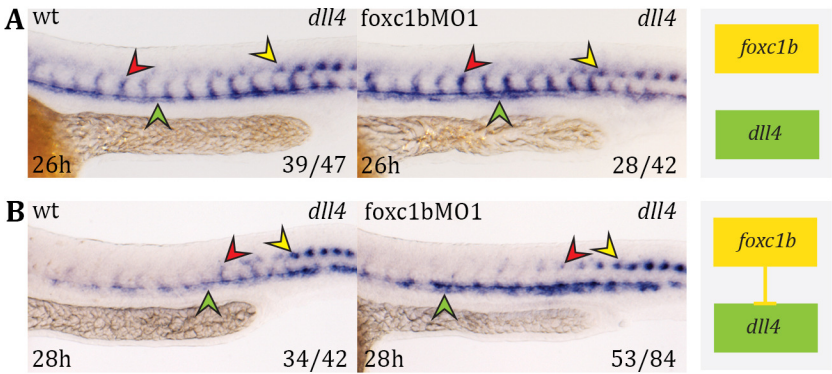


8.4 Appendix to Chapter 6 - *Foxc1b* is required for HSC emergence



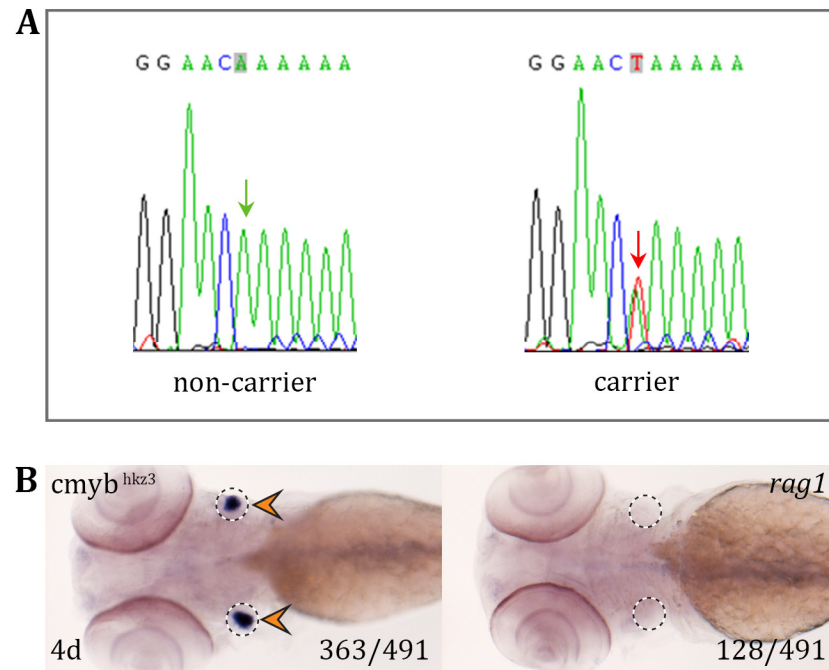
Appendix Figure 8-9. Homology analyses for vertebrate *foxc1* genes.

A. Gene tree and synteny analysis of *foxc1* across selected vertebrate species: The gene tree for *foxc1* contains speciation (yellow circles) and gene duplication nodes (pink circles). *Foxc1* is located in a syntenic region. The gene tree (re-drawn) and synteny alignment were obtained from the Genomicus browser using *D. rerio* as the reference species (Louis et al., 2013), nodes were annotated by evaluation. **B-C.** Foxc1 cross-species protein sequence comparisons: Protein sequences for sequence alignment for the human, mouse and zebrafish Foxc1 proteins were obtained from Ensembl (Flicek et al., 2014), and aligned using ClustalO (McWilliam et al., 2013), followed by manual improvement. Dark grey highlights indicate conserved amino acids across all Foxc1 proteins compared while lighter grey indicates conservation across 3 proteins compared. Dashes were introduced to optimize alignment. The highly conserved forkhead box domains are boxed (predicted by CDD (Marchler-Bauer et al., 2011)). The percentage sequence identity table (values obtained from ClustalO (Sievers et al., 2011)) shows conservation of protein sequences between any two selected species (**C**).



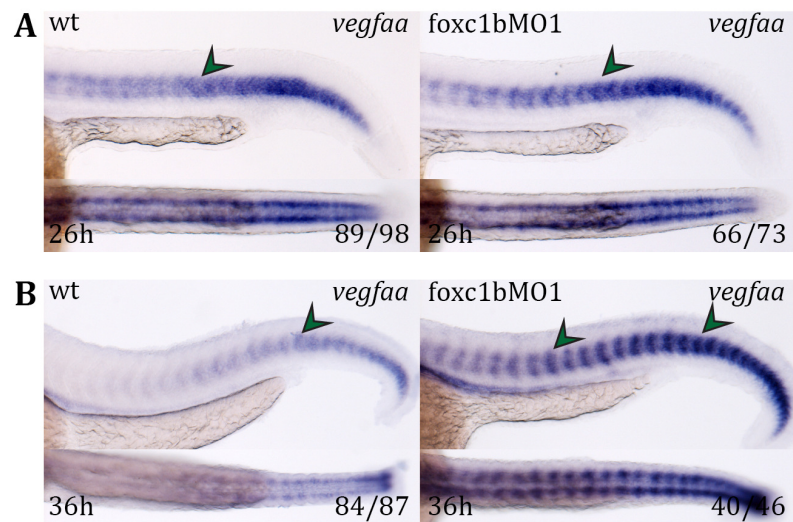
Appendix Figure 8-10. Regulation of *dll4* by *foxc1b* is highly dynamic.

At 26 hpf no effect on *dll4* expression can be seen in DA, ISVs or neural tissues of *foxc1b* morphants (**A** - data replicated from Figure 6-2, p. 197); at 28 hpf however, the *dll4* expression domain is expanded anteriorly in the DA of *foxc1b* morphants, but not in the ISVs or neurons (**C**). Arrows indicate DA (green), ISVs (red), neurons (yellow). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1, (p. 235).



Appendix Figure 8-11. Characterising *cmyb^{hkz3}* mutants.

Cmyb^{hkz3} mutant carriers are identified by PCR of the genomic locus followed by sequencing: haploid carriers have a A to T mutation resulting in a A/T double-peak at this position (A). 25% of embryos from a *cmyb^{hkz3}* heterozygous incross exhibit a complete absence of *rag1* positive T-cells at 4 dpf (B).



Appendix Figure 8-12. *Vegfaa* expression is repressed by *foxc1b* at 36 hpf but not 26 hpf.

Expression of *vegfaa* at 26 hpf is not altered in embryos injected with *foxc1bM01* (A). At 36 hpf, *vegfaa* is up-regulated and extends more anteriorly in *foxc1b* morphants (green arrows) (B). Representative embryos are shown; a detailed breakdown of the phenotype is given in Appendix Table 8-1, (p. 235).

Chapter 9

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