Transcriptional Regulators of Arterial-Specific Endothelial and Mural Cell Development

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Abstract

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The vertebrate vasculature is formed by populations of endothelial and mural cells that arrange into functionally and molecularly distinct arterial, venous and capillary beds. Although a number of signalling pathways and transcriptional regulators have been implicated in these processes of vascular differentiation, a clear picture of how arterial-specific gene regulation is achieved is yet to emerge. In this study I have investigated the transcriptional regulation of arterial identity from two different directions: characterisation of enhancers to identify the transcription factors that bind and direct arterial specification; and direct study of the function of one particular transcription factor expressed specifically in the arterial vasculature.

I have identified a novel gene enhancer that directs arterial-specific expression of *Flk1* (Vegfr2) in transgenic mouse and zebrafish models. Dissection of inputs from individual transcription factor binding sites within this enhancer shows a requirement for Gata factors for enhancer function in endothelial cells, whereas arterial-specification is directed by Rbpj-mediated repression of enhancer activity in veins. This work demonstrates that Flk1 expression in arterial endothelial cells is downstream of the Notch/Rbpj pathway, and also describes a novel transcriptional mechanism of arterial differentiation.

In parallel, I have uncovered a novel role for the transcription factor Tbx2 in the regulation of arterial mural cell identity. Histological analysis demonstrates the previously unreported expression of Tbx2 exclusively in mural cells of peripheral arteries and microvessels, and genetic deletion experiments in mice suggest a role for Tbx2 in mural cell recruitment, survival, proliferation, and differentiation upstream of Notch3 and Pdgfrβ.

Together, these results contribute valuable insights into our understanding of the establishment of vascular identity by identifying novel transcriptional regulators of arterial fate in both endothelial and mural cells.

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Finally, I need to recognize all the animals that lost their lives for my research.

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Abbreviations

BBB: blood brain barrier
CDS: coding sequence
CNS: central nervous system
ChIP: Chromatin immunoprecipitation
DBD: DNA binding domain
DLAV: dorsal longitudinal anastomotic vessels
E: Embryonic day
EC: endothelial cell
ECM: extracellular matrix
EMSA: electrophoretic shift assay
ER: endoplasmic reticulum
GFP: green fluorescent protein
GWAS: genome-wide association studies
HBVP: human brain vascular pericytes
hpf: hours post fertilisation
IHC: immunohistochemistry
ISV: intersegmental vessels
MEF: mouse embryonic fibroblasts
MO: Morpholino
NMRI: Naval Medical Research Institute
NICD: Notch intracellular domain
PC: pericyte
PCR: polymerase chain reaction
PFA: paraformaldehyde
RT: room temperature
Seq: next generation sequencing
shRNA: short hairpin RNA
SNP: single nucleotide polymorphism
TF: transcription factor
TFBS: transcription factor binding site
TSS: transcriptional start site
WT: wild-type

Chapter I - Introduction

1.1 Regulation of Gene Expression

Ever since it was established that living organisms essentially operate based on a fixed body of information encoded in nucleic acid sequences, the question of how interrogation of this information is coordinated has been a central problem in biology. While the general mechanisms that allow translation of genetic information into functional products have been well characterized, much less is known about how spatial and temporal regulation of these processes is achieved. Complex multicellular organisms utilize their largely invariant genome to generate a staggering variety of cell types and responses to environmental influences. This is especially apparent during stages of development, when the rapid differentiation and migration of various cell populations has to be orchestrated with infallible precision. It is, therefore, not surprising that the regulation of gene expression can occur at several levels and involves a multitude of players.

At this stage, it should be noted that the majority of research to this day has focused on proteins as the functional products encoded by genes. There are, however, several other, mainly RNA-based, classes of functional products for which genetic information encodes and more will almost certainly be described in the future. In this introduction, the focus will remain on the regulation of protein-coding genes, although some functions of RNA-based effectors will be discussed in the context of their role in the regulation of protein-coding gene expression.

In general, the production of a functional protein based on the information contained in a gene requires two main steps: First, DNA-dependent RNA synthesis, or transcription, by RNA polymerases (RNAPs) and its processing to a mature messenger RNA (*mRNA*). And second, translation of the mRNA template into a chain of polypeptides by the ribosome, followed by posttranslational modifications. All of these steps are subject to extensive regulation by a wide array of specialized cellular machinery. Since this process is inherently hierarchic, based on the unidirectional flow of information from gene to protein, the regulation of gene transcription is of special importance in the establishment of specific gene expression patterns associated with particular cell types and developmental stages and will be discussed in more detail.

1.1.1 Chromatin

Transcription of genetic information necessarily requires interaction of the DNA with other factors. The dynamics of such interactions are critically dependent on the nuclear environment in which they occur. In the nuclei of eukaryotes genomic DNA is associated with proteins which maintain it in an organized state. This DNA-protein composite is known as chromatin. Its core unit, the nucleosome, comprises 147 DNA-base pairs wound around a histone-protein octamer (Lelli et al 2012). Even before the genetic code is read out by the transcriptional machinery, modifications of the DNA itself, or of histone proteins, affect DNA condensation, nucleosome positioning and interactions of the chromatin with other factors; thereby controlling which information is generally available to a cell at certain point in time.

Methylation

In vertebrates, DNA methylation preferentially occurs on CpG dinucleotides. Small areas of the genome that are enriched in CpGs, known as CpG islands, have been associated with transcription initiation and are often found at annotated promoters (Illingworth et al 2010, Saxonov et al 2006). CpG islands have been shown to directly affect chromatin modification state. When non-methylated, they are thought to introduce a transcription-permissive chromatin state (Thomson et al 2010); whereas DNA methylation on CpG-rich promoters is associated with stable silencing and closed chromatin as a result of either direct inhibition of transcription factor binding or methyl-CpG-binding domain protein-dependent recruitment of histone-modifying enzymes (Ng et al 2007, Weber et al 2007).

Histone modifications

Histone modifications are extremely versatile, with at least eight different basic types of modifications and over 60 described residues at which modification can occur (Kouzarides 2007). Additionally, a number of distinct histone variants further increase the complexity with which the chromatin landscape, wherein the genetic information is embedded, can be moulded to serve as a habitat for a specific cellular fate (Maze et al 2014). Specific histone signatures have been described for different functional areas of the genome such as gene bodies, promoters and regulatory sequences as well as for particular states of activity at these sites (Harmston and Lenhard 2013). Depending on the requirements of individual cellular phenotypes they can be stable or very transient to allow for dynamic and flexible regulation of the transcriptional activity of a gene (Sawicka et al 2014). Histone signatures are of

special importance not only for function but also for the identification of regulatory DNA elements, as will be discussed in more detail later.

Mechanistically, histone modifications can remodel chromatin structure by affecting histone binding to the DNA strand as well as interactions between individual nucleosomes (Clapier and Cairns 2009). As a result, the chromatin can unravel to expose a naked DNA strand which is much more accessible to other factors. Alternatively, modifications can directly facilitate or preclude recruitment of non-histone proteins during chromatin-associated processes such as DNA replication, transcription, and repair (Zentner and Henikoff 2013). Within the global chromatin landscape, histone modifications are also responsible for the segregation of the genome into domains of eu- and heterochromatin, with the former describing areas accessible for active transcription and the latter those which are transcriptionally silent (Taverna et al 2007).

1.1.2 Transcription

Transcription of a protein coding gene depends on the interaction of a large number of sequence-specific transcription factors and co-regulators at several specialized DNA elements (Hochheimer and Tjian 2003). This extremely elaborate set-up permits a high degree of fine-tuning of the spatial and temporal patterns of transcription during cellular growth, differentiation and development; and further allows adaptation of transcriptional output to environmental influences as has been shown, for example, for cellular stress responses (López-Díaz et al 2013).

In a first step, the preinitiation complex (PIC), consisting of RNAP II (Pol II) and general transcription factors which recognize specific DNA sequences such as

TATA, forms at the promoter (Sikorski and Buratowski 2009). This is followed by local, ATP-dependent opening of the DNA helix, mediated by the multi-subunit complex TFIIH, and phosphorylation of the carboxy-terminal region of Pol II, which facilitates its release from the promoter (Dvir et al 2001). In traditional models based on experimental work in *Saccharomyces cerevisiae*, formation of the PIC followed by promoter release of Pol II are viewed as rate-limiting steps in gene transcription and therefore as the major targets of regulation. However, it is becoming increasingly clear that another critical step in transcription regulation occurs after transcription initiation in metazoans, associated with the controlled pausing of Pol II approximately 20 – 60 bp downstream of the TSS (Levine 2011). Interestingly, paused Pol II is often found at genes with roles in signal-responsive pathways involved in development, cell proliferation and cellular stress responses, suggesting it may be of particular importance for the regulation of these processes (Adelman and Lis 2012). However, the exact role of paused Pol II for transcription regulation remains to be elucidated.

While the core transcription machinery has been shown to include a number of cell-type specific components, such as isoforms of the TATA box-binding protein (TBP) family and TBP associated factors (TAF), which confer some degree of promoter selectivity, they cannot, by themselves, account for the extremely complex patterns of transcription regulation observed in metazoans (Hochheimer and Tjian 2003). An additional level of complexity with enormous potential for intricate regulation of gene expression is conferred by a vast number of tissue specific transcription factors.

1.1.3 Role of Transcription Factors in Transcriptional Regulation

The importance of transcription factors in the regulation of differential gene expression is supported by a positive correlation in the diversity of the transcription factor repertoire with the complexity of organism structure and development (de Mendoza et al 2013).

The defining feature of transcription factors is a DNA binding domain (DBD), which permits sequence-specific interactions with the genome (Fig. 1.1). Based on this loose definition, current estimates predict that transcription factors account for 5 – 10% of all protein-coding genes, which further highlights the importance of this class of molecules. The structure of the DBD is also important for the classification of transcription factors, as most have not been functionally characterized. Around 80% belong to one of the three main classes of transcription factors: C_2H_2 zinc-finger, homeodomain, and helix-loop-helix.

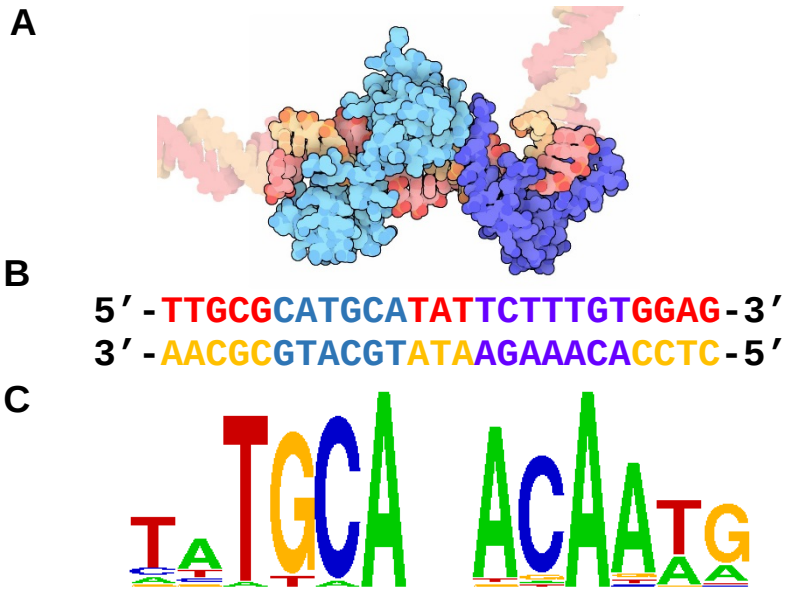

Figure 1.1: Sequence specific transcription factors bind to short degenerate DNA motifs

A: Structural model of the transcription factors Sox2 (blue) and Oct4 (purple) bound to a short, double stranded DNA molecule (from RCSB PDB).
B: Sequence of the DNA molecule with binding sites for Sox2 (blue) and Oct4 (purple).
C: Position weight matrices for Sox2 (left) and Oct4 (right) showing the plasticity of DNA binding motifs (from W ChIPMotifs)

Transcription factor function

Functionally, transcription factors are very vaguely defined as having the ability to regulate gene expression by either activating or repressing transcription. Exact mechanisms have been described only for very few transcription factors, but generally they are thought to function by: (1) interacting directly, or indirectly via co-factors, with components of the transcriptional machinery such as mediator (Yin and Wang 2014); (2) remodelling of the chromatin landscape to allow or inhibit access of the general transcription machinery or other transcription factors (Voss and Hager 2014); (3) cooperative binding with other transcription factors (Kazemian et al 2013); (4) interfering directly with the binding of other factors to prevent them from binding to the DNA. Individual transcription factors can assume several or all of these functions depending on the cellular context and the individual binding site. For

example, the forkhead box (Fox) transcription factor FoxA1 has been shown to recruit cyclic AMP-responsive element-binding (CREB) protein (CBP)/p300. This two-member family of transcriptional co-activators initiates opening of the chromatin via its histone-modifying activity and also interacts directly with members of the basal transcription machinery to promote gene expression (Koo et al 2013, Vo and Goodman 2001). FoxA1 can also act as a so called “pioneer” transcription factor that has the ability to access binding sites in condensed chromatin, facilitate nucleosome displacement and make binding sites accessible for other transcription factors (Magnani et al 2011). Moreover, FoxA1 can interact with members of the GATA family of transcription factors in a cooperative fashion, and in other cases FoxA1 has also been reported to mediate gene repression through recruitment of histone modifying enzymes (Koo et al 2013).

The function of many transcription factors is also determined by their activation state. Steroid receptors, for instance, require ligand binding to initiate translocation into the nucleus, and the well-studied tumour suppressor P53 requires phosphorylation of its transactivation domain to interact with the transcriptional machinery (Evans 1988, Lambert et al 1998).

Transcription factor binding

Identification of functional binding sites presents a major challenge in the study of transcription factors. Eukaryotic transcription factors typically recognize short (6 – 12 bp), degenerate DNA motifs. Because of the limited information contained in these recognition sites, it has been anticipated that, for the average transcription factor, there are between 10^4 – 10^6 recognition sites per genome arising purely by

chance (Wunderlich and Mirny 2009). And even assuming high (98%) genome chromatinization, 10^2 – 10^4 fortuitous sites would still be accessible (Wunderlich and Mirny 2009). This, in turn, suggests that there will be hundreds to thousands of incidental transcription factor binding events across the genome for which no functional consequence can be expected.

While this problem is widely appreciated, there are a number of different models that attempt to explain how transcription factors achieve functional specificity. In the “co-selective” DNA binding model, direct homo- or heteromeric interactions between transcription factors are thought to increase binding strength and confer specificity to the binding event (Fig. 1.2). Indeed, direct interactions have been described for several transcription factors and cooperative binding has been demonstrated in genome wide studies (Ravasi et al 2010). Additionally, it has been shown that transcription factor binding sites preferentially occur within short range (0 – 10 bp) of each other (Kazemian et al 2013). However, the importance of direct cooperation in transcription factor binding is challenged by experiments showing that, at genomic locations which are hypersensitive to DNaseI endonuclease digestion, the presence of a suitable binding motif alone can accurately predict transcription factor binding (Kaplan et al 2011, Li et al 2011). This suggests that, as long as the DNA is accessible, transcription factors can bind their recognition sites independently. This led to the adoption of the “widespread” model of transcription factor binding in which direct interactions are not, per se, required to establish binding. In this model, both recognition site occupancy by transcription factors as well as the functional outcomes of such occupancy are regarded on a continuous scale with many, low-occupancy, non-functional binding sites and few, high-occupancy sites with direct regulatory functions (Biggin 2011).

Evidence for pervasive non-functional transcription factor binding also comes from numerous ChIP studies, which regularly map transcription factor binding to sites that do not show regulatory capacity in functional assays (Whitfield et al 2012). It should also be mentioned that ChIP experiments routinely identify transcription factor binding at sites which do not contain any known consensus recognition motifs. Whether this is result of a specific interaction with a yet undescribed DNA sequence, a non-specific binding of the transcription factor to DNA directly, or an indirect interaction that involves other DNA-binding factors is mostly unclear and has to be assessed separately for each binding event. In any case, it is widely recognized that non-functional transcription factor binding is a common occurrence. However, this does not necessarily rule out a requirement for direct interaction of transcription factors at functional binding sites. Most likely, there are binding sites at which cooperative binding is required for a functional outcome and those at which a transcription factor can exert its function independently. It is also possible that the same site can sometimes be bound functionally and sometimes non-functionally by different members of a transcription factor family, which often share similar binding motifs. Additionally, there are other influences such as neighbouring sequence, chromatin environment, or interactions with co-factors that can affect transcription factor binding and functionality. But the extent to which they contribute to transcription factor function is only poorly understood and will almost certainly be different for individual binding events.

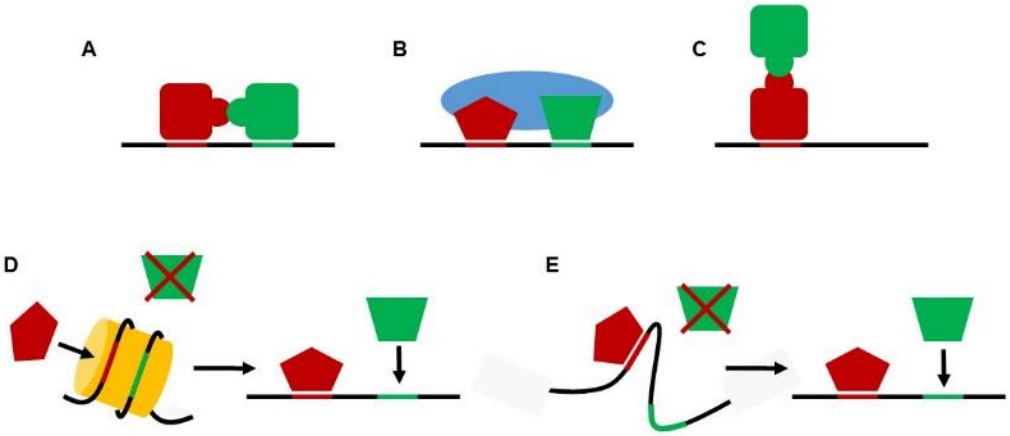

Figure 1.2: Mechanisms of Transcription Factor cooperativity

A: Direct interaction between transcription factors (TFs) allows binding of adjacent motifs. Neither factor can bind alone.
B: Indirect interaction with co-factors stabilizes binding of TFs. Both TFs and Co-factors have to present for binding to occur.
C: Direct interaction between TFs allows binding in the absence of a binding motif for one of the TFs.
D: Pioneer activity of one TF facilitates nucleosome displacement and opens up chromatin to make biding sites available for other TFs.
E: A TF induces changes in local DNA architecture which opens up an otherwise cryptic biding site for a second TF.
After (Spitz and Furlong 2012)

Expression patterns of transcription factors

Transcription factor expression varies distinctly between different tissue types and differentiation states. In a recent study of the expression patterns of 873 transcription factors in 32 major tissue types, 161 were found to be expressed in all or most tissues, whereas 349 showed tissue-specific expression, and no expression could be detected for the remaining 363 (Vaquerizas et al 2009). 226 of the tissue specific transcription factors were expressed in groups of related tissues, while 123 transcription factors had distinct expression profiles in only one tissue. This may reflect a role for these transcription factor in the regulation of features defining for that tissue group or cell type.

There is also the concept of the “master regulator” which describes a factor on the very top of the regulatory hierarchy of a particular cell type. The hematopoietic transcription factor SCL, for example, is indispensable for early hematopoietic linage specification and has the capacity to convert non-hematopoietic tissues into hematopoietic precursors when expressed ectopically (Gering et al 1998, Porcher et al 1996). However, while the idea of the master regulator is conceptually appealing, it may not be helpful to explain the dynamics of transcriptional regulation and misjudges the importance of individual transcription factors (Chan and Kyba 2013). On the contrary, the fact that transcriptional networks have evolved a high degree of robustness towards fluctuations in the levels of certain transcription factors, and redundancy is commonly observed, suggests that these transcription factors or transcription factor families have a particularly important role. In some cases, loss of a transcription factor displays incomplete penetrance and only has an effect in some individuals or in strains of knockout mice, which may further contribute to misinterpretation (Raj et al 2010).

1.1.4 The Role of Enhancers in Transcriptional Regulation

Transcription factors normally act in *cis.* And while there is often a number of binding sites for transcription factors that are involved in the regulation of a gene, they are not randomly distributed across the gene locus but tend to cluster together in *cis*-regulatory elements (CRE). In many genes, CREs can be found close to the TSS within the proximal promoter. Due to their easy identification, these promoter proximal elements have received a lot of attention and much research has focused on their characterization and role in the regulation of gene expression. There are,

however, a large number of distal CREs, which are more difficult to identify and therefore have been studied in less detail. It is becoming increasingly evident that these distal CREs, more commonly termed enhancers, are of central importance in the regulation of differential gene expression (Fig. 1.3).

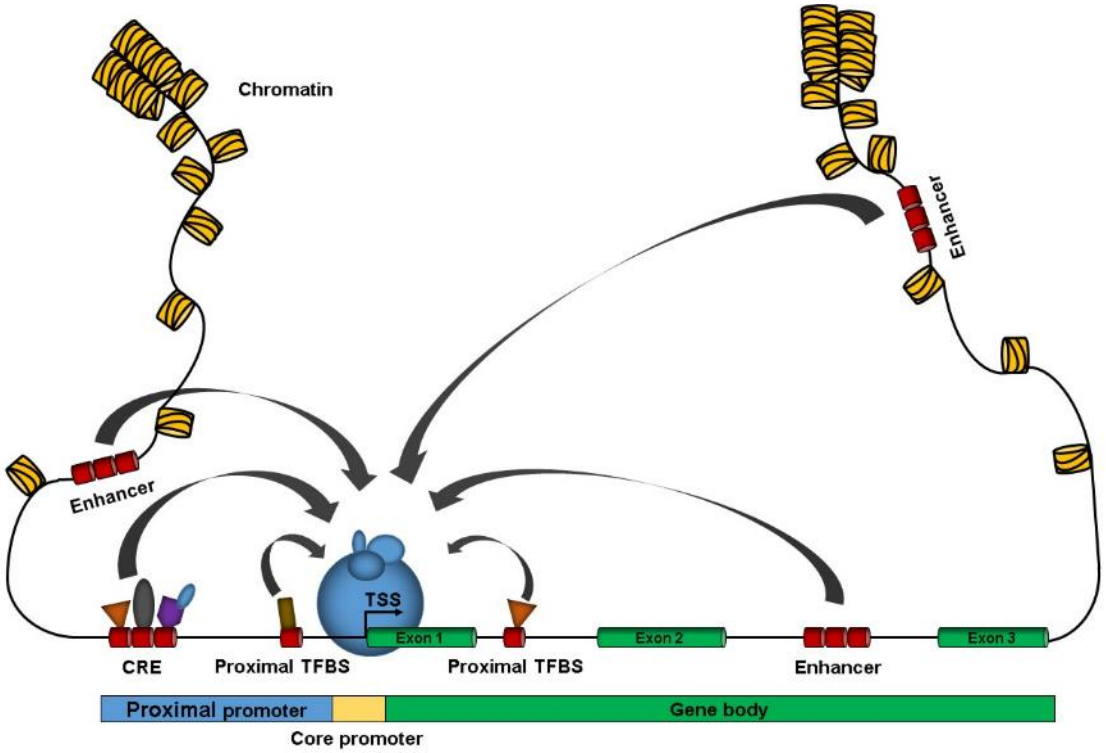

Figure 1.3: A summary of promoter elements and regulatory signals

Chromatin is comprised of DNA wrapped around histones (yellow cans) to form nucleosomes. Nucleosome density changes accessibility of the DNA to other proteins such as transcription factors. The region around the transcription start site (TSS) is often divided into a larger proximal promoter upstream of the TSS and a smaller core promoter just around the TSS. To recruit the core transcription machinery (blue globe) and to activate transcription of the gene, transcription factors bind to specific sequence motifs, transcription factor binding sites (TFBSs – red cans) that are near to the TSS (proximal elements). TFBSs often occur in clusters, forming *cis*-regulatory elements (CREs) which can occur within the proximal promoter or in more distal locations up or downstream of the coding sequence as well as in introns. These elements have important functions in the differential regulation of gene expression among various tissues and developmental stages. Figure adapted from (Lenhard et al 2012).

Enhancer architecture

Enhancers elements were first described in studies of the simian virus 40 (SV40), when it was discovered that a 366bp region upstream of the SV40 promoter dramatically increased the transcription of the rabbit β-globin gene. Remarkably, the element still increased transcription when it was inverted or placed downstream of the TSS and similarly affected transcription of a number of other genes (Atchison 1988, Banerji et al 1981). Based on this, an enhancer has been defined as a DNA element with the ability to affect the activity of heterologous, *cis*-linked promoters, independent of orientation and distance to the TSS (Atchison 1988). It is now widely accepted that these qualities of enhancers are determined by their capacity to cluster the binding of transcription factors. However, little is known about the specific requirements for the configuration of transcription factor binding sites in functional enhancers. Helpful models to understand how binding sites can be arranged include the enhanceosome model, the billboard model, and the transcription factor collective model (Spitz and Furlong 2012).

In the enhanceosome model, the transcription factors bound at the enhancer, together with co-factors, form a higher-order complex, the enhanceosome, which only becomes fully functional when all sub-units are correctly assembled (Merika and Thanos 2001, Thanos and Maniatis 1995). For this, strict arrangement as well as simultaneous transcription factor occupancy of all binding sites are necessary. Despite the fact that such enhancers have been described in certain cases, as, for example, for the interferonβ locus, they likely reflect an extreme end of a spectrum of possible enhancer architectures (Thanos and Maniatis 1995). The billboard model, on the other hand, assumes an enhancer is made up of several individual modules, which can be freely arranged and may include binding at both individual binding sites

as well as cooperative binding (Arnosti and Kulkarni 2005). Each module contributes separately to the action of the enhancer in such a way that the absence of single transcription factors does not necessarily result in a complete loss of activity. In the transcription factor collective model, a group of transcription factor binds the enhancer as a “team” in which interactions among multiple players are possible and not all factors need to bind to the DNA directly. As a result, the same transcription factor collective can be recruited to different enhancers with varying arrangements of biding sites, as has been shown for a group of cardiac enhancers in *Drosophila* (Junion et al 2012).

These models illustrate that the rules or “grammar” of enhancer architecture are unlikely to be an inherent characteristic of enhancers as such but are rather imposed by the nature of the factors that bind them. There is no experimental data suggesting that enhancers contribute anything to the regulation of gene expression besides providing a platform for the binding of transcription factors. Recently, a lot of work has focused on understanding the principles underlying binding site arrangement at enhancers. It is, however, likely that the insights provided by these studies will, to a large extend, remain limited by our incomplete knowledge of transcription factors.

Enhancer location

Since their original description, it has become clear that enhancers are a very common feature of eukaryotic genomes. In the Encyclopedia of DNA Elements (ENCODE), a map of regions of transcription, transcription factor association, chromatin structure, and histone modifications, there are almost 400,000 elements with enhancer-like features (Consortium 2012). Although only a small fraction of

these has been functionally validated, it nonetheless lends a perspective to the abundance of enhancers and their enormous potential for gene regulation (Consortium 2012). Enhancers are equally located upstream and downstream of their target promoters anywhere between a few kilobases up to megabases away and are frequently found in introns. Most are around 100bp – 1kb in size, although there are also descriptions of large clusters of enhancers which cooperate to regulate expression of a gene (Whyte et al 2013). Enhancer-driven gene regulation almost certainly requires physical interaction between the regulatory element and the target promoter. While this is supported by numerous studies using different assays, it is not entirely clear how the connection is established. Current models assume the formation of chromatin loops in order to bridge the sometimes considerable distances between the two elements and two protein complexes, the transcriptional co-activator Mediator, and Cohesin, have been implicated in this process (Kagey et al 2010).

Enhancer – promoter specificity

Most enhancers affect transcription from the nearest promoter. There are, however, cases where an enhancer skips one or more TSS to regulate a more distant gene. Further, some enhancer elements retain specificity for their target promoter even if placed closer to a different promoter. The enhancers of the juxtaposed *drosophila* genes *gsb* and *gsbn*, for example, remain in a relationship with their original promoter even if swapped around (Li and Noll 1994). Several factors may contribute to this observed enhancer-promoter specificity. For one, the chromatin environment of some promoters may make them inaccessible for some enhancers (van Arensbergen et al 2014). In other cases, biochemical compatibility

may only allow interactions between the enhancer and certain promoters. It has been shown that both the TATA-box and the downstream promoter element (DPE) play a role in establishing specificity, which may involve the binding of specific factors that mediate interaction with the enhancer (Butler and Kadonaga 2001). A different mechanisms involves the binding of CCCTC-binding factor (CTCF) to specific insulator regions in the genome, allowing large parts of the DNA strand to be "looped out", thus explaining how a distant enhancer can be brought closer to its target promoter (Herold et al 2012). Another model of insulator function assumes a direct non-functional interaction with the enhancer in which the insulator acts as a decoy (van Arensbergen et al 2014).

Based on the high numbers of regulatory elements described in genome-wide studies, it is estimated that there are on average 4-5 enhancers affecting the expression of most genes, which is consistent with experimental findings. Multiple enhancers present a mechanism for differential regulation of gene expression as they provide a framework for different groups of tissue-specific transcription factors to regulate the same gene according to the requirements of a variety of cell types and developmental stages. A well-known example is the *drosophila even skipped* locus, where five different enhancers coordinate the expression of the gene in a characteristic pattern of seven stripes across the fly embryo (Goto et al 1989). Enhancers have also been described to confer a degree of robustness to gene expression when environmental influences induce a change in transcriptional programs. In order to prevent a loss of transcription in vital genes, secondary enhancers with a similar expression pattern, also known more enigmatically as "shadow" enhancers, ensure activation of the promoter under altered conditions

(Frankel et al 2010). Interestingly, there are also reports of single enhancers affecting the expression of more than one gene (Jin et al 2013).

Enhancer Identification

For the identification of enhancers both computational and experimental approaches as well as combinations of the two can be used. Computational methods rely on sequenced-based predictions of transcription factor motifs, but also involve the training of algorithms on features of known functional enhancers to predict new elements (Hallikas et al 2006, Hardison and Taylor 2012, Kantorovitz et al 2009). Additionally, binding site clustering and phylogenetic footprinting, which assesses evolutionary conservation across orthologous gene loci in different species, can improve computational enhancer prediction (Berman et al 2002, Gotea et al 2010, Neph et al 2012). Nonetheless, computational approaches usually produce a high degree of false positive predictions.

Experimental procedures for enhancer identification can be based on detection of transcription factor or co-factor binding, DNA accessibility, and epigenetic modifications. The most widely applied technique to map DNA-protein associations is chromatin immunoprecipitation (ChIP) in which proteins are first cross-linked to the DNA, followed by fragmentation of the chromatin and extraction of the target protein DNA compound with a suitable antibody (Johnson et al 2007). After the target DNA fragment is extracted from the complex it can be analysed by sequencing (ChIP-seq) or microarray (ChIP on ChIP). This way, sequences which are bound by the target factor can be identified. An improved protocol includes exonuclease treatment after DNA fragmentation to shorten the target sequence and increase resolution (ChIP-

exo) (Stampfel et al 2014). Suitable antibodies are also available to employ ChIP in the detection of epigenetic modifications as well as general transcription factors and co-factors. A very informative assay for enhancer identification uses ChIP with antibodies directed against CBP/p300. And it has been shown that presence of these co-activators strongly correlates with enhancer activity (Visel et al 2009). An alternative method which does not require a specific antibody is DNA adenine methyltransferase identification (DamID) (van Steensel and Henikoff 2000). It involves fusing the protein of interest to DNA methyltransferase which catalyses methylation of certain DNA sites in the vicinity of the bound factor. Methyl PCR can then be used to identify methylated regions. Since this method relies on the presence of specific sites for methylation it has a much lower resolution than ChIP based protocols.

There are two commonly used methods to assess chromatin accessibility. DNaseI hypersensitivity takes advantage of the fact that open chromatin is more susceptible to enzymatic digestion with endonucleases (Boyle et al 2008). Following DNaseI digestion, small DNA fragments can be extracted and subjected to sequencing (DNase-Seq) or hybridization in microarray (DNase-Chip) to map hypersensitive sites to the genome. Formaldehyde-assisted identification of regulatory elements (FAIRE) involves DNA-crosslinking, shearing of DNA by sonication and phenol extraction (Giresi et al 2007). It exploits the fact that “naked” DNA has a higher solubility than DNA cross-linked to protein. An alternative method is the recently developed assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq), which involves the insertion of sequencing adapters with the hyperactive Tn5 transposase at regions of open chromatin (Buenrostro et al 2015).

It has been shown that epigenetic modifications are often characteristic for certain functional areas of the genome and can be very informative in for predicting regulatory elements. This includes both histone variants as well as histone modifications. Promoters and enhancers, for example, are frequently marked by H2A.Z/H3.3 histone variants (Jin et al 2009). Additionally, promoters often carry H3K4me3 and H3K27ac histone modifications, whereas H3K27ac, H3K4me1, and H3K4me2 are associated with active enhancers (Heintzman et al 2007, Ong and Corces 2011). Other histone modifications at enhancers have also been described in certain cell types and individual enhancers, such as the CD28 response element (CD28RE) and the CNS22 enhancer (Barski et al 2007, Wang et al 2008).

Interestingly, it appears that epigenetic modifications at enhancers are a highly transient, cell-type specific phenomenon that is dependent on, but also indicative of, their state of activation. Current models assume that, at least in certain cases, modifications are acquired in a gradual fashion during cellular differentiation. In embryonic stem cells (ESCs), differentiation genes are regularly occupied by paused Pol II suggesting they are “poised” and ready for transcription when differentiation is initiated. Enhancers of poised genes have been shown to be marked by H3K4me1, H3K4me2, H3K27me3 and H3K9me3 (Rada-Iglesias et al 2011, Zentner et al 2011) (Table 1.1). And it has been suggested that this reflects a “pre-patterning” of enhancers which is orchestrated by stepwise recruitment of transcription factors and chromatin remodelling throughout precursor stages (Corces and Ong 2012). In B cell development, for instance, the stem cell transcription factor Sox2 is involved in the deposition of H3K4me2 chromatin marks at B-cell-specific enhancers (Liber et al 2010). During differentiation into pro-B cells, Sox2 is replaced by the lineage-specific

transcription factor Sox4 after which the enhancers become activated to initiate gene transcription (Liber et al 2010).

Histone Mark	Poised enhancer	Active enhancer
H3K4me1	+	+
H3K4me2	+	+
H3K9me3	+	-
H3K27me3	+	-
H3K27ac	-	+
H3K36me3	-	+

Table 1.1: Histone marks commonly found at active and/or poised enhancers

Active enhancers are able to recruit RNA polymerase II (Pol II), which is absent in poised enhancers. However, none of the enhancer associated modifications alone are unique to enhancer regions. For example, H3K4me1 is present within gene bodies, whereas H3K27ac and H3K9ac are also enriched at proximal promoter regions. Importantly, nucleosomes containing marked histones flank, rather than directly overlap with, transcription factor and co-activator-occupied enhancer regions (Buecker and Wysocka 2012, Zentner and Scacheri 2012)

Another approach to identify enhancer regions takes advantage of the fact that enhancers are likely brought into physical proximity to their target promoter in order to activate transcription. By investigating the ChIP profiles of proteins involved in the establishment of enhancer-promoter interactions, such as Cohesin, regulatory elements in contact with, or about to contact, their target promoter can be detected (Kagey et al 2010). The presence of the enhancer-promoter interface also allows the use of ChIP profiles of typically promoter-associated proteins to be used in enhancer identification as has been shown, for example for Pol II or the elongation factor EIII (Kim et al 2010, Lin et al 2013).

An interesting observation in respect to the association of Pol II with enhancers is the widespread transcription of enhancer DNA into enhancer RNA (eRNA) (Natoli and Andrau 2012). Whether this is an accidental occurrence related to the close physical proximity of Pol II with the enhancer or a functional event is currently a matter of scientific debate.

Enhancer-promoter interactions can be also be detected by chromosome conformation capture (3C) and the 3C-based methods circular chromosome conformation capture (4C), chromosome confirmation capture carbon copy (5C), and Hi-C (van Steensel and Dekker 2010).

These techniques are based on crosslinking of enhancer-promoter complexes. After shearing of the genomic DNA, the enhancer DNA fragment and the promoter DNA fragment which both remain associated with the complex are ligated and sequenced. By mapping the resulting chimeric sequencing reads to the genome, both enhancer and promoter regions can be identified and connected. However, the resolution of 3C-based techniques is relatively low, which makes detection of nearby enhancers difficult (Stampfel et al 2014). Further, interactions among genomic regions do not necessarily indicate their involvement in a functional regulatory relationship (Gibcus and Dekker 2013).

Enhancer validation

The lack of a functional readout is a major limitation of all the techniques outlined so far. As we are, at this point, ignorant of the exact characteristics which confer to a sequence of nucleotides the capacity to act as a regulatory element, they cannot be identified with absolute certainty based on genomic data alone. And while

transcription factor binding, chromatin accessibility, epigenetic modification, and long-range interactions all capture certain aspects of enhancer mechanics, none are exclusive to regulatory elements. Therefore, the only way to validate the functionality of a putative enhancer is to directly test its ability to affect gene expression. Traditionally, this has been done *in vitro* by cloning the enhancer candidate into a vector containing a silent promoter and a reporter gene whose expression can be easily detected, followed by transfection of this construct into a cultured cell line. Techniques for medium through-put assays, such as site-specific integration fluorescence-activated cell sorting followed by sequencing (SIF-Seq) and high throughput validation like self-transcribing active regulatory region sequencing (STARR-Seq) have also been developed recently (Arnold et al 2013, Zhu et al 2014).

However, these assays only provide snapshots of the regulatory scene in certain cell types and developmental stages where many enhancers will be inactive. Enhancer-FACS-Seq (eFS) partially circumvents this problem by using whole transgenic *drosophila* embryos as starting material (Gisselbrecht et al 2013). Currently, the most comprehensive method to characterize an enhancer is by assessing its expression pattern throughout development in a transgenic organism carrying the enhancer-reporter construct. As this approach is both laborious and costly, especially in mice, only a fraction of putative enhancers have been assessed in this way. In one of the most extensive studies performed to date, the activity of over 7,000 genetic elements, corresponding to 13.5% of the non-coding, non-repetitive genome, was screened in the developing fly embryo (Kvon et al 2014). This study also confirms that the vast majority of enhancers is only transiently active

during certain stages of development, a phenomenon also reported in mice (Nord et al 2013).

Enhancer malfunction

Considering the significance of enhancers in the regulation of differential gene expression it can be expected that mutations affecting enhancer function may contribute to the development of disease. Whereas mutations in the genes of transcription factors or co-factor genes can be expected to affect the regulation of many genes, changes in the sequence of individual enhancers will likely have more subtle effects (Herz et al 2014, Lee and Young 2013). It has been shown that single nucleotide polymorphisms (SNP) linked to disease in genome-wide association studies (GWAS) are correlated with potential regulatory regions, suggesting a way in which SNPs may directly contribute to disease development (Schaub et al 2012). Moreover, two recent studies have demonstrated that SNPs associated with renal and colorectal cancer, respectively, are located within enhancers that drive expression of genes directly involved in the development of the particular cancers (Schödel et al 2012, Sur et al 2012). There is also evidence for a "multiple enhancer variant" hypothesis which assumes that several polymorphisms in linkage disequilibrium affect the function of multiple enhancers regulating the expression of a single gene or several genes acting in a common pathway (Corradin et al 2014). These results highlight how studies of enhancer function can contribute not only to general knowledge of gene regulation but also further our understanding of the mechanisms of disease and open up new options for treatment.

1.2 Development of the Vascular System

The cardiovascular system is involved in a number of essential processes, including the distribution and removal of metabolites, inter-organ communication through hormones and other messenger molecules, thermoregulation, and immune responses (Carmeliet 2003). In order to fulfil these versatile requirements, which can vary considerably among tissue types, the vascular system has evolved into a highly complex and locally specialized network of veins, arteries, capillaries and lymphatics (Carmeliet 2003). While the mature vasculature is invested with numerous different cell types, a single-layer inner lining of endothelial cells is the defining feature of all vascular and lymphatic structures. Endothelial cells take the centre stage during the formation of the vascular system as their development precedes and coordinates that of the remaining vascular tissue. As a key event in embryonic development, formation of the vascular system is highly similar among all vertebrates (Larrivée et al 2009).

1.2.1 Vasculogenesis

During murine embryonic development, the first endothelial cell precursors, called angioblast, appear during blood island formation in the extraembryonic yolk sac around E6.5 where they differentiate from the mesoderm (Fig. 1.4). Blood islands comprise an outer layer of angioblasts surrounding an inner mass of hematopoietic progenitors. Angioblasts have been identified as non-aggregated T-cell acute lymphocytic leukaemia protein 1 (Tal1) positive and fetal liver kinase 1

(Flk1 [also known as vascular endothelial growth factor receptor 2 (Vegfr2) or kinase insert domain protein receptor (Kdr)]) positive (Drake and Fleming 2000, Risau and Flamme 1995). Further differentiation of angioblast into endothelial cells coincides with fusion of blood islands, resulting in the assembly of a network of blood-filled endothelial tubes which becomes the vitelline circulation of the yolk sac. Endothelial cell differentiation is marked by the upregulation of lineage specific genes such as platelet endothelial cell adhesion molecule 1 (Pecam1), tyrosine kinase with immunoglobulin-like and egf-like domains 2 (Tie2 [also known as endothelial-specific receptor tyrosine kinase (Tek)]), and VE-Cadherin, as well as by the downregulation of Tal1 (Drake and Fleming 2000, Risau and Flamme 1995). This *de novo* formation of tubular endothelial structures from migrating progenitor cells is referred to as vasculogenesis. Angioblast formation in the yolk sac is also observed during avian embryonic development, whereas in zebrafish vasculogenesis occurs exclusively in two distinct intraembryonic populations of mesodermal cells in the anterior and posterior lateral plate mesoderm (Ferguson et al 2005, Gore et al 2012). The former gives rise to the head vasculature, while the later migrates to the embryonic midline to form the primordia for the axial vessels, the dorsal aorta (DA) and posterior cardinal vein (PCV) (Gore et al 2012).

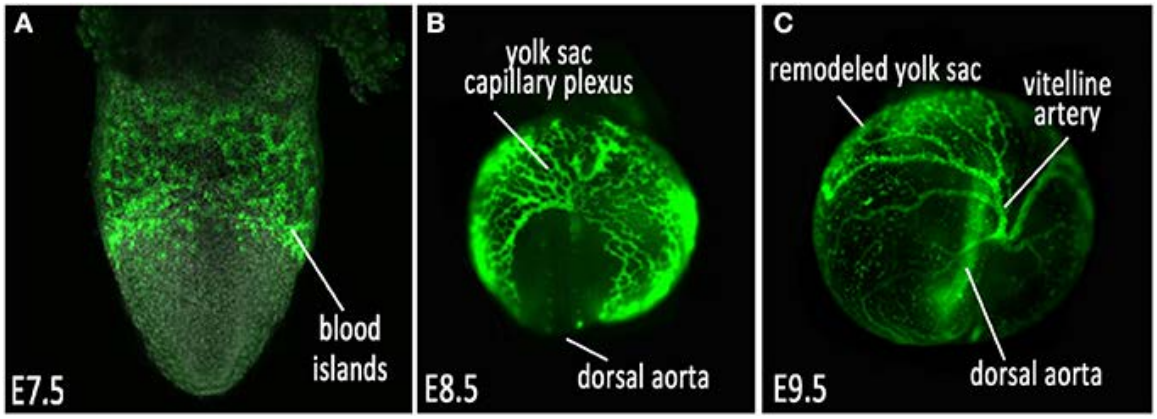

Figure 1.4: Vascular development of the mouse embryonic yolk sac

(A) Vegfr2/Flk1 expression labeling nascent vessels in the blood islands at E7.5; (B) ε-globin-GFP labeled erythroblasts filling the primitive capillary plexus at E8.5, and (C) after vascular remodeling at E9.5. Taken from (Garcia and Larina 2014)

Formation of the axial vessels

In the mouse, intraembryonic vasculogenesis begins around E7.5 when lose aggregates of angioblasts arise throughout the intraembryonic mesoderm and migrate towards the midline of the embryo to form the primitive vascular plexus (Risau and Flamme 1995, Schmidt et al 2007). This plexus undergoes remodelling to form the endocardial tube, which is the first vascular structure formed in the mammalian embryo, as well as the paired dorsal aortae and the cardinal veins, which are observed from E8.0 and begin to fuse around E9.5 (Drake and Fleming 2000, Sato 2012). In avian embryos a preliminary dorsal aorta develops from two separate endothelial cords between Hamburger-Hamilton stage 8 and 10, whereas in fish the dorsal aorta and posterior cardinal vein are formed by a single endothelial tube from the beginning (Sato 2012).

While it is widely accepted that two distinct populations of precursors contribute to the dorsal aortae and cardinal vein, respectively, the precise sequence of events that result in the establishment of two separate vessels is less clear. Experiments in

zebrafish suggest that both populations of angioblasts initially coalesce to form a single vascular tube from which the venous population segregates to form the cardinal vein (Herbert et al 2009). However, this view has been challenged by a study which reports the existence of separate medial, and lateral angioblast populations which migrate in distinct waves to form the dorsal aortae and cardinal vein (Kohli et al 2013). Most recently, it was shown that, in mice, some venous precursors do originate from the dorsal aorta and relocate to assemble the cardinal vein thus confirming earlier observations in fish (Lindskog et al 2014). Interestingly, disruption of either venous or arterial pathways results in loss of the corresponding vessel and the formation of a single tube in either case, suggesting that the dorsal aorta is not principally a prerequisite for cardinal vein assembly (Herbert et al 2009). Consequently, the inconsistent findings on cardinal vein formation may reflect a permissive developmental process during which venous precursors sometimes progress through a shared vessel and sometimes not.

Endothelium and blood cell development

The close association between hematopoietic and endothelial precursors has led to the postulation of the hemangioblast as a common precursor of both lineages. Evidence for the existence of the hemangioblast came from *in vitro* experiments which found that differentiating embryonic stem cells contain blast colony-forming cells that are able to generate both primitive hematopoietic and endothelial cells in appropriate conditions (Choi et al 1998). Later, small cell populations with the capacity to commit to either lineage were also reported in zebrafish (Vogeli et al 2006). However, the importance of these bipotential precursors for vascular development is still a matter of debate. Recently, a series of experiments in *xenopus*

leavis led to the conclusion that the hemangioblast reflects a state of bipotential competence which can result in the formation of both blood and endothelial cells under certain experimental conditions but is not actually exploited *in vivo* (Myers and Krieg 2013).

A connection between endothelium and blood cell development is also observed in the intraembryonic vasculature. At least in certain areas of the embryonic vasculature, such as in the ventral wall of the dorsal aorta in a region called the aorta-gonad-mesonephros, the endothelium appears to give rise to populations of hematopoietic stem cells. However, this hemogenic endothelium comprises only a small subset of all endothelial cells in the embryo and it is not clear whether it continues to play a role in the generation of hematopoietic stem cells in the adult (Hirschi 2012).

Vasculogenesis in the adult

Similarly, the significance of vasculogenesis in the adult is not clear. It has been proposed that bone marrow derived endothelial progenitor cells play an important role in the neovascularization during tissue repair and tumour growth in cancer (Asahara 1997, Asahara et al 1999, Nolan et al 2007, Peters et al 2005, Schmidt et al 2007). However, conflicting studies make it difficult to ascertain whether progenitor cells are directly integrated into vessels, or if they contribute indirectly by releasing localized paracrine factors that promote proliferation of endogenous endothelial cells (Udan et al 2013). There are also reports of tumour-derived endothelial progenitors in glioblastoma, although this form of vasculogenesis is likely to be confined to

tumours of a certain origin rather than a general mechanism of tumour vascularization (Ricci-Vitiani et al 2010, Rong Wang et al 2010).

1.2.2 Angiogenesis

The primary vascular network is remodelled and expanded into a functional system of veins, arteries, capillaries and lymphatics. This maturation phase is mainly driven by angiogenesis which, unlike vasculogenesis, involves vessel sprouting from existing structures and depends on the proliferation of endothelial cells *in situ* (Potente et al 2011). Sprouting is initiated in response to pro-angiogenic signalling from surrounding tissues and leads to substantial changes in endothelial cell behaviour. The tile-like, sedentary endothelial cells forming the vascular lumen lose their apicobasal polarity, extend filopodia and then migrate into the tissue along a gradient of signalling molecules. For this purpose, tip cells secret matrix metalloproteases such as MT-Mmp which facilitate the proteolytic breakdown of the basement membrane and the extracellular matrix (Potente et al 2011). However, this specialized “tip-cell” phenotype is tightly regulated so that only few endothelial cells break out of the endothelial collective to form sprouts (Phng and Gerhardt 2009). The endothelial cells in the immediate neighbourhood of the tip cell are instead selected to become “stalk cells”. The stalk cells form the base of the protruding angiogenic sprout and, unlike tip cells, they are non-invasive and readily proliferate to maintain a continuous row of endothelial cells that extends from the advancing tip cell back to the original vessel. The stalk cells also secret proteins such as collagen IV and laminin to start deposition of a new basement membrane and re-establish

apicobasal polarity by forming a lumen within the newly formed sprout (Potente et al 2011).

Angiogenesis initially involves the formation of many individual sprouts. However, when migrating tip cells meet they can connect and initiate a fusion event that ultimately leads to anastomosis of the newly formed lumina (Carmeliet and Jain 2011). These small endothelial loops again extend sprouts and gradually a circuitry network is established which is subsequently re-shaped into a mature vascular bed (Carmeliet and Jain 2011).

Vascular beds can be further extended by intussusception, or splitting angiogenesis, in which new vessels are formed by the separation of an existing vessel into two individual pillars (Mentzer and Konerding 2014). This morphogenetic process allows for the rapid expansion of existing microvascular networks without compromising blood flow. Intussusceptive angiogenesis appears to be a main mechanism for vascular expansion in the lung and exercise-induced increase of vascular capacity; it is also a prominent mechanism of tumour-induced angiogenesis (Mentzer and Konerding 2014).

1.2.3 Hemodynamics

Experiments show that blood flow is critically important for this vascular maturation phase and ablation of flow results in defective remodelling (Lucitti et al 2007). As a fluid flows through a tube, it exerts a force tangential to the tube, called shear stress, while another force, called circumferential stretch, is perpendicular to the tube wall and caused by pressure (Hahn and Schwartz 2009). As the innermost cell layer of the cardiovascular system, the endothelium is directly exposed to these

forces. While many studies describe an impact on endothelial cell behaviour, morphology, cytoskeleton organization, ion channel activation, or gene expression, the exact role of flow in vessel development is not clear (Jones et al 2006). It seems, however, that endothelial cells are very responsive to altered hemodynamics. Laminar flow, for example, has been shown to promote endothelial cell survival and inhibit proliferation, whereas turbulent blood flow has been implicated in vascular pathologies such as atherosclerosis (Bartling et al 2000, Cunningham and Gotlieb 2005). Shear stress also is a driver of intussusception (Chouinard-Pelletier et al 2013). It is attractive to conceptualize a situation where perfusion provides a live-feedback mechanisms by which particularly favourable connections are reinforced, while vessels which experience lower conductive demand are not maintained and eventually lost. However, definite proof of this mechanism is so far lacking.

1.2.4 Arteriovenous specification

Despite the fact that morphological variances in the endothelia of vascular beds of different organs had been observed, endothelial cells were long considered a homogenous, and relatively inert population of cells. In a landmark study, Wang et al. provided evidence that endothelial cells of veins and arteries are molecularly distinct by showing that the Eph family transmembrane ligand Efnb2 is expressed exclusively by arterial endothelial cells, whereas the receptor Ephb4 is only present on venous endothelial cells (Wang et al 1998). Mice lacking Efnb2 undergo vasculogenesis of the major arterial and venous trunk vessels but display defective angiogenic remodelling without proper intercalation of arterial and venous vessels (Swift and Weinstein 2009). Moreover, deletion of Ephb4 results in an almost

identical vascular phenotype suggesting that the interaction between the two proteins in the vasculature is highly specific (Gerety et al 1999, Helbling et al 2000).

Genetic basis of vessel identity

After the discovery of distinct endothelial phenotypes, studies addressed the question whether arteriovenous identity is genetically imprinted and already established before the formation of the earliest vessels during vasculogenesis or the result of environmental influences such as hemodynamic forces. In fish, it was shown that the basic helix-loop-helix protein *gridlock* is expressed first in the lateral plate mesoderm and is later specific to the aorta (Zhong et al 2000). Further, reduction of gridlock levels decreased the size of the aorta while enlarging the vein, whereas gridlock overexpression resulted in a stunted vein (Zhong et al 2001). Other studies in fish, murine, and avian models identified a requirement for hedgehog signalling in the establishment of arterial endothelial fate (Gering and Patient 2005, Lawson et al 2002, Vokes et al 2004). In zebrafish, deletion of hedgehog signalling inhibits angioblast migration during the formation of the dorsal aorta and results in the engagement of the entire angioblast population in cardinal vein assembly (Williams et al 2010).

Environmental factors in vessel identity

Since these early studies, other markers of arterial and venous endothelial cells have been described, many of which are expressed at the progenitor stage and therefore prior to the onset of flow. Despite numerous studies arguing that arteriovenous fate is indeed genetically predetermined, hemodynamic forces are

required for the expression of the full set of arterial and venous specific genes, indicating that endothelial cell identity is acquired in a stepwise fashion that integrates multiple inputs (Chong et al 2011). Graft experiments have shown that endothelial cells display a certain degree of plasticity when introduced into a different environment (le Noble et al 2004). During early stages of avian development, endothelial cells are capable of colonizing vessels of different identities. And in mice, vein grafts gradually lost expression of venous markers but did not acquire arterial identity of the host vessel (Kudo et al 2007).

Vascular heterogeneity

It is important to consider that the simplistic segregation in venous and arterial endothelial cell phenotypes based on the direction of blood flow does not adequately reflect endothelial cell heterogeneity. Endothelia in different organs are highly specialized and diverse, as exemplified by the high endothelial venules of the spleen, the blood brain barrier of the central nervous system, or the glomerular capillaries of the kidney (Aird 2007, Nolan et al 2013). If, as current models assume, these vascular structures are indeed formed by angiogenic growth from pre-existing vessels, endothelial cells must embrace a high degree of plasticity to adapt to their environment. Tissues, on the other hand, must also have the means to instruct the vasculature on their specific requirements. So far, little is known about this crosstalk between endothelial cells and tissues. Without a doubt, it is an area of research in which many interesting discoveries on endothelial cell behaviour can be made in the future.

1.2.5 Mural Cells

As already mentioned, the endothelium is the innermost layer of cells lining the vascular lumen. Depending on vessel type, diameter and location, the endothelium will be associated with further cell types that form additional layers. The first cells to be recruited to newly formed endothelial tubes are vascular pericytes. Pericytes are loosely associated with the microvascular network of arterioles, capillaries and post capillary venules, where they extend long processes which engulf the vessel tube and often cross endothelial cell boundaries. As a characteristic feature, pericytes share a basement membrane with the endothelium, whereas the continuous layer of vascular smooth muscle cells which covers larger arteries and veins is separated from the endothelium by the internal elastic membrane. In contrast to pericytes, vascular smooth muscle cells display a spindle-like morphology and concentrically enclose the vessel perpendicular to direction of flow. Pericytes and vascular smooth muscle cells are jointly referred to as mural cells. A clear distinction between the two cell types is challenging and not necessarily practical in all cases, as mural cell morphology and coverage changes gradually between the typical smooth muscle layer of large vessels and the solitary pericytes associated with the capillaries (Armulik et al 2005). In addition, there are no available biomarkers which are exclusive to either cell type and ultrastructural analysis is not always feasible (Armulik et al 2011).

In larger veins and arteries, the layer of contractile vascular smooth muscle can be many cells strong (up to 50 in an adult human aorta) and has important functions in the regulation of vascular tone. As such, it is also referred to as tunica media, as opposed to the tunica intima, the endothelium, and the tunica adventitia, a third layer of connective tissue which contains fibroblasts, immune cells, nerves and smaller

vessels (Fig. 1.5). This outer layer offers further structural support and anchors the vessel in the surrounding tissue. Recently, the tunica adventitia has also been sparked interested as a potential source of smooth muscle and endothelial progenitor cells (Majesky et al 2011).

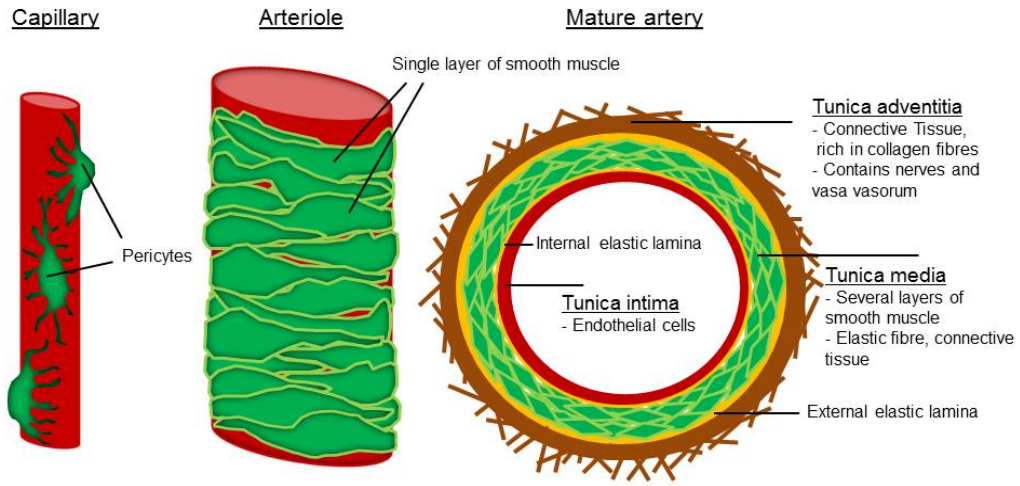

Figure 1.5: Mural cells coverage in the arterial circulation

Capillaries are only loosely covered by individual pericytes. Coverage density varies greatly among different tissues. Equally, contractility and the ability to set capillary tone may differ between capillary beds in different organs.
Arterioles are covered by a single layer of contractile smooth muscle cells which play a central role in setting vascular tone.
In mature arteries, the tunica media is invested with several layers of vascular smooth muscle, as well as elastic and collagen fibres which confer both contractility and elasticity to the vessel. An outer tunica adventitia, rich in collagen fibres, anchors the artery in the surrounding tissue. It also contains a blood supply of its own, the vasa vasorum.

Mural cell origins

It is not clear if pericytes and vascular smooth muscle cells arise from a common progenitor. Although a common ancestry of both cell types is widely assumed in the field of vascular biology, due to their similar gene expression profiles

and anatomical location, investigations are complicated by a complex ontogeny. Fate mapping studies have described at least eight different origins for vascular smooth muscle cell progenitors (Majesky et al 2011). The developing aorta, for example, is first associated with smooth muscle cells derived from the lateral plate mesoderm before it is colonized by somite-derived smooth muscle cells around E10.5 (Wasteson et al 2008). Later, somite-derived vascular smooth muscle cells are found in the ascending aorta, while smooth muscle cells of the descending aorta and the aortic arch originate from the splanchnic mesoderm and the neural crest, respectively (Majesky et al 2011). Recruitment of pericytes and vascular smooth muscle cells also does not seem to follow an entirely uniform mechanism. In central nervous system development, for example, pericytes migrate and proliferate along with the expanding vasculature (Winkler et al 2011). In this longitudinal recruitment all pericytes originate from a common pool of progenitors. In other situations, pericytes are locally differentiated from a variety of different cell types, including mesenchymal stem cells, fibroblasts or epithelial cells (Berthod et al 2012, Díaz-Flores et al 2009, Mellgren et al 2008). There are also reports describing a mechanism by which pericytes invade avascular tissues prior to endothelial cells and form endothelium-free tubes which provide a scaffold for the angiogenic sprout (Ozerdem and Stallcup 2003). It is not clear, however, if this alternative mechanism is a widespread occurrence or specific to certain tissues and developmental stages.

In zebrafish, mural cells are first observed around 3.5dpf in the bulbus arteriosus and the ventral aorta. Mural cell coverage then steadily spreads to other parts of the vasculature over the next ten days of development (Santoro et al 2009, Whitesell et al 2014). While it is assumed that, like in other vertebrates, mural cells in

the zebrafish trunk originate from the lateral plate mesoderm, definite evidence from lineage tracing experiments is lacking so far (Santoro et al 2009).

Pericyte function

Pericytes have important functions in vascular development. Upon recruitment, they stabilize immature endothelial tubes by contributing to extracellular matrix deposition and possibly by offering direct structural support (von Tell et al 2006). Paracrine signalling between pericytes and endothelial cells triggers endothelial growth arrest while promoting survival (Armulik et al 2005). Therefore, pericytes may render the endothelium less responsive to angiogenic signalling and instead promote vessel maturation. This is supported by observations in platelet-derived growth factor receptor beta (Pdgfrβ) – deficient mice which lack pericyte coverage in large parts of the vasculature (Lindahl et al 1997). While the vascular network in these animals develops to normal dimensions, it shows irregularities in vessel calibre as well as endothelial hyperplasia, indicating dysfunctional maturation and remodelling (M. Hellström et al 2001). Conversely, in the quiescent vasculature of the adult where angiogenic growth factors are not present, pericyte deficiency leads to vessel regression suggesting endothelial dependency on pericyte-derived survival signals (Winkler et al 2011). The mice die perinatally as a result of widespread haemorrhage, partly caused by micro aneurysms and edema. This suggests that apart from their role in vessel remodelling and stability, pericytes also have an effect on endothelial barrier function (Díaz-Flores et al 2009). This is further supported by the fact that pericyte coverage of capillary beds differs considerably among organs and correlates inversely with vessel permeability. While the pericyte to endothelial cell ratio is, for example, around 1:1 in the nervous system, it is 1:10 in lung capillaries, and only 1:100 in striated muscle (Shepro and Morel 1993).

Pericytes and the blood brain barrier

In the central nervous system, pericytes are involved in the maintenance of a very low permeability endothelium referred to as the blood brain barrier. The blood brain barrier is realized by limiting both intercellular permeability through increased numbers of tight junctions between endothelial cells, as well as transcellular passage of molecules by tightly regulating endocytosis. It was shown that reduced pericyte coverage compromises blood brain barrier function in embryonic and adult mice, and affects both tight junction formation and transcytosis (Armulik et al 2010, Daneman et al 2010). In the adult brain, mural cell loss can lead to brain vascular damage, including diminished capillary perfusion, impaired cerebral blood flow-response to stimuli and blood brain barrier breakdown (Bell et al 2010). Pericyte deficiency also has an effect on endothelial gene expression causing upregulation of genes associated with increased vascular permeability such as *Angpt2*, *Plvap* and leukocyte adhesion molecules (Daneman et al 2010). Therefore, pericytes do not merely present an additional physical barrier but also modulate endothelial behaviour.

Pericytes in the regulation of blood flow

While the function of vascular smooth muscle cells in the regulation of vascular tone and blood flow is widely appreciated, it has been a matter of debate whether pericytes also play a role in the regulation of blood flow in capillary beds (Armulik et al 2011). While it has been shown that pericytes are contractile and can affect capillary diameter, physiological effects could only be shown for vascular smooth

contraction at arterioles (Fernández-Klett et al 2010, Peppiatt et al 2006). Recently, a study in rats demonstrated that increased neuronal activity triggers the release of messengers which activate an outward membrane current in pericytes that results in capillary dilation and increased blood flow (Hall et al 2014). Moreover, the pericyte-response preceded that of the arterioles, underscoring the importance of pericytes in regulating blood supply to neural tissues. Interestingly, the response can be propagated between adjacent pericytes, but it is not clear whether the signal then extends onto arterioles or if delayed arteriolar dilation is the result of greater average distance from the signalling tissues (Hall et al 2014). So far, direct regulation of blood flow by pericytes has only been shown in the brain. Since the cerebral capillaries have the highest pericyte coverage of all tissues, this effect may well be specific to the central nervous system. In fact, it is difficult to conceptualize how a similar mechanism could be in place, for example, in striated muscle where there is only a single pericyte for every 100 endothelial cells. Further, since pericytes do not express the same set of genes in all tissues, not all pericytes necessarily possess the ability to contract. For example, *Acta2*, the gene coding for alpha smooth muscle actin (αSMA), a major constituent of the contractile apparatus, is absent in certain capillary beds (Nehls and Drenckhahn 1991). An interesting question to address is whether expression of genes involved in contractility in pericytes depends on the hemodynamic environment of the capillary bed in which they reside.

In general, it is important to consider that pericytes are classified as such mainly based on their location in immediate proximity to the endothelium and not necessarily based on functional characteristics. In fact, it may be useful to think of pericytes as a histovascular interface, the functional characteristics of which reflect the specific requirements of the tissue from a broad, pericyte-inherent repertoire.

1.3 Regulatory Pathways in Endothelial Development

1.3.1 The Notch signalling pathway in endothelial cell development

The Notch pathway is an evolutionary conserved, intercellular signalling pathway with central functions in the regulation of cell-to-cell communication (Fig. 1.6). Notch signalling plays a key role in the development and homeostasis of the vasculature and coordinates several aspects of both endothelial and mural cell behaviour.

In mammals there are four Notch receptors (Notch1, Notch2, Notch3, and Notch4) and five Delta/Serrate/Lag-2 (DSL) ligands: Delta-like1 (Dll1), Delta-like3 (Dll3), Delta-like4 (Dll4), Jagged1 (Jag1), and Jagged2 (Jag2) (D'Souza et al 2010). Both receptors and ligands are transmembrane proteins with a large extracellular domain that consist primarily of epidermal growth factor-like repeats (D'Souza et al 2010). Accordingly, for a signalling event to take place, direct physical contact of receptor and ligand-expressing cells is necessary. Notch receptor is synthesized as a precursor protein, which is cleaved in the endoplasmic reticulum (ER) to yield a large ectodomain and a membrane-tethered intracellular domain (Andersson et al 2011). Both domains associate in a non-covalent, calcium-dependent interaction with a third juxtamembrane heterodimerization domain (Kopan and Ilagan 2009). Ligand binding to Notch receptors allows proteolytic processing, including a metalloprotease-mediated break within the extracellular juxtamembranous region, followed by Presenilin/γ-secretase-dependent cleavage to release Notch intracellular domain (NICD) from the membrane (Kopan and Ilagan 2009). Subsequently, NICD

translocates to the nucleus where it forms a complex with Rbpj and the co-activator Mastermind (Kopan and Ilagan 2009). Rbpj is a sequence specific transcription factor which facilities transcriptional repression by recruiting histone deacetylases and other co-repressor proteins. Upon Notch receptor activation, interaction with NICD displaces the histone deacetylase/corepressor complex from Rbpj and thus allows transcriptional activation to occur at the target site (Kao et al 1998). Therefore, repression and activation via Rbpj involves the recruitment of distinct protein complexes, which influence transcription of target genes in a positive or negative fashion (Borggrefe and Oswald 2009).

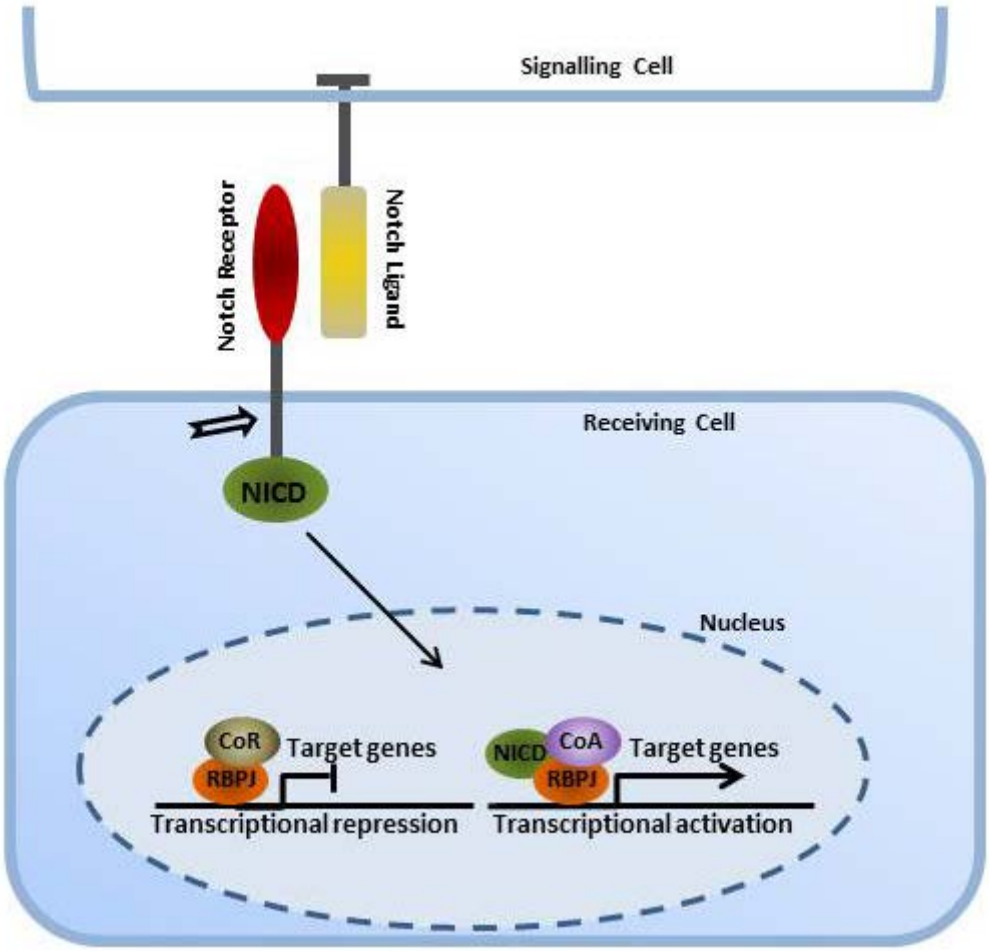

Figure 1.6: Canonical Notch Signalling

In the absence of Notch receptor activation, Rbpj and corepressor (CoR) complexes inhibit the transcription of target genes.
Upon Notch ligand binding, proteolytic cleavage (hollow arrow) releases the Notch intracellular domain (NICD) from the cell membrane. NICD translocates to the nucleus, where it forms a complex with Rbpj, displaces corepressors and recruits coactivators (CoA), thereby inducing the transcription of target genes.

While Rbpj can bind at many regulatory regions throughout the genome, certain direct Notch targets such the basic helix–loop–helix (bHLH) transcription factors Hairy/Enhancer of Split (Hes) and Hes-related proteins (Hey/Hrt/Herp) are of special importance as they broaden the gene regulatory range downstream of Notch signalling (Guruharsha et al 2012). The Notch pathway itself is subject to extensive positive and negative regulation. As the effect of Notch signalling varies with dosage

and chromatin accessibility in signal-receiving cells, its output is highly dependent on cellular and developmental context (Andersson et al 2011).

The Notch receptors, Notch1 and Notch4, as well as the ligands Dll1, Dll4, Jag1, and Jag2 are all expressed in certain populations of endothelial cells and control various aspects of vascular development (Gridley 2010). Deletion of Notch1 in mice leads to embryonic lethality around E9.5 and causes defects in somitogenesis and severe cardiovascular anomalies (Conlon et al 1995, Swiatek et al 1994). The critical role of Notch1 is even more apparent in the endothelial-specific knockout, which is lethal after E10.5 due to substantial irregularities in vessel remodelling in placenta, yolk sac, and the embryo proper (Limbourg et al 2005). Loss of Notch4, on the other hand, does not result in an obvious vascular phenotype and mice are viable and fertile (Krebs et al 2000). However, combined loss of both receptors produced a more severe phenotype than Notch1 deletion alone, suggesting partial redundancy (Krebs et al 2000). During vasculogenesis, Notch is required for the specification and migration of angioblast subpopulations from the lateral mesoderm during formation of the dorsal aorta in chicken and zebrafish (Sato et al 2008, Zhong et al 2001). In fish, the Notch target gene *gridlock* has been described as major downstream effector in this process (Zhong et al 2001). In mice, the gridlock homologue Hey2 and Hey1 could be identified as key mediators of Notch signalling in the vasculature (Fischer et al 2004).

Notch signalling also controls subsequent endothelial specification towards an arterial or venous phenotype. In zebrafish and mice, absence of Notch signalling leads to a reduction in arterial endothelial cell numbers accompanied by decreased expression of Efnb2 and ectopic expression of Ephb4 on arterial endothelial cells and it has been shown that Efnb2 is a direct transcriptional target of Notch (Grego-

Bessa et al 2007, Kim et al 2008, Zhong et al 2001). In venous endothelial cells Notch signalling appears to be repressed by the transcription factor chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) and loss of COUP-TFII leads to arterial endothelial cell expansion during early embryonic development in the mouse (You et al 2005). However, while knockdown experiments in *Xenopus laevis* and zebrafish confirmed the importance of the COUP-TFII homologue Nuclear Receptor 2F2 (*nr2f2*), no evidence was found for Notch-mediated repression as an expansion of arterial markers into venous vessels was not observed (Aranguren et al 2011).

Within the vasculature both Notch1/4 and the Notch ligand Dll4 are predominantly expressed in arteries (Shutter et al 2000, Villa et al 2001). A null mutation of Dll4 results in haploinsufficiency with lethality at E9.5 in certain breeding backgrounds (Duarte et al 2004, Gale et al 2004, Krebs 2004). As in Notch1 mutants, Dll4 knockout mice fail to remodel the primitive vascular plexus in both the yolk sac and the embryo proper. In addition, the phenotype was characterized by reduced aorta size and ectopic expression of venous markers in the aorta, indicating impaired arterial specification. Other defects include reduced vascular sprouting, endothelial cell proliferation, and migration. These findings suggest that Dll4 is an early and critical ligand for Notch1/4 signalling in the vasculature, while also highlighting the importance of dosage in Notch signalling. This is supported by experiments in which overexpression of Dll4 in mice was lethal around E10.5 and produced premature arteriovenous fusion, while veins displayed ectopic expression of arterial markers (Trindade et al 2008).

Dll4-Notch signalling also plays a central role for tip/stalk cell dynamics during sprouting angiogenesis. Close examination of the angiogenic front revealed elevated

levels of Dll4 expression in the tip cells of wild-type mice and a hypersprouting phenotype with increased tip cell formation in Dll4 knockouts (Hellström et al 2007, Leslie et al 2007, Lobov et al 2007, Siekmann and Lawson 2007, Suchting et al 2007). Expression of Notch1, on the other hand, is higher in stalk cells and ectopic Notch activation in tip cells results in decreased sprouting. Further, it has been shown that endothelial cells with active Notch signalling are rarely found at the tip of the angiogenic sprout, whereas Notch deficient endothelial cells preferentially assume this position (Phng and Gerhardt 2009). Consequently, current models assume that tip cell expression of Dll4 induces Notch signalling in adjacent cells to promote the stalk cell phenotype (Phng and Gerhardt 2009). Tip/stalk cell selection is further modulated by the Notch ligand Jag1, which is expressed in stalk, but not in tip cells (Benedito et al 2009). Endothelial-specific deletion of Jag1 in mice resulted in impaired retinal angiogenesis with a lower density of blood vessels and fewer tip cells compared to wild-type (Benedito et al 2009). Conversely, overexpression of Jag1 in retinal endothelial cells leads to increased sprouting and tip cells numbers. The investigators further demonstrated that expression of the Notch target genes *Hey1* and *Hes1* increases in Jag1-deficient endothelial cells, indicating that Notch signalling is repressed by Jag1 (Benedito et al 2009). This regulatory effect of Jag1 on Notch signalling has been shown to involve the glycosaminyl transferases Manic Fringe. Fringe family proteins mediate posttranslational modification of Notch and reportedly enhance Notch activation by Delta family ligands but reduce Notch activation by Jagged family ligands (Andersson et al 2011). It is assumed that Jag1 on stalk cells actively represses Notch signalling in tip cells, whereas Dll4 directly activates Notch in the stalk, thus maintaining a sharp separation of the two phenotypes in the sprout (Suchting and Eichmann 2009). However, experiments

using time-lapse microscopy revealed that tip/stalk cell designation involves a high degree of plasticity that allows for dynamic tip-stalk cell shuffling as a result of constant re-evaluation of environmental cues (Jakobsson et al 2010).

1.3.2 Vegf signalling in endothelial cell development

While Notch signalling is key to controlling the angiogenic response among endothelial cells through regulation of sprouting and establishment of tip/stalk cell borders, the signal for the initiation of this process is delivered mainly through vascular endothelial growth factors (Vegfs) from extra-endothelial sources. The Vegf family is composed of five structurally related factors: VegfA, VegfB, VegfC, VegfD and placenta growth factor (Pigf), which, in their mature state, are predominantly present as homodimers (Olsson et al 2006). Their complexity is further increased by alternative splicing (for VegfA, VegfB, and Pigf) and posttranslational modification (for VegfA, VegfC, and VegfD), resulting in altered receptor affinities and bioavailability (Olsson et al 2006). For example, in humans, VegfA is alternatively spliced to generate $VegfA_{121}$, $VegfA_{145}$, $VegfA_{165}$, and $VegfA_{189}$ (numbers indicating the number of amino acid in the polypeptide chain) (Olsson et al 2006). Whereas $VegfA_{121}$ is freely diffusible, the heparan sulphate binding isoforms $VegfA_{165}$ and $VegfA_{189}$ can be retained in the extracellular matrix or on cell surfaces (Ferrara 2010). VegfA has a critical function in endothelial development and is the most extensively studied member of the Vegf family. It is expressed by the extraembryonic visceral endoderm of mice as early as E7.5, which coincides with blood island formation in the yolk sac (Risau and Flamme 1995). Heterozygous loss-of-function mutants of VegfA are lethal between E11.5 and E12.5 due to defective vascular

development and VegfA deficient embryonic stem cell failed to develop vessel-like patterns in an *in vitro* vasculogenesis assay (Ferrara et al 1996).

Three Vegf tyrosine kinase receptors have been identified in mammals (Vegfr1-3), all of which are expressed by populations of endothelial cells: The fms-like tyrosine kinase Flt1 (Vegfr1), the kinase domain region (Kdr), more commonly referred to as fetal liver kinase-1 (Flk1/Vegfr2), and fetal liver kinase-4 (Flt4/Vegfr3) (Olsson et al 2006). A fourth member of the Vegf receptor family has been described in other vertebrates. In zebrafish, this fourth member was initially described as a *Kdr* orthologue, *kdra,* due to its high similarity with the mammalian gene (Habeck et al, Schulte-Merker 2002, Liao et al 1997). However, a second *Kdr* orthologue, *kdrb*, was also described later. More recent studies revealed a common ancestry between *kdrb* and the human *Kdr* gene, whereas *kdra* is thought to have been lost in mammalian evolution (Bussmann et al 2008). Therefore, studies on zebrafish *kdra* cannot be considered meaningful for the biology of the mammalian Vegfr2. To avoid further confusion in the literature, *kdra*, which has been cited as the zebrafish orthologue of the human Vegfr2 gene in many studies, was recently renamed *kdr-like,* and *kdrb* has been named *kdr* to reflect its relation to the mammalian gene (Bussmann et al 2008).

Each Vegf receptor has seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence interrupted by a kinase insert domain (Olsson et al 2006). VegfA signals through its main receptors, Vegfr1 and Vegfr2. Although Vegfr2 has a lower affinity for VegfA than Vegfr1, it has stronger tyrosine kinase activity, and VegfA responses in endothelial cells and their precursors are usually attributed to Vegfr2 activation (Koch

and Claesson-Welsh 2012). Mice lacking Vegfr2 die at E8.5 to 9.5 and lack blood island and vascular plexus development, despite normal angioblasts formation in mouse chimeras (Shalaby et al 1995, 1997). Consistent with this, Vegfr2 deficient embryonic stem cells can generate endothelial cells *in vitro*, however, receptor signalling is required for their subsequent migration and proliferation (Schuh et al 1999). The role of Vegfr1 seems to be in the negative regulation of Vegf receptor signalling. While Vegfr1 deletion in mice results in embryonic lethality after E9.5, arteriovenous differentiation is not affected. Instead, endothelial hyperproliferation leads to abnormal angiogenic development in these animals (Fong et al 1995). Further, it has been shown that mice lacking Vegfr1 kinase activity are viable and display normal vascular development (Hiratsuka et al 1998). An alternatively spliced, soluble isoform of Vegfr1 without an intracellular domain has also been described, the primary function of which appears to lie in the sequestration of Vegf (Kendall and Thomas 1993). Together, these results implicate that the main function of Vegfr1 in vascular development is to buffer the effect of pro-angiogenic VegfA.

Upon dimerization, Vegfr2 is phosphorylated at different tyrosine residues, allowing binding of several different factors (Koch and Claesson-Welsh 2012) (Fig. 1.7). Recruitment and activation of PLCγ catalyses the generation of diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which culminates in the release of intracellular calcium and activation of protein kinase C (PKC). Intracellular calcium levels are involved in the regulation of vascular tone and permeability (Ando and Yamamoto 2013). PKC stimulates the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase-1/2 (Erk1/2) cascade (Raf–MEK–Erk), which promotes endothelial cell proliferation (Takahashi et al 1999). Mice that express a mutated version of Vegfr2, unable to bind PLCγ, die at E8.5 to E9.5 due to

vascular abnormalities that resemble those observed in Vegfr2 knockouts, suggesting an essential function for the respective tyrosine residue (Sakurai et al 2005). Activation of PI-3 kinase (PI3K) by Vegfr2 induces the serine/threonine kinase Akt/PKB to mediate cell survival (Karkkainen and Petrova 2000). Interestingly, Akt activation may also antagonize signalling through PLCγ and the Raf–MEK–Erk pathway to block arterial endothelial differentiation (Hong et al 2006). Other interactions of Vegfr2 with, for example, Ras GTPase-activating protein and Src family proteins are less clear. Overall, these observations indicate that Vegfr2 activation feeds into intracellular signalling pathways which elicit distinct effects on endothelial cells.

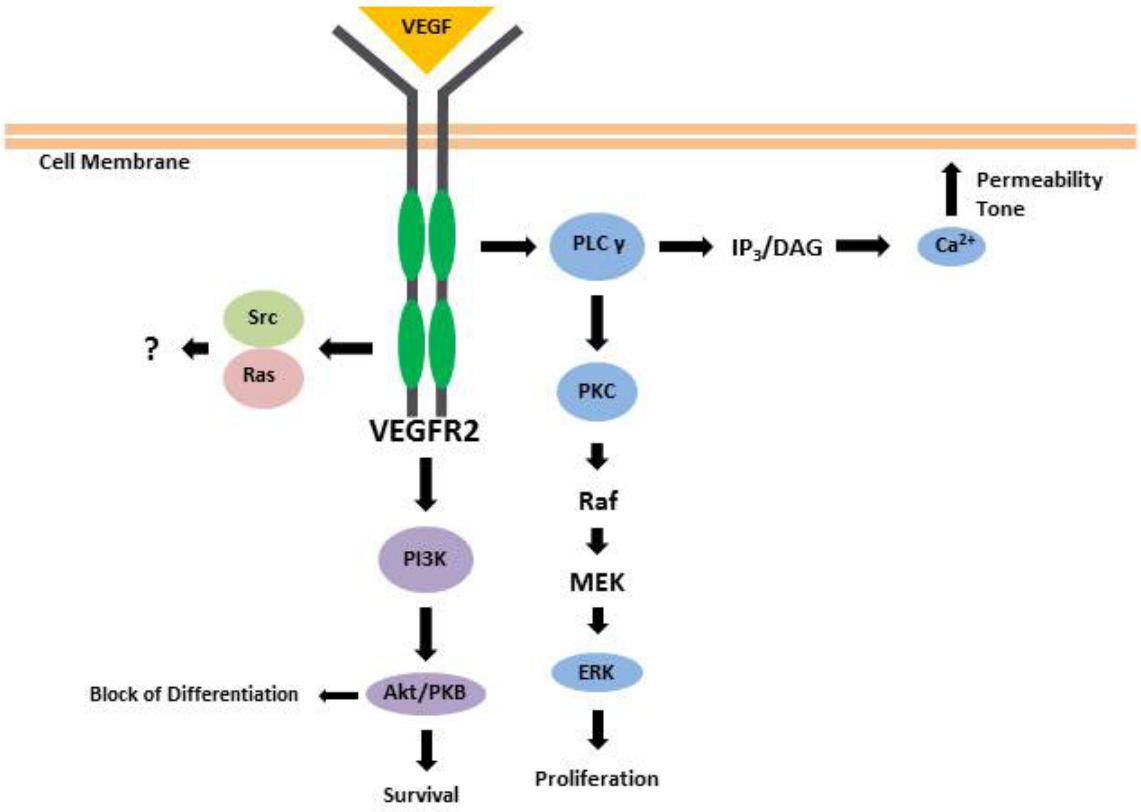

Figure 1.7: Vegfr2 signalling

Receptor dimerization upon binding of Vegf leads to activation of PLCγ, which catalyses production of IP_3/DAG and subsequent release of intracellular calcium. Calcium levels are involved in the regulation of vascular tone and permeability.
PLCγ also activates PKC which triggers Raf–MEK–Erk cascade and promotes endothelial cell proliferation.
Vegfr2 activation further induces PI3K to activate the Akt/PKB pathway, which promotes cell survival and may also block differentiation.
The roles of Src and Ras activation by Vegfr2 are less clear.

Apart from its critical function in the expansion of angioblasts during vasculogenesis, Vegf signalling is also implicated in the establishment of the arterial endothelial cell phenotype. In mice and zebrafish, Vegf signalling has been reported to act downstream of hedgehog and upstream of Notch (Coultas et al 2010, Lawson et al 2002). This is supported by experiments demonstrating that, while suppression of hedgehog activity leads to Vegf down-regulation and prevents arterial differentiation, this can be rescued by overexpression of Vegf, indicating that hedgehog acts upstream of Vegf (Lawson et al 2002). On the other hand, Vegf is

unable to rescue arterial marker gene expression in embryos lacking Notch function, whereas exogenous Notch activity can induce arterial differentiation in the absence of Vegf (Lawson et al 2002). However, Vegf-mediated induction of Notch receptors requires intact Notch signalling itself. Therefore, Vegf alone is not sufficient to induce gene expression in this case, and control of arterial gene expression by Vegf and Notch is likely to involve complex transcriptional inputs acting at artery-specific gene regulatory elements (Siekmann et al 2008).

VegfA is also a key inducer of sprouting angiogenesis. Hypoxic cells secret VegfA in a process involving hypoxia-inducible factors (HIFs). Under normoxic conditions, the alpha subunit of the heterodimeric transcription factor HIF1 is hydroxylated by prolyl-hydroxilases, which targets them for ubiquitination and subsequent degradation by the proteasome (Kaelin and Ratcliffe 2008). In hypoxia, however, oxygen-dependent hydroxylation is diminished allowing HIF1 heterodimer stabilization and activation of target genes including Vegfs. The importance of Vegf gradients in directing the formation of new capillaries has been elegantly shown in the vascularization of the mouse retina (Gerhardt et al 2003). In this model, extracellular Vegf gradients stimulate endothelial sprout formation through induction of tip cells, which migrate along the gradient. Interestingly, mutational restriction of VegfA expression to individual isoforms resulted in distinct vascular phenotypes (Ruhrberg et al 2002, Stalmans et al 2002). Expression of only the most soluble isoform $VegfA_{120}$ causes dilated and poorly branched vessels and was fatal in all animals within two weeks after birth (Stalmans et al 2002). Conversely, expression of only the heparin-binding $VegfA_{188}$ isoform results in thin and overly branched vessels (Stalmans et al 2002). On the other hand, mice expressing $VegfA_{164}$ exhibited normal vessel networks. Remarkably, double heterozygous mice, which expressed

both $VegfA_{120}$ and $VegfA_{188}$, but no $VegfA_{164}$, also displayed a normal vessel development (Stalmans et al 2002). This strongly suggests that regulated spatial Vegf presentation is essential for proper vessel morphogenesis (Gianni-Barrera et al 2011). One model assumes that Vegf gradient integrity is necessary to stimulate sprouting and branching, whereas overall Vegf levels regulate endothelial proliferation (Bautch 2012). This is supported by experiments in tumours, where expression of a form of VegfA that cannot be cleaved and released once bound to the extracellular matrix resulted in thin, highly branched vessels, whereas expression of the cleavable version of VegfA lead to dilated tumour vessels (Lee et al 2005). Studies further indicate that matrix-bound Vegf promotes sustained signalling through Vegfr2 and activation of downstream pathways distinct from those activated by soluble Vegf (Chen et al 2010). This might explain how Vegfr2 mediated proliferation and branching can integrate extracellular matrix properties which influence Vegf bioavailability. Finally, endothelial cells themselves express low levels of Vegf that contribute to vessel homeostasis, and perhaps also to sprouting migration via integrin regulation of Vegfr2 (Bautch 2012, da Silva et al 2010). Taken together, these data highlight that VegfA presentation is important for proper vessel morphogenesis and is controlled by several mechanisms that spatially regulate ligand availability.

Vegfr3 is present in the entire endothelium in early embryonic development, but becomes restricted to the lymphatic vasculature at later stages, where it plays a key developmental role through activation by VegfC (Kaipainen et al 1995, Schulte-Merker et al 2011). However, Vegfr3 knockout mice, which die around E10.5, display cardiovascular malformations even before the onset of lymphatic development, indicating a role for this receptor in blood vessel formation (Dumont et al 1998).

Vegfr3 is highly expressed in angiogenic sprouts, and blocking of Vegfr3 signalling with monoclonal antibodies results in decreased sprouting, vascular density, vessel branching, and endothelial cell proliferation in the mouse retina (Tammela et al 2011). Therefore, Vegfr3 signalling contributes to vascular sprout formation by activation through VegfC. In the same model, however, deletion of Vegfr3 caused a hypersprouting phenotype reminiscent of the hyperplastic vascular pattern observed in Dll4/Notch signalling-deficient animals, suggesting that it also has an opposing effect on vessel sprouting, independent of VegfC (Tammela et al 2011). Indeed, decreased levels of Dll4 were detected upon Vegfr3 deletion but not after blocking antibody treatment (Tammela et al 2011). Further supporting this model, it was shown that the tyrosine kinase domain of Vegfr3 can be phosphorylated following endothelial cell binding to Collagen I, which is involved in cell adhesion during migration (Tammela et al 2011). Interestingly, activated Vegfr3 was capable of inducing Notch target gene expression even in the presence of a potent Notch inhibitor (Tammela et al 2011). It was shown that this non-canonical Notch signalling is dependent on PI(3)K/Akt and involves induction of Notch target genes through the transcription factor FoxC2 (Tammela et al 2011). Therefore, Vegfr3 can be activated by distinct inputs which trigger different cellular outcomes. While active induction via VegfC induces a stalk cell phenotype in adjacent endothelial cells via upregulation of Dll4, it also contributes to the establishment of the tip cell phenotype. In contrast, passive Vegfr3 signalling through binding of Collagen I only upregulates Dll4. Consequently, antibody blocking of Vegfr3 results in fewer tip cells, whereas loss of the entire receptor is accompanied by reduced stalk cell formation and hypersprouting (Thomas et al 2013). Surprisingly, Notch inhibition leads to upregulation of Vegfr3, but had no significant impact on Vegfr2 expression and

induced deregulated endothelial sprouting and proliferation even in the absence of Vegfr2 (Benedito et al 2012). It appears, therefore, that Vegfr2 and Vegfr3 are regulated in a highly differential manner by Notch and that, while Vegfr3 is normally an antagonist of sprouting, in a situation of minimal Notch activity, it may become an agonist (Benedito and Hellström 2013).

1.3.3 Wnt signalling in endothelial cell development

In general, cooperation and coordination of different inputs, as seen for Vegf and Notch signalling, is essential for meaningful biological responses. Another example of this is the involvement of the Wnt signalling pathway is the regulation of vascular bed maturation. The Wnt family contains 19 highly conserved glycoproteins, which signal via Frizzled receptors on the cell surface to trigger various intracellular downstream cascades controlling cell fate, proliferation, apoptosis, migration and polarity (MacDonald et al 2009). Wnt signalling pathways can be categorized into canonical (β-catenin dependent) and non-canonical (β-catenin-independent) branches, which include planar cell polarity (PCP) and Wnt/Ca^{2+} signalling (MacDonald et al 2009). In the canonical pathway, receptor activation leads to an accumulation of the transcriptional cofactor β-catenin, which interacts with the Tcf/Lef family of transcription factors to activate transcription of target genes. Endothelial-specific deletion of β-catenin in mice is lethal after E12.5, with embryos displaying altered patterning of the vasculature and irregular vascular lumen morphology (Cattelino et al 2003). Similarly, an endothelial-specific gain-of-function mutation for β-catenin was embryonically lethal with signs of defective vessel sprouting, branching, and lumen formation. Additionally, arteriovenous specification

was affected, which was linked to elevated levels of Dll4 (Corada et al 2010). On the other hand, Notch-regulated ankyrin repeat protein (Nrarp), a direct downstream target of Notch promotes canonical Wnt signalling via stabilization Tcf/Lef-1 (Phng and Gerhardt 2009). Nrarp also acts as a negative regulator of Notch and thereby coordinates Notch and Wnt signalling in the stalks of angiogenic sprouts to promote stabilisation of the nascent vessel and endothelial cell proliferation (Phng and Gerhardt 2009). This is in line with observations from *Nrarp*$^{-/-}$ mice, in which endothelial cell proliferation and vessel density are reduced (Phng et al 2009). Moreover, it has been shown that β-catenin transcriptional activity also contributes to vessel stabilization through the direct induction platelet-derived growth factor-B (PdgfB) expression in endothelial cells and subsequent recruitment of mural cells (Reis et al 2012).

There is also evidence for interactions of the Wnt and Vegf pathways. In mouse retinas, Wnt ligands secreted by retinal myeloid cells induce expression of Vegfr1 through non-canonical Wnt/Ca^{2+} signalling to suppress angiogenesis and vessel branching. A similar mechanism was also described in macrophages during the regulation of angiogenesis during wound healing (Stefater et al 2013). In a different study the receptor Frizzled4 (Fzd4) was shown to signal through the non-canonical Wnt/PCP pathway and impair vascular cell proliferation and migration (Descamps et al 2012). More specifically, Fzd4 deletion in post-ischemic neoangiogenesis is characterized by a disorganized, non-directional, and non-functional arterial network suggesting a role for Fzd4 in the organisation of arterial vessels.

1.3.4 BMP signalling in endothelial cell development

Yet another input that contributes to the regulation of endothelial cell behaviour during vascular development is delivered by bone morphogenic protein (Bmp) / transforming growth factor-beta (Tgfβ) signalling. Evidence for this comes from studies that identified a genetic link between mutations in this pathway and hereditary haemorrhagic telangiectasia, a condition which is accompanied by arteriovenous malformations (Johnson et al 1996, McAllister et al 1994). Bmps are a group of cytokines with over 20 identified members and play essential roles in the regulation of tissue architecture throughout the body (Cai et al 2012). They can signal through the Tgfβ signalling pathway, which commonly culminates in the activation of intracellular Smad transcription factors (Pardali et al 2010). Smads regulate target gene expression by directly binding to Smad-binding elements, or indirectly through interactions with other transcription factors or association with coactivators/corepressors and histone-modifying factors. The type I Tgfβ receptors, activin receptor-like kinase Alk1 and Alk5, which activate Smad1/5 and Smad2/3, respectively, are expressed in both endothelial cells and mural cells, where they appear to trigger different, seemingly opposing, effects (Pardali et al 2010). In endothelial cells, a complex interplay between Alk1 and Alk5 signalling has been suggested, in which Alk1 inhibits Alk5, whereas Alk5 is required for Alk1 signalling (Goumans et al 2003). Overall, Alk5 seems to promote vessel maturation, whereas Alk1 has the opposite effect. The net effect may depend on the relative levels of Alk1/5 expression, as well as on the strength and duration of their activation (Goumans et al 2003). While Alk1 signalling may stimulate proliferation and migration at early stages, Alk5 may be more important for differentiation and stabilization of endothelial cells at later stages.

Knockout of most of the different Tgfβ signalling pathway genes in mice leads to embryonic lethality at midgestation (Pardali et al 2010). Deletion of endothelial Alk1, which binds Bmp9 and Bmp10, in mice is lethal around E11.5 and results in the development of arteriovenous shunts, lowered levels of arterial Efnb2 and failure to confine intravascular haematopoiesis to arteries (Urness et al 2000). In zebrafish, Alk1 has been shown to be important for shear stress-related vascular remodelling by limiting arterial vessel calibre (Corti et al 2011). Blocking of Alk1 signalling during vascularisation of the mouse retina resulted in a hypersprouting phenotype similar to that of reduced Dll4 expression (Larrivée et al 2012). Indeed, Bmp9 signalling through Alk1 was shown to induce expression of Notch target genes *Hey1* and *Hey2* as well as Jag1. Therefore, Alk1 signalling has been proposed to restrict tip cell formation by induction of Notch signalling, thus revealing an interesting collaboration of the two pathways in angiogenesis.

Endothelial-specific co-inactivation of the Alk1 downstream effectors Smad1 and Smad5 also results in defective vascular remodelling, excessive sprouting, impaired tip cell polarity, and embryonic lethality. Smad1/5 were shown to induce expression of Notch target genes both directly, and indirectly through Id proteins, and thereby promote the stalk cell phenotype (Moya et al 2012). This role for Alk1 is supported by studies reporting anti-angiogenic effects for Bmp9. However, other studies using lower doses of Bmp9 or different methods of Alk1 inhibition demonstrated contrary effects, suggesting that precise regulation of signalling input levels critically affects vascular growth (van Meeteren et al 2012, Scharpfenecker et al 2007).

In zebrafish, Bmps have been identified as a mediator of venous endothelial sprouting from the axial vein (Wiley et al 2011). The angiogenic signal was delivered

by Bmp2 independently of Vegf signalling, and involved intracellular activation of Smads and the Erk pathway (Kim et al 2012). The existence of a separate venous sprouting mechanisms is especially interesting as many factors described in the regulation of angiogenic sprouting, such as the Notch pathway components, are often restricted to the arterial tree in mature vascular beds. Therefore, current models of angiogenic sprouting may be specific to arterial angiogenesis, while venous vessel growth may rely on different mechanisms.

1.3.5 Transcription factors in endothelial development

Ets factors

E-26–specific (Ets) transcription factors are a family of at least 27 known proteins which all share a winged helix-turn-helix motif, the ETS DNA-binding domain, that binds the consensus sequence (5′-GGA(A/T)-3′) to regulate expression of target genes (Wei et al 2010). All Ets factors contain a transactivation domain, and some members of the family, Ets1, Fli1 (Friend leukemia integration 1), and Erg (Ets-related gene), contain the highly conserved PNT domain that mediates protein–protein interactions (Sharrocks 2001). Ets factors play important roles in development and in differentiated tissues and cells (Bartel et al 2000, Sharrocks 2001). They are critical for vasculogenesis, angiogenesis, haematopoiesis and neuronal development (Park et al 2013).

The importance of Ets factors for endothelial development is highlighted by the fact that almost all known endothelial enhancers and promoters contain multiple, essential ETS binding sites (Bernat et al 2006, De Val et al 2008). Within the Ets

family, Ets1, Elf1, Fli1, Tel, and Erg each have well characterized roles in endothelial gene expression, and all bind to enhancers involved in the activation of the expression of numerous endothelial genes (De Val and Black 2009). Surprisingly, for of the majority of individual Ets factors, germline deletion did not result in severe vascular phenotypes. However, this is likely to indicate redundancy among Ets factors in endothelial development, an idea supported by research of combined Ets deletions, which tend to have greater severity (Pham et al 2007, De Val and Black 2009, Guo Wei et al 2009).

An exception to this is Etv2 (also known as Ets-related 71, Er71), which has emerged as a critical regulator of vascular development on its own (Lammerts van Bueren and Black 2012). Gene knockout studies showed an indispensable function of Etv2 in both vessel and blood development. Mice deficient in Etv2 die at E9 with complete absence of vascular structures and hematopoietic cells. Studies suggest an essential role for Etv2 in the specification of $Flk1^{+}$ mesodermal progenitor cells (Ferdous et al 2009, Dongjun Lee et al 2008, Sumanas et al 2008). Etv2 expression recedes in later stages of endothelial development indicating that it is mainly involved in vasculogenesis. Another Ets factor Fli-1, expressed during the earliest stages of endothelial development, has been implicated as a crucial regulator of vasculogenesis, upstream of Gata2, although this has only been directly demonstrated in lower vertebrates (Liu et al 2008).

Gata factors

Transcription factors of the Gata family are essential regulators of the specification and differentiation of numerous tissues (Zheng and Blobel 2010). They all share 2 highly conserved zinc fingers of the C2H2 type that mediate DNA binding. Gata factors typically recognize the element (5'-(A/T)GATA(A/G)-3'). Gata2 is the

most abundantly expressed Gata factor in endothelial cells, and is also expressed in hematopoietic cells (Orkin 1992). Gata2 null mutant embryos die at E9.5 from a failure of primitive and definitive haematopoiesis (Tsai et al 1994). Several key endothelial genes contain GATA binding sites and it has been shown that Gata factors have a role in the regulation of many endothelial specific genes including endothelin-1, von Willebrand factor, and VE-cadherin, metalloproteinase-2 and Endomucin (Han et al 2003, Kanki et al 2011, Park et al 2013, De Val and Black 2009). However, endothelial specific deletion of *Gata2* in mice, and mutation in fish, has limited effects on early vascular formation, suggesting that its function in the vasculature may be redundant. Other Gata factors, including Gata3, are also widely expressed in the vasculature but to date have not been studied in depth (Song et al 2009, De Val 2011).

Sox factors

Sox7, Sox17 and Sox18 comprise the SOXF sub-group of transcription factors within the extensive Sox transcription factor family. SoxF factors have a pivotal role in cardio-vascular development. In zebrafish, where there are only two SoxF factors expressed in the vasculature, they have been demonstrated to play a crucial role in arteriovenous differentiation (Cermenati et al 2007, Herpers et al 2008, Pendeville et al 2008, Sakamoto et al 2007). Combined Morpholino-induced knock-down of *sox7* and *sox18* results in severe arterial defects and partial loss of arterial identity, although initial formation of the dorsal aorta does occur. However, when knockdown of *sox7* and *sox18* is performed in the absence of Notch signalling, arterial specification is abolished, suggesting that the SoxF factors regulate arterial identity in a convergent pathway with Notch (Sacilotto et al 2013). The role of SoxF factors in

mice has been difficult to establish, as Sox7, Sox17 and Sox18 are all transiently expressed in endothelial cells with distinct but partially overlapping patterns. Sox18 has been implicated in lymphatic development in mice, whereas Sox17 null mice have arterial defects (François et al 2008). However, SoxF factors are also important for haematopoiesis, complicating analysis, and combinatorial deletion of all SoxF factors in mice has not yet been effectively achieved (Cermenati et al 2007, Herpers et al 2008, Pendeville et al 2008, Sakamoto et al 2007).

Fox factors

Several Forkhead box (Fox) transcription factors play essential roles in vascular development and members of the FoxC, FoxF, FoxH, and FoxO families are all expressed in endothelial cells (Papanicolaou et al 2008, De Val and Black 2009). Targeted disruption of *FoxO1* in mice causes midgestational lethality with defects in vessel remodelling (Furuyama et al 2004, Hosaka et al 2004, Kume et al 2001). FoxO factors act as positive and negative regulators of transcription and both FoxO1 and FoxO3 have been shown to inhibit tube formation and migration of endothelial cells *in vitro* (Matsukawa et al 2009).

FoxF1 is expressed in the splanchnic mesoderm prior to endothelial cell specification and its inactivation in mice results in a severe vascular phenotype and embryonic lethality in mice (Mahlapuu et al 2001). In zebrafish, FoxH1 has been shown as a negative regulator of *kdr-like* expression, suggesting that it may act as an inhibitor during vascular development (Choi et al 2007).

Combined deletion of FoxC1 and FoxC2 in mice is lethal at midgestation, and leads to severe defects in vessel formation and repression of arterial marker

expression (Seo et al 2006). In addition, FoxC1 and FoxC2 are important for arterial and lymphatic endothelial cell specification and may function downstream of Notch signalling (Hayashi and Kume 2008). In zebrafish, loss of *foxc1a* alone causes severe vascular abnormalities and depletion of *foxc1b* causes defects in arteriovenous differentiation (De Val 2011). It has been shown that a composite binding motif which is recognized jointly by FoxC2 and Etv2 (FOX:ETS motif) is found in many endothelial-specific regulatory elements and can be used to predict novel endothelial enhancers (De Val et al 2008). Therefore, it has been suggested that Fox and Ets factors have a synergistic role in the regulation of gene expression in endothelial development (De Val 2011).

1.4 Regulatory Pathways in Mural Cell Development

1.4.1 PDGF signalling

The Pdgf family of glycoproteins has four members (PdgfA, B, C, and D), encoded by four different genes. These form dimers to create five different isoforms (PdgfAA, -BB, -CC, -DD, and -AB), which bind to one of two Pdgf receptor tyrosin kinases, Pdgfrα and Pdgfrβ (Fredriksson et al 2004). Pdgfrα has high affinity for PdgfAA, PdgfBB and PdgfAB, whereas Pdgfrβ preferentially binds PdgfBB and PdgfAB (Fredriksson et al 2004). Binding facilitates receptor dimerization which is required for receptor activation.

During angiogenesis, sprouting endothelial cells secrete PdgfBB, which signals through Pdgfrβ expressed by pericytes and induces activation of several well-characterized signalling pathways such as Ras-MAPK, PI3K, and PLC-γ (Andrae et al 2008). Rather than each mediating specific cellular events, these pathways cooperatively promote pericyte growth, migration and differentiation. Evidence for this comes from a study using a series of targeted Pdgfrβ mutations, which inhibit activation of individual downstream signalling pathways by replacing critical tyrosine residues with phenylalanine (Tallquist et al 2003). Surprisingly, the requirements for different signalling pathways appeared to be additive rather than specific for pericyte recruitment, which was gradually reduced with increasing numbers of mutations (Tallquist et al 2003). Additionally, it was found that even disruption of all major known downstream signalling pathways did not result in lethality even in hemizygous combination with the *Pdgfrβ*-null allele, although homozygous *Pdgfrβ*-nulls rarely survive until birth (Tallquist et al 2003). These results point to a yet undescribed

downstream signalling pathway in Pdgfrβ activation, or a different, elusive function of the receptor. Moreover, conditional knockout of Pdgfrβ in mural cells, using the mural cell-specific sm22α-Cre did not phenocopy global Pdgfrβ ablation but resulted in viable, fertile animals, although lack of mural cell coverage was observed (French et al 2008). However, when both Pdgfrβ and Pdgfrα were deleted, embryos died after E10.5 and showed defective development of the yolk sac vasculature, suggesting that Pdgfrα can compensate for a loss of Pdgfrβ to some extent (French et al 2008). This may also explain why, even in *Pdgfrβ* knockout mice, pericytes are not entirely absent, although this does not rule out the existence of alternative mechanisms of recruitment, which may be active at least in certain tissues or developmental stages (Hellström et al 2001). Further, Pdgfrβ signalling is likely not required for initial pericyte differentiation but critically important for expansion of the pericyte pool and pericyte migration along the growing vessel. This would explain why the effects of *PdgfB* deletion are most striking in the central nervous system, which lacks mesenchymal cell populations from which pericytes could be induced and instead depends on longitudinal pericyte recruitment (Armulik et al 2005). Differences in mural cell recruitment between the nervous system and other tissues have also been reported by a study addressing the effect of selective deletion of heparan sulphate in mural cells (Stenzel et al 2009). Heparan sulphate proteoglycans are important for the retention of Pdgf on the cell surface. Mural cells lacking heparan sulphate die in late gestation with reduced mural cell coverage, abnormal vessel morphology and compromised vessel stability (Stenzel et al 2009). The brain vasculature, however, is not affected by a loss of heparan sulphate suggesting that Pdgf retention is less crucial for longitudinal pericyte recruitment.

The importance of Pdgfrβ for pericyte proliferation was further demonstrated in a study which investigated the effects of constitutively active Pdgfrβ mutants in mice (Olson and Soriano 2011). The investigators observed increased pericyte coverage in vessels of the retina and the brain but did not detect higher numbers of cells expressing regulator of G-protein signaling 5 (Rgs5), an established marker of mature pericytes in the nervous system. Similarly, constitutive Pdgfrβ signalling leads to smooth muscle hyperplasia in the wall of the dorsal aorta, accompanied by a reduction in expression of markers of mature smooth muscle, such as αSMA (Magnusson et al 2007). This suggests that Pdgfrβ signalling is important for the proliferation and maintenance of immature mural cell populations but inhibits further differentiation into mature mural cells.

Intriguingly, in contrast to the global loss of *PdgfB* or *Pdgfrβ*, the endothelial-specific deletion of PdgfB was still compatible with basic vascular functions and postnatal life, despite the reduction of pericyte coverage by 90% (Enge et al 2002). While this observation does indicate that the endothelium is a major source of Pdgf, the results also imply that other sources or alternative mechanisms of recruitment are involved and further illustrate that even a severe loss of pericytes is tolerated relatively well.

As previously mentioned, the angiogenic front is exposed to gradients of Vegf that guide vessel sprouting. Surprising experiments on vascularization of matrigel plugs in chick embryos show that a combination of pro-angiogenic doses of Vegf and Pdgf results in robust suppression of angiogenesis. At the molecular level, this effect is caused by formation of a Vegfr2/ Pdgfrβ complex on mural cells in which Vegf-mediated activation of Vegfr2 blocks Pdgfrβ signalling by inhibition of receptor phosphorylation (Greenberg et al 2008). This mechanism could ensure that pericyte

recruitment is only effective in areas of lower Vegf concentrations to prevent pericyte-induced endothelial quiescence at the angiogenic sprout.

1.4.2 Notch signalling in mural cells

Notch2 and Notch3, and to a lesser extent Notch1, are all expressed in certain populations of mural cells, although only Notch3 expression is specifically concentrated in mural cells (Domenga et al 2004, Gaengel et al 2009). Similar to observations in endothelial cells, expression of Notch receptors seems to be a feature of arterial mural cells rather than venous ones (Domenga et al 2004). The importance of Notch signalling in the development of mural cells was originally recognized in studies of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Joutel et al 1996). This condition is characterized by a progressive loss of smooth muscle cells in small cerebral arteries, causing recurrent transient ischemic attacks and strokes. A genetic basis for CADASIL has been identified in dominant mutations of the Notch3 locus, which affect receptor trafficking and specifically impair the clearance of the Notch3 ectodomain from the cell surface (Joutel et al 1996, 2000). Interestingly, a loss of Notch3 does not recapitulate a similar pathology in mice. *Notch3* nulls are viable and fertile and do not show decreased proliferation or an increase in apoptosis of vascular smooth muscle cells (Domenga et al 2004). However, these mice displayed thinner arterial smooth muscle layers, and auto-regulation of cerebral blood flow in response to blood pressure increases was impaired (Domenga et al 2004). Further, arterial vascular smooth muscle cells displayed venous morphological characteristics in these mice, suggesting that Notch3 is important for the proper differentiation of

arterial smooth muscle cells (Domenga et al 2004). Interestingly, this observation was restricted to peripheral arteries, while the major trunk vessels appeared unaffected. However, in another study, abrogation of all Notch signalling in cardiac neural crest cells by expression of a dominant negative form of Mastermind resulted in decreased smooth muscle coverage of the outflow tract (High et al 2007). This observation can potentially be explained by reports of a role for the Notch2 receptor in the proliferation of neural crest-derived vascular smooth muscle cells (McCright et al 2001, Varadkar et al 2008). A combination of the Notch2 hypomorph with a loss of Notch3 is lethal around E12.5 and shows enlarged blood vessels with thin walls as well as reduced smooth muscle coverage (Wang et al 2012). However, it is not clear whether this reflects a redundant role for these two receptors or an accumulative effect in different mural cell populations. Interestingly, smooth muscle cell-specific abrogation of all Notch signalling using the dominant negative form of Mastermind did not result in an obvious cardiovascular phenotype and animals were viable despite abnormal cerebrovascular patterning, suggesting that defective mural cell development does not necessarily result in a strong phenotype (Proweller et al 2007).

A role for Notch3 in mural cell development has also been described in zebrafish, where deletion of Notch3 lead to reduced numbers of pericytes, while overexpression of the intercellular domain of Notch3 (N3ICD) leads to increased mural cell numbers (Wang et al 2014). Further, pharmacological inhibition of Pdgfrβ signalling negates the effect of overexpression of N3ICD, suggesting a role for Notch3 in the regulation of Pdgfrβ expression. This is supported by experiments in which both Notch1 and Notch3 signalling directly induced transcription of Pdgfrβ in cultured vascular smooth muscle cells (Jin et al 2008). The investigators also report

decreased levels of Pdgfrβ in the arteries of *Notch3* nulls as well as in cultured vascular smooth muscle cells of CADASIL patients. Furthermore, they describe a potential negative feedback loop in which Pdgfrβ decreases its own expression as well as expression of Notch3 after prolonged exposure to Pdgfrβ (Jin et al 2008). This could provide a potential mechanism by which mural cells first proliferate upon PdgfBB exposure and later become refractory to this growth factor in order to allow differentiation.

Of the Notch ligands, only Jag1 is expressed on mural cells, while all other ligands are found only on endothelial cells. Jag1 nulls die around E10.5 as a result of cardiovascular failure, including pericardial effusions, dilated, blood-filled vessels, and localized haemorrhage (Xue et al 1999). Closer investigation revealed decreased mural cell coverage caused by deficient vascular smooth muscle cell differentiation, which was apparent in reduced expression of markers such as SM22α and αSMA (Xue et al 1999). Jag1 expression in mural cells has also been shown to be a directly upregulated by Notch signalling through a regulatory element in its second intron (Manderfield et al 2012). Therefore, current models assume a positive feedback loop in which endothelial expression of Jag1 induces its own expression via activation of Notch signalling in adjacent mural cell precursors which then induces differentiation. Thus, the endothelial-derived “maturation signal” can spread across multiple layers of smooth muscle. Vascular smooth muscle cell-specific deletion of Jag1 seems to support this model (Feng et al 2010). In these mice, the first layer of vascular smooth muscle develops normally but subsequent layers fail to mature into contractile smooth muscle cells. However, this effect was only observed in the ductus arteriosus and the descending aorta and the mice died in the early postnatal period as a result of patent ductus arteriosus (Feng et al 2010).

1.4.3 Ang/Tie signalling in mural cells

Another pathway in endothelial-mural cell communication is the angiopoietin–Tie system. The growth factor angiopoietin 1 (Ang1) is expressed by perivascular mesenchymal cells including pericytes, while its main receptor, Tyr kinase with Ig and EGF homology domains 2 (Tie2), is found on endothelial cells. Loss of Ang1 or Tie2 in mice leads to midgestational lethality and produces very similar phenotypes, including cardiovascular defects and a lack of mural cells, suggesting non-redundant role for the receptor-ligand pair. Ang2 reportedly has an antagonistic effect on Ang1 signalling and is also expressed by endothelial cells (Maisonpierre et al 1997). This is supported by experiments showing that local application of Ang2 in the rat retina results in a dose-dependent loss of vessel associated pericytes (Hammes et al 2004).

The role of Ang/Tie signalling between pericytes and endothelial cells is not entirely clear. Although a paracrine loop including PdgfBB/Pdgfrβ and Ang1/Tie2 has been proposed to mediate endothelial maturation and stability, no molecular link between Ang-Tie and Pdgf receptor signalling has been formally established and pericyte recruitment takes place even in the absence of Tie2 or Ang1 (Augustin et al 2009). On the other hand, there is evidence supporting a model in which Ang/Tie signalling is more important for vessel integrity. When Pdgfrβ was blocked to inhibit pericyte recruitment in the developing retina, Ang1-mediated Tie2 signalling could restore survival and vascular network architecture in the absence of mural cells (Uemura et al 2002). Further, Ang1 overexpression promotes circumferential vessel enlargement by stimulating endothelial cell proliferation in the absence of angiogenic

sprouting (Thurston et al 2005). Overall, it appears pericyte-secreted Ang1 delivers an important maturation cue to endothelial cells which coincides with, but is mechanistically independent of mural cell recruitment.

1.4.4 BMP signalling in mural cells

As discussed earlier, Bmp/Tgfβ signalling plays an important role in vascular development. Manipulations that reduce Bmp levels usually result in reduced mural cell coverage and vessel dilation. However, few studies have so far focused on ablating components of the Bmp/Tgfβ pathway specifically in mural cells. Conditional inactivation of Tgfβ receptor 2 (*Tgfbr2*), using sm22α-Cre, is lethal between mid to late gestation and causes impaired vascular smooth muscle cell differentiation in the coronary vessels and the descending thoracic aorta (Carvalho et al 2007). Likewise, conditional knockout of *Alk3* using sm22α-Cre resulted in embryonic death around E11.5, with defects in vessel stabilization and severe vascular and pericardial haemorrhages (El-Bizri et al 2008). This effect was linked to a drastic reduction in expression of the Bmp target genes *Mmp2* and *Mmp9*. A similar reduction in the expression of these genes along with a comparable vascular phenotypes was observed after sm22α-Cre-driven co-deletion of the *Msx1* and *Msx2* genes, which have been implicated in Bmp signalling (Lopes et al 2011). Interestingly, mural cell-specific knockout of individual Bmp/ Tgfβ pathway components display more severe phenotypes than conditional the Pdgfrβ knockout. One explanation may involve deleterious effects on heart development, although these have been ruled out by some studies. Bmp/Tgfβ signalling has been implicated in the induction of mural cell formation from undifferentiated mesenchyme, in mural cell proliferation and differentiation, whereas Pdgf signalling appears to be required for recruitment of differentiated mural cells (Armulik et al 2011). Consequently, it seems likely that

Bmp/Tgfβ signalling acts upstream of Pdgf signalling in mural cells, which may be the basis of the more pronounced phenotype. In support of this, regulation of Pdgfrα by Tgfβ signalling has previously been described in fibroblasts (Yamakage et al 1992).

1.5 Aim of the study

The question of how distinct morphological and molecular identities of arteries, veins, capillaries and lymphatics are established and maintained is a central problem within the field of vascular biology. While it is clear that transcriptional regulation of gene expression plays a central role in the development of the vasculature, a comprehensive picture of the transcriptional regulatory pathways which govern cellular fates in blood vessels is so far lacking. Additionally, the study of these pathways has primarily focused on endothelial cells as the central cellular unit of all vessels, although it is becoming increasingly clear that vascular mural cells also possess distinct identities in different branches of the vasculature.

This study aims to investigate transcriptional regulation of arterial blood vessel identity by two different approaches:

1. The identification and characterization of an arterial specific gene enhancer to identify transcription factors involved in arterial patterning. Both *in silico* and *in vivo* methods, including zebrafish and mouse transgenics, were used to inspect the *Flk1* locus for functional endothelial enhancers driving expression to different sub-populations in the vasculature. The expression pattern of a functional, arterially restricted enhancer was then analysed during development in zebrafish and mouse transgenic models. Targeted mutations were made to transcription factor binding sites in the novel enhancers to identify binding motifs with key functions in enhancer activity, and gene silencing was employed to confirm the upstream transcription factors controlling enhancer activity and specificity.

2. The study of the functional role of a single transcription factor expressed in only the arterial vasculature. The expression pattern of Tbx2 in the vasculature was examined throughout murine embryonic development where it was established to be expressed specifically in arterial vessels. Consequently, the effects of a genetic deletion of Tbx2 were investigated to elucidate potential functions of Tbx2 in the vasculature, particularly in mural cells where it was shown to be highly expressed. To identify downstream targets of Tbx2, expression analysis of known regulators of mural cell development was performed after *in vitro* knockdown of Tbx2 and followed by *in silico* analysis of the *cis*-regulatory landscape of potential direct target genes. Further, regulation of Tbx2 expression in mural cells was investigated *in vivo* using transgenic mouse models and *in silico* analysis of the *Tbx2* locus.

Chapter 2 – Materials and Methods

2.1 Mice

Mice were bred and housed in the Functional Genomics Facility in the Henry Wellcome Building of Genomic Medicine. All animal procedures were supervised under local ethical review and approved by Home Office of the UK (PPL 30/2783). Tbx2-flox mice were previously described and a kind gift from V. Christoffels (Wakker et al 2010). Tie2-Cre mice were obtained from the Jackson Laboratory (008863) (Kisanuki et al 2001). Rosa26-CreERT2 mice were a gift from X. Lu (Ventura et al 2007). Animals were kept in individually ventilated cages with a maximum of eight mice per cage. All transgenic lines were generated on a C57BL/6 background.

2.1.1 Breeding

The breeding strategy to obtain $Tbx2^{flox/flox}$; $Tie2Cre^{+}$ mice is summarised in Fig. 2.1. The first round of mating gave rise to F1 progeny heterozygous for the Tbx2 floxed locus, and either negative or positive for the presence of the Tie2Cre expression gene ($Tie2^{cre/+}$; $TBX2^{flox/+}$;). F1 mice were genotyped to confirm heterozygosity at the Tbx2 flox locus and to identify Cre positive mice. $Tie2^{cre/+}$; $Tbx2^{flox/+}$ mice were then back-crossed with $Tbx2^{flox/flox}$ mice in order to obtain $Tie2^{Cre/+;}$ $Tbx2^{flox/flox}$ progeny in a ratio of 1:3 in the F2 generation. The inducible $Rosa26^{creERT2}$ allele was introduced in an

analogous fashion, however, to obtain Rosa26$^{creERT2/+}$;Tbx2$^{flox/flox}$ mice in a higher Medelian ratio, these mice were again back-crossed with Tbx2$^{flox/flox}$ to give rise to an F3 generation with a ratio of 1:1, Rosa26$^{creERT2/+;}$ Tbx2$^{flox/flox}$ to Tbx2$^{flox/flox}$ mice (Fig. 2.1).

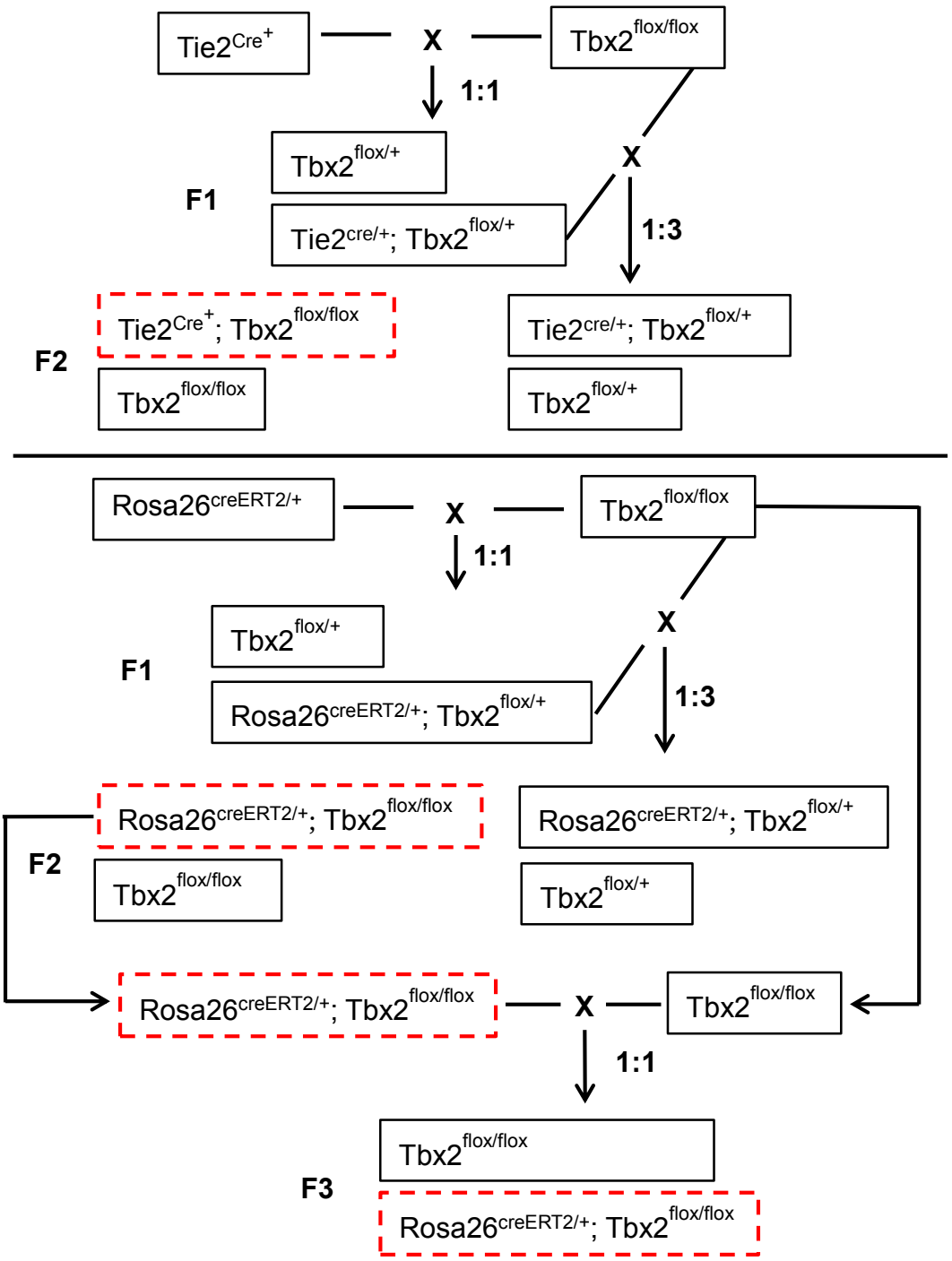

Figure 2.1: Breeding Strategies for Tie2$^{cre/+}$; Tbx2$^{flox/+}$ and Rosa26$^{creERT2/+}$; Tbx2$^{flox/flox}$

For description see text.

2.1.2 Genotyping

Genotyping was carried out by PCR followed by agarose gel electrophoresis. For this, tissue was harvested either from the auricles of newly weaned mice by ear punching or, *post mortem*, from embryonic yolk sacs. Placenta tissue was used in cases where the embryonic genotype was clearly distinguishable from that of the mother (e.g. when collecting transient transgenic embryos), in order to preserve the yolk sac for other purposes.

Tissue samples were incubated over night at 55°C with 100 - 500µl GNT buffer (50mM KCL, 1.5mM $MgCl_2$, 10mM Tris-pH8, 0.01% gelatin, 0.45% nonidet P40, 0.45% Tween) and 2% proteinase K (10mg/ml) depending on sample size. Afterwards, the solution was heated to 95°C for 30 minutes, then cooled to room temperature and centrifuged for one minute at maximum rpm in a benchtop centrifuge to obtain a clear supernatant free of insoluble tissue debris. 0.2 - 2µl of this supernatant were subsequently used in a PCR reaction with the GoTag Green master mix (Promega, M7122). Genotyping PCR conditions and primers are listed below in Tables 2.1 and 2.2.

***LacZ* genotyping**				
	35 cycles			
3 min at 94°C	30 sec at 94°C	30 sec at 58°C	30 sec at 72°C	5min at 72°C
***Cre* genotyping**				
	29 cycles			
3 min at 94°C	40 sec at 94°C	40 sec at 60°C	40 sec at 72°C	5min at 72°C
Rosa26*LacZ* genotyping				
	34 cycles			
3 min at 94°C	30 sec at 94°C	30 sec at 63°C	30 sec at 72°C	5min at 72°C
Tbx2 flox genotyping				
	34 cycles			
5 min at 94°C	30 sec at 94°C	30 sec at 58°C	45 sec at 72°C	5min at 72°C
Tie2 cre				
	34 cycles			
5 min at 94°C	30 sec at 94°C	30 sec at 60°C	30 sec at 72°C	5min at 72°C

Table 2.1: PCR conditions for genotyping

Primers	
Name	Sequence
LacZFw	GTTGCAGTGCACGGCAGATACACTTGCTGA
LaczRv	GCCACTGGTGTGGGCCATAATTCAATTCGC
CreFw	CATTTGGGCCAGCTAAACAT
CreRw	ATTCTCCCACCGTCAGTACG
TBX2floxFw	GGGGAGCATTAGTTGAACACC
TBX2floxRv	CTTGACCCATTCCTTACAGCA
Tie2CreFw	CGCATAACCAGTGAAACAGCATTGC
Tie2CreRv	CCCTGTGCTCAGACAGAAATGAGA

Table 2.2: Primers for genotyping

2.1.3 Mouse Transgenesis

For mouse transgenesis, the enhancer-Hsp68*LacZ* constructs (see below, 2. Cloning) were linearized by restriction digest with XhoI (NEB, R0146S) and NotI (NEB, R3189S as NotI-HF) and purified by agarose gel electrophoresis (0.8%(w/v) agarose in TBE, 200V) and gel extraction using QIAquick gel extraction kit (Qiagen, 28704). The pronuclear injection procedure was performed by K. Liu at the Institute of Ageing and Chronic Diseases, University of Liverpool as previously described (Plück and Klasen 2009). Briefly, zygotes were harvested from oviducts of female donor mice and immobilized at the opening of a holding capillary. The diluted linearized DNA was injected into zygote nuclei under a stereoscopic microscope and re-implanted into the oviduct infundibulum of an anesthetized, pseudopregnant female that had previously been mated with a vasectomized male. For analysis of transgenic embryos, foster mothers were sacrificed at the desired stage of pregnancy and embryos harvested by hysterectomy.

2.1.4 Tamoxifen treatment for Cre Induction

For inducible, Cre-mediated, gene knock-out in mouse embryos, pregnant females were injected intraperitoneal with a solution of 10mg/ml tamoxifen, 10% (v/v) ethanol in peanut oil (Sigma, P2144) on two consecutive days. If the injections were performed prior to E14.5, progesterone was added to the solution at 5mg/ml to avoid pregnancy abortion.

2.2 Cloning

2.2.1 Gateway

The 826bp fragment of the 10th intron of the mouse Flk1 gene was generated by PCR from mouse genomic DNA using Bio-X-act Short DNA polymerase (Bioline, BIO-21065) with the following primers: Fw 3' - gcatgtcaagatttgacttc - 5' and Rv 3' – cacgatggcaagtgaaacag - 5' (PCR: 5min, 94°C; 30X: 30sec, 94°C; 45sec 58°C; 60sec 72°C).

TOPO-TA cloning using the pCR8/GW/TOPO TA Cloning Kit (Invitrogen, K2500-20) was performed to insert the PCR product into the vector pCR8 to form a GateWay Entry Vector (Fig. 2.2). This was done following the recommended Invitrogen protocol but using only 0.5µl of TOPO vector, as previous experiments had proven this to be just as effective. In the TOPO-TA reaction a topoisomerase ligates adenine overhangs left by certain Taq polymerases at the end of the polymerisation to unpaired thymine residues on a linear TOPO vector to create a circular Entry Vector.

Following the TOPO-TA reaction, the Entry Vector was used for bacterial transformation. For this, 3µl of the reaction was carefully mixed with 50µl chemically competent *E.coli* on ice and incubated for a further 10 minutes. The mixture was heat-shocked in a water bath at 37ºC for two minutes and immediately cooled on ice. 200µl LB medium (1% Tryptone, 0.5% Yeast Extract, 1.0% Sodium Chloride) was added to the mixture which was then incubated for 45 minutes at 37ºC in a shaking incubator. Afterwards, 50µl bacteria were plated on an LB agar plate (1% Tryptone, 0.5% Yeast Extract, 1.0% Sodium Chloride, 1.5% agar) containing 0.001% spectinomycin

(100µg/ml, MerckMilipore, 567570-10GM) to select for successfully transformed bacteria carrying the pCR8 plasmid. Plates were incubated at 37ºC overnight, after which bacterial colonies were picked and cultured in a shaking incubator at 37ºC overnight in 4ml LB medium containing spectinomycin.

DNA miniprep was performed according to the manufacturer's instructions (Qiagen, 27106). Concentration of the extracted DNA was measured using a Nanodrop spectrophotometer followed by restriction digest and agarose gel electrophoresis (1%(w/v) agarose in TBE, 200V) to check correct insertion and orientation of the PCR product in the vector. For this, DNA was digested using a combination of PstI and XbaI restriction enzymes (NEB, R0140 and R0145) following standard NEB protocol for restriction enzyme digest.

Next, the fragment containing the Flk1i10 enhancer was transferred from the entry to a destination vector by *in vitro* recombination using the Gateway LR Clonase II Enzyme mix (Life Technologies, 11791-100) following manufacturer's instructions to obtain an Expression Vector (Fig. 2.2) which was then used for bacterial transformation as described above. Successful recombination was confirmed by restriction enzyme digestion with XhoI (NEB, R0146) and PstI (NEB, R0140). For mouse transgenesis the destination vector Hsp68-*LacZ*-Gateway (gift from N. Ahituv) was used, while pE1b-GFP Tol2 was used for zebrafish transgenesis.

DNA extraction from bacterial cultures (50 – 100ml in LB medium) was performed with the QIAfilter Plasmid Midi Kit (Qiagen, 12245) according to manufacturer's instructions to obtain highly pure, concentrated plasmid DNA.

Cloning was repeated as described above for a 1012bp fragment of the chicken Flk1 intron 10, generated by PCR using Roche Taq polymerase, the following primers:

Fw 3 ’- gctgatcaacaaatcagatg - 5’ and Rw 3’ – agcacatatggcatagggag - 5’ and genomic chicken DNA as a template. Entry Vector insertion and orientation were analysed using EcoRI and BanII restriction enzymes, respectively (NEB, R0101T and R0119S), and successful destination vector creation was confirmed using XmnI (NEB, R0194S).

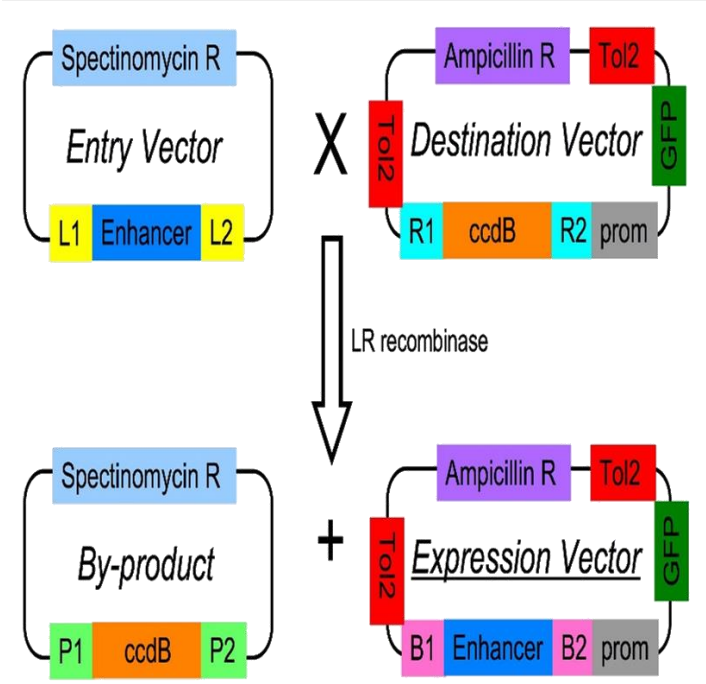

Fig. 2.2: GateWay Cloning

Successful cloning of the Entry Vector can be selected for using the spectinomycin resistance cassette. Crossing with the Destination Vector using LR recombinase allows recombination of the attL and attR sites to exchange the PCR product and ccdB regions. The ccdB region inhibits DNA gyrase in E.coli to prevent survival of bacteria transformed with the byproduct. The expression vector can be selected for using the Ampicillin Resistance gene. The flanking Tol2 sites allow transfection of the entire cassette into the zebrafish embryo genome.

2.2.2 Mutagenesis

For mutation of binding motifs within the Flk1i10 enhancer construct, PCR-based site directed mutagenesis was used. This particular variation of the technique required two non-mutagenic oligonucleotide primers, P_{Fw} and $P_{Rv,}$ which were located directly outside of two convenient restriction sites up- and downstream of the target mutation site, in this case SfoI and PstI (NEB, R0606 / R0140). Furthermore, two partially overlapping mutagenic primers, $^{\Delta}P_{Fw}$ and $^{\Delta}P_{Rv}$, were required to introduce the

sequence alteration. In a first step, two regular PCR reactions were performed using the primer pairs P_{Fw} and $^{\Delta}P_{Rv}$ as well as $^{\Delta}P_{Fw}$ and P_{Rv} resulting in two double stranded products which, at one end, covered a short overlapping sequence that also contains the mutation. Afterwards, the products were purified using QIAquick PCR Purification Kit (Qiagen, 28104) and subsequently used in a second PCR reaction. This reaction included an initial denaturing step in the absence of both primers and polymerase. Polymerase was then added and extension was initiated without a prior annealing step. This allowed for the formation of some DNA dimers which anneal over the short overlapping sequence and then serve as both primer and template for the polymerase. The product of this reaction was a mutated DNA strand extending from the P_{Fw} primer site to the P_{Rv} primer site. This DNA fragment served as a template for the P_{Fw}/P_{Rv} primer pair and was subsequently amplified in a regular PCR reaction (Fig. 2.3). Both the original vector and the mutated PCR product were then subjected to restriction digest with SfoI and PstI, followed by dephosphorylation of the vector DNA with calf intestinal phosphatase (NEB, M0290S) for 1h at 37°C. Afterwards both vector and mutated insert were run on an agarose gel (0.8%(w/v) agarose in TBE, 200V) and bands of the appropriate size were excised and purified using QIAquick gel extraction kit (Qiagen, 28704). This was followed by ligation with T4 DNA ligase (NEB, M0202) for 1h at 37°C and a molar ratio of 1:3 vector to insert. The ligated plasmid was then transformed into chemically competent Top10 *E. coli* from which DNA was later extracted using QIAfilter Plasmid Midi Kit (Qiagen, 12245) as described above.

Mid-way through the research project, the GeneArt (Life Technologies) gene synthesis service became available and financially viable. Some of the Flk1i10 enhancer mutation constructs were therefore generated by direct synthesis of DNA fragments containing the desired mutations. The synthetic DNA strings were incubated

for 15 minutes with Taq polymerase (LifeTechnologies, 10342-053) at 72°C to add A-overhangs and allow for subsequent TOPO clonation as described above for PCR products.

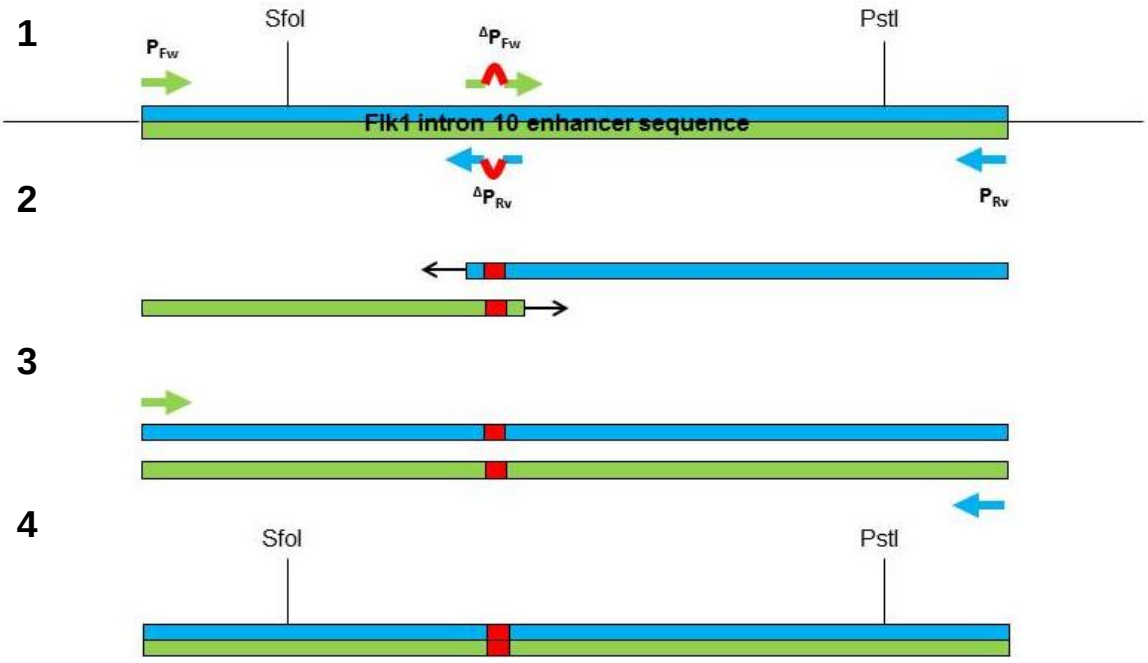

Figure 2.3: Site directed mutagenesis

1: Two separate PCRs are performed with P_{Fw} / $^{\Delta}P_{Rv}$ and $^{\Delta}P_{Fw}$ / P_{Rv} and plasmid DNA as a template.
2: The resulting double stranded products are denatured and anneal with low efficiency at the short ovderlap containing the mutated binding site where they function as primer for DNA polymerase to produce low amounts of a full length mutated template.
3: Primers P_{Fw} and P_{Rv} are added to amplify the mutated template in a PCR reaction.
4: The mutated sequence is cut in a restriction digest with SfoI and PstI and ligated back into the original vector.

2.2.3 Flk1i10 enhancer sequences

Mouse Flk1i10 WT

TGCATGTCAAGATTTGACTTCTCTCTCGTTCAGGAGTGCCGGAAAGGGTCAGCCTCTGGTTATCACGTTCCTAGTGATAACCCTCGACACACTCGAACAC
TTCGCAGAACTTGGCGCCAATTAAAAATAGATGCCTATACACAACAGTGCGAAGGTCCTGAGGATACAGGAGGGAAGCAGCTATTCTGGGAACAAGTCT
CCATTCATAAATTGGATCGACAAGACAATTCAAGCTCACTTAGTTCAAAGGAAGGTAAGGAAACTTGGAAGCCATTGGGGCTTCTTAAAAGTCACCTCTCT
GGGACGGACCGACTGCGGGCTTTTGTTTAGGAAATGGCCAGCAGCAGAGGAAGAAACTCGGGTTTGCTATTTCAAAAACAACAACAGGAAGTGGAATGC
TTGGGGTGGTAGGTTGAAGGTGGTGGCTGTTGTTTTCCCTAAGGATGTCTGCACTTGTGGTAGACCTTGATAAGCCTGGGCCTGAGACTCTCGAGGCCT
GGGCTAGGTTTATCACTGCCTCGCATCCGCCAGCACTTCCTCTGAGAGATGGACATTCCCACAGATAAGGAGGAGCAGTGTGGTCCTCTGCAGTCCACA
GACAGAAGATGATCCGATGATTGGCATCTAGCAAACGCAGCAAGTAGTATCCCTTTGGAGAGGAATCCACCAGGGCTAACTAAGGAGAAAGAGCTGACT
CGCATAGTGGGATACGGGGGAATGAGTCCAAGTACCACTGAAGGGGTAACCTTGGAGACAGCCTTCCGGGTTTCGACGGAACCCTGGCATGGCTTGGC
AAGAAGGGACGCACGATGGCAAGTGAAACAGA

mutGATA-All

TGCATGTCAAGATTTGACTTCTCTCTCGTTCAGGAGTGCCGGAAAGGGTCAGCCTCTGGTCCCCACGTTCCTAGTGGGGACCCTCGACACACTCGAACA
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GACTCGCATAGTGGGGGGCGGGGGAATGAGTCCAAGTACCACTGAAGGGGTAACCTTGGAGACAGCCTTCCGGGTTTCGACGGAACCCTGGCATGGC
TTGGCAAGAAGGGACGCACGATGGCAAGTGAAACAGA

mutGATA-b, c

TGCATGTCAAGATTTGACTTCTCTCTCGTTCAGGAGTGCCGGAAAGGGTCAGCCTCTGGTTATCACGTTCCTAGTGATAACCCTCGACACACTCGAACAC
TTCGCAGAACTTGGCGCCAATTAAAAATAGATGCCTATACACAACAGTGCGAAGGTCCTGAGGATACAGGAGGGAAGCAGCTATTCTGGGAACAAGTCT
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GGGACGGACCGACTGCGGGCTTTTGTTTAGGAAATGGCCAGCAGCAGAGGAAGAAACTCGGGTTTGCTATTTCAAAAACAACAACAGGAAGTGGAATGC
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CAGACAGAAGATGATCCGATGATTGGCATCTAGCAAACGCAGCAAGTAGTATCCCTTTGGAGAGGAATCCACCAGGGCTAACTAAGGAGAAAGAGCTGA
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mutRBPJ-a, b

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GGGCTAGGTTTATCACTGCCTCGCATCCGCCAGCACTTCCTCTGAGAGATGG**C**CA**GG**C**T**CACAGATAAGGAGGAGCAGTGTGGTCCTCTGCAGTCCAC
AGACAGAAGATGATCCGATGATTGGCATCTAGCAAACGCAGCAAGTAGTATCCCTTTGGAGAGGAATCCACCAGGGCTAACTAAGGAGAAAGAGCTGAC
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CAAGAAGGGACGCACGATGGCAAGTGAAACAGA

mutGATA-b, c ΔRBPJ-a, b

TGCATGTCAAGATTTGACTTCTCTCTCGTTCAGGAGTGCCGGAAAGGGTCAGCCTCTGGTTATCACGTTCCTAGTGATAACCCTCGACACACTCGAACAC
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CTCGCATAGTGGGATACGGGGGAATGAGTCCAAGTACCACTGAAGGGGTAACCTTGGAGACAGCCTTCCGGGTTTCGACGGAACCCTGGCATGGCTTG
GCAAGAAGGGACGCACGATGGCAAGTGAAACAGA

mutRBPJ-a, bΔSox-a, b, c

TGCATGTCAAGATTTGACTTCTCTCTCGTTCAGGAGTGCCGGAAAGGGTCAGCCTCTGGTTATCACGTTCCTAGTGATAACCCTCGACACACTCGAACAC
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TGACTCGCATAGTGGGATACGGGGGAATGAGTCCAAGTACCACTGAAGGGGTAACCTTGGAGACAGCCTTCCGGGTTTCGACGGAACCCTGGCATGGC
TTGGCAAGAAGGGACGCACGATGGCAAGTGAAACAGA

mutAll

TGCATGTCAAGATTTGACTTCTCTCTCGTTCAGGAGTGCCGGAAAGGGTCAGCCTCTGGTTATCACGTTCCTAGTGATAACCCTCGACACACTCGAACAC
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GCTGACTCGCATAGTGGGATACGGGGGAATGAGTCCAAGTACCACTGAAGGGGTAACCTTGGAGACAGCCTTCCGGGTTTCGACGGAACCCTGGCATG
GCTTGGCAAGAAGGGACGCACGATGGCAAGTGAAACAGA

mutSmallAll

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AGACAGAAGATGATCCGATGATTGGCATCTAGCAAACGCAGCAAGTAGTATCCCTTTGGAGAGGAATCCACCAGGGCTAACTAAGGAGAAAGAGCTGAC
TCGCATAGTGGGATACGGGGGAATGAGTCCAAGTACCACTGAAGGGGTAACCTTGGAGACAGCCTTCCGGGTTTCGACGGAACCCTGGCATGGCTTGG
CAAGAAGGGACGCACGATGGCAAGTGAAACAGA

Chicken Flk1i10 WT

GCTGATCAACAAATCAGATGGATCACTGGCTACAGCCTACATTTCAGAAGGAAAAAGTATGTTTCTTGAAATATTTCTATTAAATTGAACCCCATAACCTTC
TGAGACCTTTGTGCTCTGTAAAATAAGAAGCCCCCAAGCTGAAAGGTGAATTCCCTAATTAATCTGCCTGTGTCAGCTCTATCAGGGTGCTTGGAGGGAA
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AACCCCCTCTCAACTCCCTGCCCATGAGTCAGCAGAGCCTGACTGCCTACTGAGTACAACGATCTTCTTTGTTGGCAAGTGAAGTCATTTTGAGCCTACA
GGTGTCATGAGCAAAATCTCTCATGACTTAAGAGGGAGCAAAACCAAATCCTAGGGCTGAATCCTTCAGTGTCCACCACTCTCTGTCCAGCACATATGGC
ATAGGGAG

2.3 Histology

2.3.1 X-Gal staining for mouse embryos

Transgenic mouse embryos were harvested at specified time points by hysterectomy after cervical dislocation of pregnant females. The uteri were transferred to a Petri dish, covered in chilled PBS, and carefully opened with a pair of fine forceps. The placentae were separated from the embryos and placental tissue was used for genotyping if required. Finally, embryos were gently freed from the surrounding yolk sacs which were left intact and attached to the embryos unless embryonic tissue was needed for genotyping. For embryos older than E13.5 the skin was removed and chest cavity was opened with fine forceps to facilitate X-Gal infiltration.

After dissection, samples were rinsed in cold PBS and fixed in FIX solution (2% PFA, 0.2% glutaraldehyde in PBS) at 4°C. Fixation times depending on embryionic stages are shown in Table 2.3.

Age	Fixation time	Further processing
E7.5-8.5	10 min	Embryos were rinsed twice in 1x PBS, than incubated in PBS for 30 min at 4°C
E8.5-9.0	20 min	
E9.5	30 min	
E10-11.5	60 min	
E12.5	1.5 h	Embryos were rinsed twice in PBS, than incubated 2x30 min in Rinse Solution on rotor at 4°C, abdomen was opened for better perfusion
E13.5	2 h	
E14.5	2.5 h	
E15.5	3 h	
E16.5-17.5	4 h	

Table 2.3: Fixation times for embryos

The embryos were then rinsed twice in PBS and, depending on age, washed for another 30 minutes in PBS at 4°C, or washed twice for 30 minutes in RINSE solution (for 500ml: 50ml 10X PBS, 1mL 1M $MgCl_2$, 500mg sodium deoxycholate, 1ml NP40, distilled water) on ice and were then incubated in STAIN solution (500ml RINSE solution, 825mg potassium ferricyanide, 920mg potassium ferrocyanide; add 1% 100mg/ml X-Gal in N,N,dimethylformamide to working solution) overnight in the dark at room temperature to allow the X-Gal reaction to occur. After X-Gal staining, embryos were rinsed in 1X PBS and fixed overnight in 4% PFA at 4°C.

Imaging of whole embryos was performed using a stereo microscope (Leica M165C) equipped with a ProGres CF Scan camera (Jenoptik) and ProgRes CapturePro software (Jenoptik).

2.3.2 Paraffin embedding and sectioning

For paraffin embedding, whole embryos or organs were processed in an Excelsior ES Tissue Processor (Thermo Scientific). In brief, the samples were first dehydrated by sequential incubation in an ethanol series (in water) at 40%, 70% , 80%, 95%, 100% (twice) ethanol, for 1h each. Dehydrated tissues were incubated twice in xylene for 1h each; and twice in liquid paraffin (at 56-58°C), for 1.5h each. The paraffin infused tissues were then embedded into paraffin blocks on a HistoStar Embedding Workstation (Thermo Scientific, A81000002) and left to set. Sectioning was performed on a HM 355S Automatic Microtome (Thermo Scientific) by S. De Val and I. Ratnayaka

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Paraffin embedding and sectioning as described above was also used to process non X-Gal stained embryos for *in situ* hybridization, immunohistochemistry or immunofluorescence analysis.

2.3.3 Histological analysis

Sections were first cleared from paraffin by two 5 minute incubations in Histo-clear II (National Diagnostics, HS-202). This was followed by sequential rehydration in an ethanol series starting with two 3 minutes incubations in 100% ethanol, 1 minute in 90% ethanol; 1 minute in 70% ethanol; and 1 min in water. For counterstaining, X-Gal-stained embryo sections were placed in nuclear fast red (Electron Microscopy science, 26356-02) for 30 - 90 seconds, and thoroughly rinsed in water. Afterwards, sections were again dehydrated in a sequential alcohol series starting with 1 minute in 70% ethanol; 1 minute in 90% ethanol; 3 minutes in 100% ethanol; twice and 5mins in Histoclear II, twice. Finally, the slides were mounted using Vectashield HardSet mounting medium (Vetorlabs, H-1004). After the mounting medium had set, imaging was performed using a Zeiss Axioplan 2 Upright Microscope (Carl Zeiss) fitted with a ProgRes C5 microscope camera (Jenoptik) and ProgRes CapturePro software (Jenoptik).

2.3.4 Immunohistochemistry and immunofluorescence

All stainings were performed on PFA-fixed, paraffin embedded tissues. Unless stated otherwise, sections were cut at 5µm. Rehydration of the sectioned tissues was performed as described earlier. Afterwards, optional antigen retrieval was achieved by incubation in 10mM sodium citrate buffer, pH 6, and boiling in a commercial pressure cooker for 3 minutes. The slides were then rinsed in PBS and briefly blotted dry. Blocking of free, promiscuous binding sites was accomplished by applying 500µl of 1% normal horse serum (in PBS) per section for 30 minutes. Primary antibodies (Table 2.4) were diluted in 1% normal horse serum (in PBS), and after the blocking solution was tipped off, 100µl primary antibody solution was applied to each section, which were then covered with a glass cover slip and incubated over night at 4°C. Next, sections were washed in PBS for 10-15 minutes and incubated with either biotinylated or Alexa Fluor (Molecular Probes) labelled secondary antibodies (Table 2.4) in PBS for 2h. The Elite ABC kit (Vectorlabs, PK-6100) was used according to manufacturers' instructions to amplify the signal from biotinylated secondary antibodies. The DAB peroxidase substrate kit (Vectorlabs SK-4100) or a Tyramide signal amplification kit (LifeTechnologies, T20922, T20924) was then used for visualization. Afterwards, sections were washed in PBS for 5 minutes. DAB stained sections were dehydrated and embedded as described above, while fluorescently labelled sections were embedded using Vectashield mounting medium for fluorescence (Vector Laboratories, H-1200).

Antibody	Source	Manufacturer	AR	Dilution	Detection
Primary Antibodies					
Nrp1	Rabbit	Abcam	Yes	1:100	αRab.-AF568 / αRab.-Biotin
Eph-B4	Goat	R&D Systems	Yes	1:50	αGoat-Biotin
Endomucin	Rat	unknown	No	1:300	αRat-AF568
Tbx2	Mouse	Gift from C. Goding	Yes	1:100	αMouse-Biotin Reagent
ERG	Rabbit	Santa Cruz	Yes	1:100	αRab.-Biotin
α-smooth muscle actin	Mouse	Santa Cruz	No	1:200	αMouse-AF488/568
NG2	Rabbit	Millipore	No	1:200	αRab.-AF488/568
Isolectin B4-Biotinylated	-	Vector	No		
Secondary Antibodies					
αRabbit-Biotin	Goat	Vector Laboratories	-	1:300	TSA
αGoat-Biotin	Horse	Vector Laboratories	-	1:300	TSA
αRat-AF568	Goat	Invitrogen	-	1:300	-
αRabbit-AF568	Donkey	Invitrogen	-	1:300	-
αMouse-Biotin Reagent	-	Vector	-	Stock solution	TSA
Streptavidin AF568	-	Invitrogen		1:300	

Table 2.4: Antibody List for IHC

AR = Antigen retrieval

2.3.5 In situ hybridization

In situ hybridization was performed as described previously (Edmondson et al 1994). Vectors for generation of *in situ* probes for Tbx1, Tbx2, and Tbx3 were gifts from M. Pontecorvi (Table 2.5). To generate DNA templates for both sense and antisense RNA probes, vectors were linearized by restriction digest using restriction sites at either end of the target DNA insert and purified by gel extraction as described above. RNA probes were made by *in vitro* transcription with T3, T7, or T3 RNA polymerase and labelled with digoxigenin-UTP using the DIG RNA Labeling Kit (Roche, 11175025910) following the recommended protocol.

A dilution series of labelled probes was quantified against a known standard. For this, a 1:200 dilution was prepared for both probe and standard which was then further diluted 1:10 for four times. A 2µl drop of each dilution was spotted on a nitrocellulose transfer membrane (Abcam, ab133412) and cross-linked for one minute in a CL-1000 ultraviolet crosslinker (UVP). The membrane was then soaked in MABT (150 mM NaCl, 100 mM Maleic acid, 0.1% Tween20, pH 7.5) and blocked for five minutes in a 2% (w/v) solution (in MABT) of blocking reagent (Roche, 11096176001). Next, Fab fragments from polyclonal anti-digoxigenin antibodies, conjugated to alkaline phosphatase (Roche, 11093274910) were added at a concentration of 1:2500 and incubated for 20 minutes. The membrane was washed twice for 2 minutes in MABT and subsequently soaked in NTMT (100mM Tris pH 9.5, 100mM NaCl, 10mM $MgCl_2$, 1% Tween20) for 5 minutes. For staining, the membrane was covered in BM Purple (Roche, 11442074001) and left at room temperature until desirable staining intensity had developed. Afterwards, the membrane was briefly rinsed in water and left to dry on tissue paper. Probe concentration was then estimated by comparing staining intensity against the standard.

For *in situ* hybridization, 5µm sections of PFA-fixed, paraffin embedded tissue were cleared from paraffin by two consecutive 5 minute incubations in Xylene, followed by rehydration in an ethanol gradient (in water) (2 x 100%, 90%, 70%, 40%; 1 minute each). Sections were incubated in PBS for 5 minutes and then treated with proteinase K (20µg/ml, in: 50mM Tris pH 8.0, 5mM EDTA) for 7.5 minutes at room temperature. Afterwards, slides were washed in PBS for 5 minutes and fixed in 4% PFA for 20 minutes at room temperature, followed by 5 minute washes in PBS and 2X SSC (300mM NaCl, 30mM sodium citrate, pH 7.0), before dehydration in an alcohol series (40% - 20 seconds, 70% - 7 minutes, 90% - 1 minute, 100% - 2 minutes). Slides were briefly left to dry afterwards.

In situ probes were diluted to 1µg/ml in hybridization solution and 100µl diluted probe was applied to each slide which were then covered with a glass cover slip and incubated in a sealed moist chamber filled with a 1:5 mixture of 20X SSC and formamide and incubated over night at 65°C.

On the next day, sections were washed in 50% formamide / 1X SSC/ 0.1% Tween20 at 65°C for 20 minutes and twice for 15 minutes in 2X SSC at 37°C before RNase A was added at a concentration of 10µg/ml and left for 15 minutes. After RNase treatment, sections were washed once more for 15 minutes in 2X SSC and for 15 minutes in 0.1X SSC at 37°C. This was followed by a 20 minute wash in MABT followed by incubation with blocking solution (see above, + 10% sheep serum; 700µl per slide) for 1.5h. Anti-digoxigenin antibody was diluted 1:400 in blocking solution (1% sheep serum) before 100µl was added per slide which was covered with a glass cover slip and incubated over night at room temperature in a water based moist chamber.

On the following day, slides were washed twice for 15 minutes in MABT and then 5 minutes in NTMT, before each slide was covered with 500µl BM Purple (add levamisole at 3mM) and incubated at 37°C a water based moist chamber until desired staining intensity had developed (up to three days). Afterwards, sections were rinsed in water, dehydrated, embedded and imaged as previously described.

In situ probes				
Probe	**Vector**	**Target**	**Antisense**	**Sense**
Tbx1	pCMV-SPORT6.1	mRNA (31>1497)	T7/EcoR1	Sp6/Xho1
Tbx2	pCMV-SPORT6.1	mRNA (59>2164)	T3/EcoR1	T7/HindIII
Tbx3	pCMV-SPORT6.1	mRNA (1348>3573)	T7/Kpn1	Sp6/Xho1

Table 2.5: Vectors for *in situ* probes

2.4 Zebrafish

All Home Office regulated zebrafish work was performed by N. Sacilotto or A. Neal under PPL 30/2783. All embryo analysis was performed by the candidate.

Zebrafish were bred and maintained at 28.5°C in systemically circulating water. Embryos were maintained in E3 medium (5mM NaCl, 0.17mM KCl, 0.33 mM $CaCl_2$, 0.33mM $MgSO_4$.) at 28.5°C. Typically, the embryos were obtained by timed spawning of wild type adult zebrafish.

2.4.1 Generation of Tol2-Mediated Mosaic Transgenic Fish

Transient mosaic transgenic zebrafish embryos were generated using the Tol2 transposon system (Kawakami 2005). Embryos were injected at the late one-cell or early two-cell stage. First, Tol2 transposase capped mRNA was generated from the linearized pCS-TP plasmid using the mMESSAGE mMACHINE SP6 kit (LifeTechnologies, AM1340), according to the manufacturer's protocol. Next, injection solution was prepared with 1µl pE1b-enhancer-GFP expression vector (60 ng/µl), 1µl of 50ng/µl Tol2 transposase capped mRNA, 0.5µl of Phenol red and 2.5µl of RNase-free water. A 1nl drop of this solution was microinjected into the embryo under a Leica Wild M8 stereomicroscope (Leica) using a Leitz Micromanipulator M (Leica). Standard glass wall capillaries (Clark Electromedical Instruments, GC100F-15) were pulled using a Narishige PB-7 needle puller (David Kopf) to allow backfilling of the sample and fine insertion into the fish egg.

2.4.2 Morpholinos and Chemical Treatments

Morpholinos (Table 2.6) were injected into 1- to 2-cell *tg(Flk1i10:GFP)* embryos, using volumes of 0.5 – 1.5nl of the corresponding MO solution at a concentration depicted in figure legends. Injections were performed with the same equipment as described for Tol2 transgenesis. For pharmacological inhibition of Vegf and Notch signalling pathways, embryos were manually dechorionated and incubated with 1μM of SU5416 (Sigma) or 100μM DAPM (Calbiochem), respectively, in E3 medium starting at 10hpf.

Morpholinos			
Gene	Sequence	Target	Ref.
SOX7	ACGCACTTATCAGAGCCGCCATGTG	AUG	Cermenati et al 2008
SOX18	TATTCATTCCAGCAAGACCAACACG	AUG	Cermenati et al 2008
RBPJ	CAAACTTCCCTGTCACAACAGGCGC	intron/exon boundary	Sieger et al 2003
GATA1	CTGCAAGTGTAGTATTGAAGATGTC	intron/exon boundary	Galloway et al 2005
GATA2	CATCTACTCACCAGTCTGCGCTTTG	intron/exon boundary	Galloway et al 2005
scrambled	CCTCTTACCTCAGTTACAATTTATA	N/A	

Table 2.6: MO sequences

2.4.3 Zebrafish Analysis and Imaging

Reporter gene expression analysis was typically carried out between 24 – 72hpf. Prior to this, the embryos were dechorionated and anesthetized with 0.01% tricaine mesylate treatment, to minimize fish movement and allow image capture.

Single embryos were transferred into a flat bottom 96-well plate well, and GFP reporter gene expression screened using fluorescence microscopy. The total number of injected fish, the total number of GFP expressers and the number of vascular expresser were all noted, and images of representative transgenic embryos were obtained by confocal microscopy. Whole fish were imaged using the “tile scan” command, combined with Z-stack collection under a confocal microscope Zeiss LSM 710 MP (Carl Zeiss) at 488nm excitation and 509nm emission. The fish were then transferred into a petri dish and recovered from anaesthesia in E3 or killed using an overdose of tricaine mesylate.

2.5 Electrophoretic mobility shift assay

2.5.1 Making protein for EMSA

Recombinant proteins were made *in vitro* with the TNT T7/SP6 Coupled Reticulocyte Lysate System (Promega, L5020) according to manufacturer's instructions. Mammalian expression vectors containing the coding sequence for the tested TF (or its DNA binding domain) were used as template DNA for *in vitro* transcription with either T7 or Sp6 RNA polymerases. Depending on the DNA binding, protein vectors used were pCITE2, pcDNA, pCS2 or pCR2.1 vector. All constructs were sequenced to ensure that the coding sequence was in frame with the ATG start codon.

Briefly, 25µl TNT lysate, 2µl reaction buffer, 1µl RNA polymerase (T7 or Sp6), 0.5µl leucine amino acid mix, 0.5µl methionine amino acid mix was incubated with 1µg vector DNA at 30°C for 1.5 hrs.

2.5.2 EMSA probe preparation

Short double stranded DNA fragments containing the binding motif to be tested (Table 2.7) were generated by mixing 5µl top strand oligo (1mg/ml) and 5µl bottom strand oligo (1mg/ml) with 10µl 2X annealing buffer and incubated for 10 minutes at 95°C. The heat block was then switched off and left to cool to RT. For radiolabelling, 1µl of the annealed oligo was incubated with 2.5 µl buffer 2 (NEB, B7002S), 1µl 0.5mM dGTP, 1µl 0.5mM dATP, 1µl 0.5mM dTTP (all from dNTP set, LifeTechnologies,

10297-018), 17µl distilled water, 0.5µl Klenow (Promega, #9PIM220) and 1µl ^{32}P-dCTP (PerkinElmer EasyTides, NEG502A250UC) at RT for 30-60 minutes. The radiolabeled EMSA probe was then run on a non-denaturing 10% acrylamide gel in 0.5X TBE buffer for 1-2 hours at 150V, covered in Saran wrap, and briefly exposed to Fuji RX X-ray film (Fuji Medical Systems) which was developed using a radiographic film processor. Location of the labelled probe in the gel was marked by overlaying of the developed film using previously applied pen marks for correct alignment. The labelled probe was excised from the gel using a surgical blade, placed in a tube with 100µl 40mM KCl, and incubated at 37°C overnight for elution. Afterwards, the sample was centrifuged briefly and the supernatant was collected in a new tube. 2µl of the supernatant were added into 1ml liquid scintillation cocktail (PerkinElmer) and analysed in a microplate counter (PerkinElmer 2450 MicroBeta2 LumiJet). The labelled probe was then adjusted to 10-40,000 cpm of alpha by dilution with 40mM KCl.

2.5.2 Gelshift

All samples were run on 6% acrylamide gels. Gels were poured and allowed 10-20 minutes to set, then pre-run in 0.5X TBE buffer at 150V at RT, or 200V at 4°C. For each EMSA reaction 2µl 10X binding buffer, 0.2-1.0µl poly dIdC (Roche, Cat10108812001), 1-5µl protein from TNT reaction, 1µl competitor annealed oligo (optional), and distilled water up to 18µl were mixed. Samples were incubated for 10 minutes at RT, after which 2µl hot probe at 10-40,000 cpm/µl was added, followed by incubation for an additional 20-30 minutes at RT. 2ul 10X DNA loading buffer was added to each sample which was then loaded onto the gel and run for 2 – 3h at either

150V at RT or 200V at 4°C. After electrophoresis, gels were dried at 80°C for 45 - 60 minutes on a GD2000 Vacuum Gel Dryer (Hoefer), and exposed to X-ray film over night or up to 7days at -80°C followed by development of the film in a radiographic film processor.

Flk1 intron 10 EMSA Oligo Sequences				
RBPJ Sites			**Sox Sites**	
RbpjFw	ctagagctattctgggaacaagtctcca		SoxAFw	ctagagtctccattcataaattgga
RbpjRv	ctagtggagacttgttcccagaatagct		SoxARv	ctagtccaatttatgaatggagact
Rbpj2Fw	ctagacttagttcaaaggaaggtaag		SoxBFw	ctagaagacaattcaagctcactta
Rbpj2Rv	ctagcttaccttcctttgaactaagt		SoxBRv	ctagtaagtgagcttgaattgtctt
ΔRbpj2Fw	ctagacttagtttgacggaaggtaag		SoxCFw	ctagtcacttagttcaaaggaaggta
ΔRbpj2Rv	ctagcttaccttccgtcaaactaagt		SoxCRv	ctagtaccttcctttgaactaagtga
POU Site			SoxDFw	ctagtttcaaaaacaacaacaggaagtgg
POUFw	ctagagtctccattcataaattggat		SoxDRv	ctagccacttcctgttgttgtttttgaaa
POURv	ctagatccaatttatgaatggagact		ΔSoxAFw	ctagagtctccatgggtaaattgga
Lef Site			ΔSoxARv	ctagtccaatttacccatggagact
Lef1Fw	ctagctcacttagttcaaaggaaggtaagg		ΔSoxBFw	ctagaagacaatgggagctcactta
Lef1Rv	ctagtccttaccttcctttgaactaagtga		ΔSoxBRv	ctagtaagtgagctcccattgtctt
ΔLef1Fw	ctagctcacttagtttgacggaaggtaagg		ΔSoxCFw	ctagtcacttagtgggaaggaaggta
ΔLef1Rv	ctagccttaccttccgtcaaactaagtga		ΔSoxCRv	ctagtaccttcctcccactaagtga
GATA Sites			ΔSoxDFw	ctagtttcaaaagggaggacaggaagtgg
GataWTFw	ctagtagaccttgataagcctgggc		ΔSoxDRv	ctagccacttcctgtcctccctttgaaa
GataWTRv	ctaggcccaggcttatcaaggtcta		**Fox:Ets Site**	
ΔGataFw	ctagtagaccttgggaagcctgggc		Fox:EtsWTFw	ctagatttcaaaaacaacaacaggaagtggaatg
ΔGataRv	ctaggcccaggcttcccaaggtcta		Fox:EtsWTRv	ctagcattccacttcctgttgttgtttttgaaat
Gata2fw	ctagtcccacagataaggaggagc		Fox:EtsΔFoxCFw	ctagatttcaaaaacaacagccggaactggaatg
Gata2rv	ctaggctcctccttatctgtggga		Fox:EtsΔFoxCRv	ctagcattccacttccggctgttgtttttgaaat
ΔGata2Fw	ctagtcccacagggaaggaggagc		Fox:EtsΔFoxOFw	ctagatttcacaggcgacaacaggaagtggaatg
ΔGata2Rv	ctaggctcctccttccctgtggga		Fox:EtsΔFoxORv	ctagattccacttcctgttgtcgcctgtgaaat
Gatacon2Fw	ctaggctaggtttatcactgcctc		Fox:EtsΔEtsFw	ctagatttcaaaaacaacaacatagtactgtacg
Gatacon2Rv	ctaggaggcagtgataaacctagc		Fox:EtsΔEtsRv	ctagcgtacagtactatgttgttgtttttgaaat
ΔGatacon2Fw	ctaggctaggttccccactgcctc		Fox:EtsΔAllFw	ctagatttcacaggcgacagccggaagtggaatg
ΔGatacon2Rv	ctaggaggcagtggggaacctagc		Fox:EtsΔAllRv	ctagcattccacttccggctgtcgcctgtgaaat

Table 2.7: Oligos for EMSA

2.6 shRNA knockdown

2.6.1 Lentiviral vector production

For shRNA knockdown of Tbx2 *mRNA* in cultured human brain vascular pericytes (HBVP; Science Cell, 1200), six different clones were obtained from the GIPZ Lentiviral shRNAmir Individual Clone library (from A. Ceroni at the Target Discovery Institute, University of Oxford) (Elbashir et al 2001). Lentiviral particles were produced by L. Nikitenko through cotransfection of HEK293T cells with 1.5µg envelope plasmid, 1.5µg packaging plasmid and 2µg transfer plasmid (Fig. 2.4). A GIPZ non-silencing lentiviral shRNA control was produced at the same time.

In brief, cells were seeded the day prior to transfection and cultured in DMEM (+10% FBS, 100U/ml penicillin, 100µg/ml streptomycin) in 10mm culture dishes at 37°C, 5% CO_2, to reach about 70% confluency. Before transfection, medium was replaced with 8ml Opti-MEM (Life Technologies, 11058021). A DNA solution was prepared by diluting the appropriate amounts of each plasmid in a total of 50µl Opti-MEM. Next, a mixture of 15µl Fugene 6 reagent (Promega, E2691) and 35µl Opti-MEM was incubated for five minutes and then and then mixed with the DNA solution and incubated for a further 15 minutes at room temperature. This transfection solution was added dropwise to the culture followed by gentle swirling of the dish. The cells were subsequently cultured for five hours after which the medium was removed and replaced with DMEM (+10% FBS, 100U/ml penicillin, 100µg/ml streptomycin) after a single wash with PBS. Following 48h incubation, lentivirus containing supernatants were harvested, passed through a 45µm filter and frozen at -80°C until use. Lentivirus infectivity was assessed by adding 1ml of viral supernatant to a 35mm dish culture of

70% confluent HEK293T cells and estimating the percentage of GFP-positive cells after 48 – 72h using an inverted microscope.

2.6.2 Lentivirus infection and shRNA knockdown

For infection, supernatant volumes were adjusted according to infectivity (2.4 – 2.9ml) and added to a T75 flask of 70% confluent HBVPs, which were seeded the day before. Once this culture reached about 80% confluency, it was trypsinized, split 1:7 and cultured in the presence of 4µg/ml puromycin over 3 days for selection. A non-selected culture was also maintained at the same time.

To assess the percentage of infected cells for each GIPZ clone FACS analysis was performed (Fig. 2.5). A low infection rate is desirable to promote insertion of only a sigle copy of the transgene per infected cell. Both non-selected and selected HBVPs were trypsinized, washed once in PBS, and resuspended in 400µl PBS. 20,000 live, single cells from each infection were analysed for GFP expression on a FACSCanto cell analyser (BD) using FACSDiva 8.0 sofware (BD). Gating for live, single and GFP positive cells was performed manually.

To isolate RNA, 10^4 cells were seeded per well in a 6-well plate and cultured for 2 days. Isolation was then performed using illustra RNAspin Mini Kit (GE Gealthcare, 25-0500-70) according to manufacturer's instructions. Aterwards, RNA concentration was measured using a NanoDrop UV-Vis spectrophotometer (Thermo Scientific) and stored -80°C.

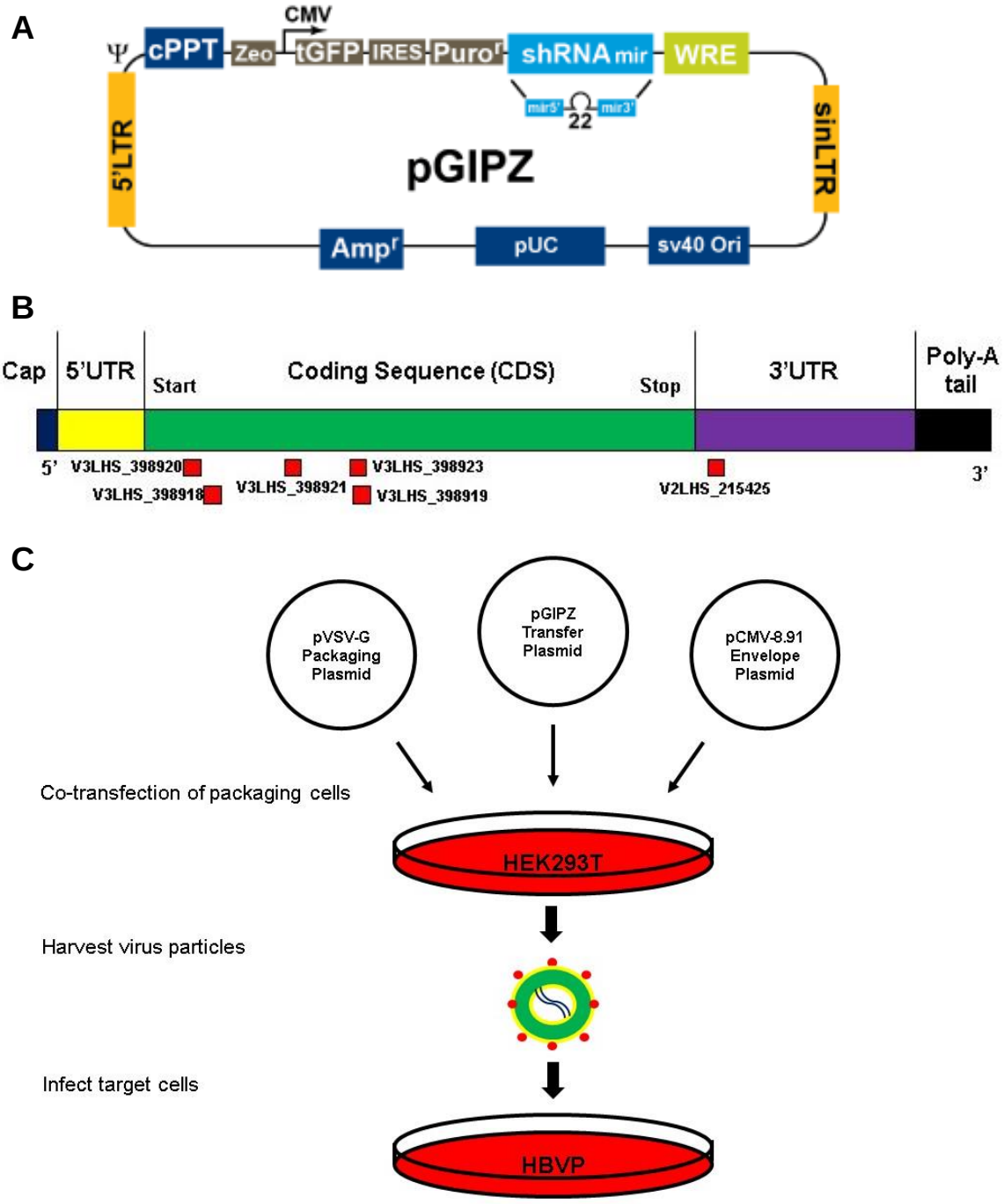


Figure 2.4: shRNA knockdown with GIPZ Lentivirus

A: Plasmid map of pGPIZ containing GFP reporter and puromycin resistance gene for selection.
B: Target sites for GIPZ Lentiviral shRNAmir clones on mature Tbx2 mRNA.
C: General principle of lentivirus production in cultured cells.

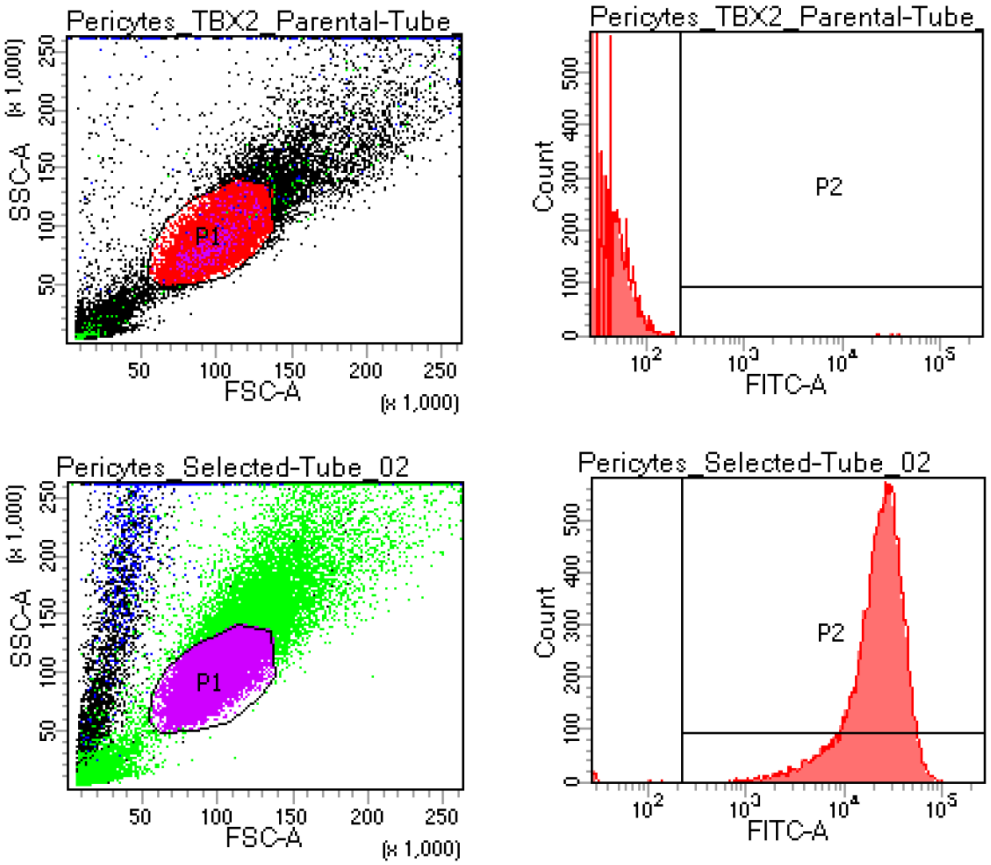


Figure 2.5: FACS analysis of GIPZ Lentiviral shRNA clone infection of HBVPs

Non-selected cells (upper panel, parental) were gated for single, live cells (P1) as well as GFP expression (P2). Onle 1.3% percent of non-selected cells fall into P2.
In contrast, 99.1% of puromycin selected cells (lower panel) fall into P2 indicating highly effective selection of infected cells.

2.6.3 Real-Time qRT-PCR

Reverse transcription was achieved using SuperScript III Reverse Transcriptase (Life Technologies, 18080-044) following the recommended protocol and using 500ng of RNA for each reaction. Subsequently, 1µl of this reaction was used as template for qPCR together with 1.25µl Single Tube TaqMan Gene Expression Assays (Life Technologies, 4331182) and 12.5µl TaqMan Universal PCR Master Mix (Life Technologies, 4304437) in a 25µl reaction. The reaction was run in a 7500 Real-Time PCR System (Life Technologies) starting with a 12 minute incubation at 95°C for polymerase activation, followed by 40 cycles of denaturing for 15 seconds at 95°C and annealing and extension for 60 seconds at 60°C. System software was used to automatically produce threshold cycle numbers (CT). Comparative CTMethod (ΔΔCT Method) was then used to compare gene expression between Tbx2 shRNA and the non-silencing control. For this, ΔCT values were calculated by subtracting ACTB housekeeping control CT values from target gene CT values for each of the different shRNA clones. ΔΔCT was then determined by subtracting non-silencing ΔCT values form the respective Tbx2 shRNA ΔCT values. ΔΔCT values indicate a fold difference between the expression of a gene in the control and the test sample.

ΔΔCT Method formula:

$$\Delta C_T = C_{T\ \text{target gene}} - C_{T\ \text{housekeeping gene}}$$

$$\Delta\Delta C_T = \Delta C_{T\ \text{test shRNA}} - \Delta C_{T\ \text{non-silencing shRNA}}$$

Gene	Amplicon length	Assay ID
TBX2	60	Hs00911929_m1
Notch3	81	Hs01128541_m1
ACTA2	105	Hs00426835_g1
PDGFRB	62	Hs01019589_m1
ACVRL1	88	Hs00953798_m1
TGFBR1	73	Hs00610320_m1
CSPG4	60	Hs00361541_g1
ACTB	63	Hs01060665_g1

Table 2.8: Single Tube TaqMan Gene Expression Assays

3 Reagent List

All chemicals were purchased form Sigma Aldrich, unless stated otherwise

10X PBS (1L)

80g	NaCl
2g	KCl
2g	KH2PO4
11.5g	Na2HPO4
	to 1L with distilled water

20X SSC (1L)

175g	NaCl
88g	sodium citrate
	to 1L with distilled water

5M NaCl (1L)

292.2g	NaCl
	to 1L with distilled water

1.0M Tris pH 7.4 (500ml)

60.57g	Tris base
	adjust pH with conc. HCl
	to 500ml with distilled water

1.0M Tris pH8.0 (500ml)

60.57g	Tris base
	adjust pH with conc. HCl (approx. 15ml)
	to 500ml with distilled water

0.5M EDTA pH8.0 (1L)

186.1g	disodium EDTA.2H2O
	dissolve in 700ml dH2O
	adjust pH with 10M NaOH (approx 50ml)
	to 1L with distilled water

5X TBE (1L)

54g	Tris base
27.5g	boric acid
20ml	0.5M EDTA pH 8.0

GNT buffer

50mM	KCL
1.5mM	$MgCl_2$
10mM	Tris-pH8
0.01%	gelatin
0.45%	nonidet P40
0.45%	Tween

LB medium

1%	Tryptone,
0.5%	Yeast Extract
1%	Sodium Chloride
	make up with distilled water

Rinse solution (500ml) – store at 4°C:

50ml	10 X PBS
1ml	1M MgCl2,
500mg	sodium deoxycholate (deoxycholic acid)
1ml	NP40
	to 500ml with distilled water

Stain solution (500ml) – store at 4°C in the dark:

825mg	potassium ferricyanide,
920mg	potassium ferrocyanide
	to 500ml with Rinse Solution

Working stain solution:

10ml	Stain Solution,
100ul	X-Gal (100mg/ml in N,N,dimethylformamide)

10% paraformaldehyde (1L) – store at -20°C:

600ml	water, heat in microwave to around 60-70°C
100g	paraformaldehyde, stir on a hot plate (in fume hood)
1M NaOH	until powder goes into solution (around 1-2mls)
100ml	10 X PBS
	to 1L with distilled water, aliquot

4% paraformaldehyde (10ml):

4ml	10% paraformaldehyde
6ml	1X PBS
	adjust to pH 7.6

FIX Solution (10ml):

2ml	10% paraformaldehyde
80µl	glutaraldehyde (25%)
8ml	1X PBS
	adjust to pH 7.6

60X E3 Medium (2L)

34.8 g	NaCl
1.6 g	KCl
5.8 g	$CaCl_2 \cdot 2H_2O$
9.78 g	$MgCl_2 \cdot 6H_2O$
	adjust pH to 7.2 with NaOH
	up to 2L with distilled water

1X E3 medium

16.5 ml	60X E3 medium
100µl	1% methylene blue
	to 1L with distilled water

10X Tricaine stock Solution (100ml) – store at -20°C:

400mg	tricaine
97.9ml	E3 medium
0.26g	Tris in 2.1 ml E3,
	adjust to pH 7

10X Black buffer

400mM	KCl
150mM	HEPES (pH 7.9)
10mM	EDTA
5mM	DTT
50%	glycerol
	make up with distilled water

10X DNA loading buffer (50ml)

10g	Ficoll (20%)
50mg	bromophenol blue
15mg	xylene cyanol
	up to 50ml with distilled water
	1hr at 55°C to dissolve

Acrylamide gel 6%:

8ml	30% Acrylamide/Bis, 37.5:1 (Biorad #1610158)
4ml	5XTBE buffer
28ml	distilled water
450µl	10% APS
26µl	TEMED

Non-denaturing acrylamide gel:

10ml	40% acrylamide/bis 19:1 (Biorad #1610144)
4ml	5 X TBE
26ml	distilled water
450µl	10% APS
25µl	TEMED

Formamide/SSC/Tween wash (600ml)

300ml	formamide
30ml	20X SSC
600µl	Tween 20

5X MAB (500ml)

29g	maleic Acid
20.5g	NaCl
	pH to 7.5 with approx. 50ml 10M NaOH

1X MABT (1L)

200ml	5X MAB
800ml	distilled water
10ml	Tween 20

Blocking Solution (100ml)

100ml	1X MABT
2g	blocking reagent (Roche)

To dissolve, heat in microwave but be very careful not to allow to boil (volume must not change) – heat on full power at 8-10 sec intervals. Aliquot and freeze until needed.

NTMT (200ml)

4ml	5M NaCl
10ml	2M Tris pH 9.5
10ml	1M $MgCl_2$
2ml	Tween 20
174ml	distilled water
	make fresh

Proteinase K solution (200ml)

10ml	1M Tris pH 8.0
2ml	0.5M EDTA
400µl	proteinase K (20mg/ml)

Hybridization mix (10ml) – store at -20°C

10X salt	1X 1ml
Formamide	50% 5ml
Dextran sulfate	10% 1g
1ml	Yeast tRNA(10mg/ml, filtered)
100µl	100x Denhardts
	to 10ml with distilled water
	heat at 55 degrees to dissolve dextran

10X salt (200ml)

22.8g	NaCl
286mg	Tris Base
2.8g	Tris HCL
1.14g	NaH_2PO_4
1.42g	Na_2HPO_4
5ml	0.5M EDTA
	make up with distilled water

Moist Chamber Solution (50ml)

25ml	Formamide
5ml	20XSSC
20ml	distilled water

Chapter 3 – The Flk1i10 enhancer

3.1. Introduction

3.1.1 Vegfr2 signalling

The tyrosine kinase receptor Vegfr2, encoded by the gene *Flk1* in mice, is the main mediator of Vegf signalling during angiogenesis in mammals and a prime target for anti-angiogenic therapy (Olsson et al 2006). *Flk1*$^{-/-}$ mice die at E8.5 to 9.5 and lack blood island and vascular plexus development, despite normal angioblast formation, while mice with a heterozygous deletion of *Flk1* were viable and fertile (Shalaby et al 1995). Apart from a fundamental role in endothelial cell proliferation, migration, and survival, Vegf signalling is also implicated in the establishment of the arterial endothelial cell phenotype and tip/stalk cell selection during angiogenic sprouting (Benedito and Hellström 2013, Phng and Gerhardt 2009, Swift and Weinstein 2009). Despite its developmental and clinical importance in cardiovascular tissues, the regulation of *Flk1* transcription is still incompletely understood, although both promoter and enhancer regions have been previously studied.

3.1.2 The *Flk1* promoter region

Both the human and murine *Flk1* extended promoter regions have been closely investigated, primarily in tissue culture, and shown to contain regulatory elements with

binding sites for Sp1, AP-2, NFkB, and E-box/Ets proteins (Patterson et al 1995, 1997, Rönicke et al 1996) *In vitro* studies suggest that the promoter can be activated by the transcription factor NANOG in response to Wnt signalling, by HIF-2α in response to hypoxia, and by Gata2 . Mutational analysis using *in vitro* activity assays has further revealed a critical role for two E-twenty-six (ETS) binding sites for promoter activity (Kappel et al 2000). Moreover, the promoter can be negatively regulated *in vitro* by Kruppel-like factor 2 (KLF2), which competes with the activating transcription factor specific protein 1 (Sp1), and by Tgfβ signalling which has been shown to inhibit Gata2 binding to the 5' untranslated region (Bhattacharya et al 2005, Minami et al 2001). However, these promoter-proximal regulatory elements were not sufficient to drive endothelial specific expression of a reporter gene in transgenic mouse embryos, suggesting that distal regulatory elements are important for endothelial *Flk1* expression (Kappel et al 1999).

3.1.3 First intron enhancer

A 510 bp enhancer was identified in the first intron of the mouse *Flk1* locus. This enhancer, in combination with either a 939bp fragment of the endogenous promoter region or the heterologous, minimal thymidine kinase promoter, drives reporter gene expression specifically in endothelial cells during early murine development (Kappel et al 1999). In a mouse reporter line, activity of this enhancer was detected in angioblasts of the yolk sac at E7.8. At E10.5 activity was confined to the vascular endothelium and at E11.5 reporter expression was observed in arteries, veins, and capillaries (Kappel et al 1999). Enhancer activity was further detected in the vasculature of various organs of new-born mice. Comparison of reporter gene

expression pattern with heterozygous *Flk1*$^{+/-}$ mouse embryos, which express the *LacZ* gene from the endogenous *Flk1* locus suggested that enhancer activity closely resembles the expression pattern of the endogenous Vegfr2 (Kappel et al 1999). Mutational analysis of potential binding sites indicated a role for Scl/Tal1, Gata and Ets transcription factors in the regulation of enhancer activity (Kappel et al 2000). However, deletion of the entire first intron enhancer in the context of a *Flk1-LacZ* knock-in model did not result in a discernible loss of vascular reporter gene expression compared to the wild-type. This suggests that the first intron enhancer is sufficient, but not necessary for the regulation of endothelial Vegfr2 expression, and strongly suggests that elements elsewhere in the locus make significant contributions to the regulation of *Flk1* gene expression in the endothelium (Ema et al 2006).

3.1.4 5' Upstream Enhancer

A second regulatory element was also recently identified approximately +30kb upstream of the *Flk1* TSS (Ishitobi et al 2011). This element only directs mesodermal expression during development and no endothelial activity was detected in transgenic mice. The +30kb enhancer can be activated by Bmp, Wnt and FGF, and contains conserved binding sites for Gata, Cdx, Tcf/Lef, ER71/Etv2- and Fox-transcription factors (Ishitobi et al 2011). Deletion of this upstream enhancer resulted in a drastic reduction of Vegfr2 expression in the primitive streak of mouse embryos. However, mice were born in Mendelian ratios, indicating that the enhancer is not essential for embryonic vascular development (Ishitobi et al 2011). Surprisingly though, mice deficient for the +30kb enhancer died shortly after birth for reasons unknown, demonstrating that this enhancer has an essential function.

3.1.5 Identification of a novel enhancer of *Flk1*

Together, these studies demonstrate that regulation of Vegfr2 expression is coordinated by different *cis*-regulatory elements which control expression of Vegfr2 in different tissues and developmental stages. However, it is also apparent that the picture of the *cis*-regulatory landscape of the *Flk1* locus is incomplete and that additional *cis*-regulatory elements are likely to be involved in the regulation of *Flk1* gene transcription. Identification and analysis of such elements can provide valuable insights in the regulatory pathways that are upstream of Vegfr2 expression in endothelial development.

This study describes a *cis*-regulatory element within the tenth intron of the *Flk1* gene (Flk1i10 enhancer) which, in combination with a minimal promoter, is sufficient to drive expression of a reporter in arterial endothelial cells in both mice and zebrafish. The element contains conserved ETS, RBPJ, SOX, GATA, FOX, and TCF/LEF binding sites. This study demonstrates a crucial role for the transcription factor Gata2 for the activation of the Flk1i10 enhancer. It further shows that this activity is modulated by the Notch downstream effector Rbpj, such that it represses enhancer activity in the venous endothelium to mediate differential expression of Flk1 in arteries and veins. Furthermore, the enhancer activity is itself dependent on Vergfr2 signalling, arguing for the existence of an auto-regulatory Vegf-signalling loop.

3.2. Results

3.2.1 Encode Analysis of *Flk1* locus reveals two intronic candidate enhancer regions

Non-coding, regulatory elements such as enhancers are often under purifying selection (Karolchik et al 2014, Kent, Sugnet, et al, Zahler and Zahler 2002). As a result, mutation rates in these elements are much lower when compared to other non-coding sequence. As a first step in the identification of potential *cis*-regulatory elements in the *Flk1* locus, its non-coding, upstream, downstream and intronic regions were screened for islands of high evolutionary conservation. For this, sequence alignments were examined for different mammalian species available on the UCSC genome browser (Karolchik et al 2014). Several intronic regions, including the previously described enhancer in the first intron of the *Flk1* gene, showed peaks of mammalian conservation (Fig. 3.1, A).

To further narrow down the search for potential enhancers, ChIP-Seq data on histone modifications associated with regulatory elements compiled by the ENCODE project was examined (Rosenbloom et al 2012). Since epigenetic signatures at regulatory elements are generally tissue-specific they need to be examined in the cell type or tissue in which the element is active. However, appropriate ChIP-Seq data sets were not available for mouse endothelial cells. Therefore, epigenetic signatures were investigated in the highly vascularized tissues of liver, lung and brain, as well as in the heart, which often shares histone signatures with endothelial cells, and mouse embryonic fibroblasts (MEFs). H3K4me1, which has been shown to mark both poised and active enhancers, was found to be increased at regions in the third and tenth

intron of the *Flk1* gene in heart, liver, and lung but not in brain or MEFs (Fig. 3.1, B). The tenth intron additionally showed peaks in H3K27ac, a marker of active enhancers, in heart and liver, which further implies this region may be a potential enhancer (Fig. 3.1, C). As the same regions were also marked by peaks in mammalian conservation, this suggests they may be functional regulatory elements. The previously identified endothelial enhancer in the first intron of *Flk1* was also conserved and showed small but noticeable peaks in H3K4me1 and H3K27ac, suggesting that these parameters are suitable to identify endothelial enhancers (Fig. 3.1).

As endothelial-specific epigenetic signatures were unavailable for the mouse sequence of the *Flk1* locus, epigenetic signatures of the human sequence were also investigated. In human umbilical vein endothelial cells both regions in the third, and the tenth intron were marked by peaks of H3K4me1 but not H3K27ac, suggesting the enhancers are poised in these cells (Fig. 3.2, B).

DNaseI hypersensitivity and FAIRE data is available for a panel of human endothelial cells (Rosenbloom et al 2012). These techniques identify regions of open chromatin characteristic of active sites such as enhancers (Tsompana and Buck 2014). Both the third and the tenth intron, as well as the previously identified endothelial enhancer in the first intron, were marked by regions of open chromatin in the majority of the endothelial cells analysed (Fig. 3.2, A). This indicates that these regions are bound by transcription factors in endothelial cells, further corroborating their identity as potential enhancers.

A combined survey of ChIP-Seq profiles for 161 transcription factors in 91 human cell lines indicates binding of the transcription factor Gata2 to both the third and the tenth intron (Fig. 3.2, C). Gata2 is known to be involved in the regulation of endothelial

specific genes (De Val and Black 2009). Apart from GATA binding motifs, close examination of the sequence of the conserved elements in the third and tenth intron further revealed the presence of multiple ETS binding motifs. Ets factors play a critical role in the regulation of endothelial gene expression and clusters of ETS binding sites have been found at all known vascular enhancers (De Val and Black 2009). Furthermore, both regions contained a FOX:ETS motif, which has been shown to be a common feature of endothelial enhancers and has been used for the successful identification of novel endothelial gene enhancers (De Val et al 2008).

Together, the analysis of *Flk1* locus discovered two regions within the third and tenth intron of the *Flk1* gene that are evolutionary conserved and marked by enhancer-associated histone modifications in highly vascularized mouse tissues and human endothelial cells *in vitro*. These regions are further found in an open chromatin conformation in human endothelial cells *in vitro* and can be bound by the transcription factor Gata2. Moreover, both regions contain transcription factor binding motifs typically found at regulatory elements involved in endothelial gene expression. Therefore, both regions are attractive candidates for functional regulatory elements in the regulation of endothelial *Flk1* expression.

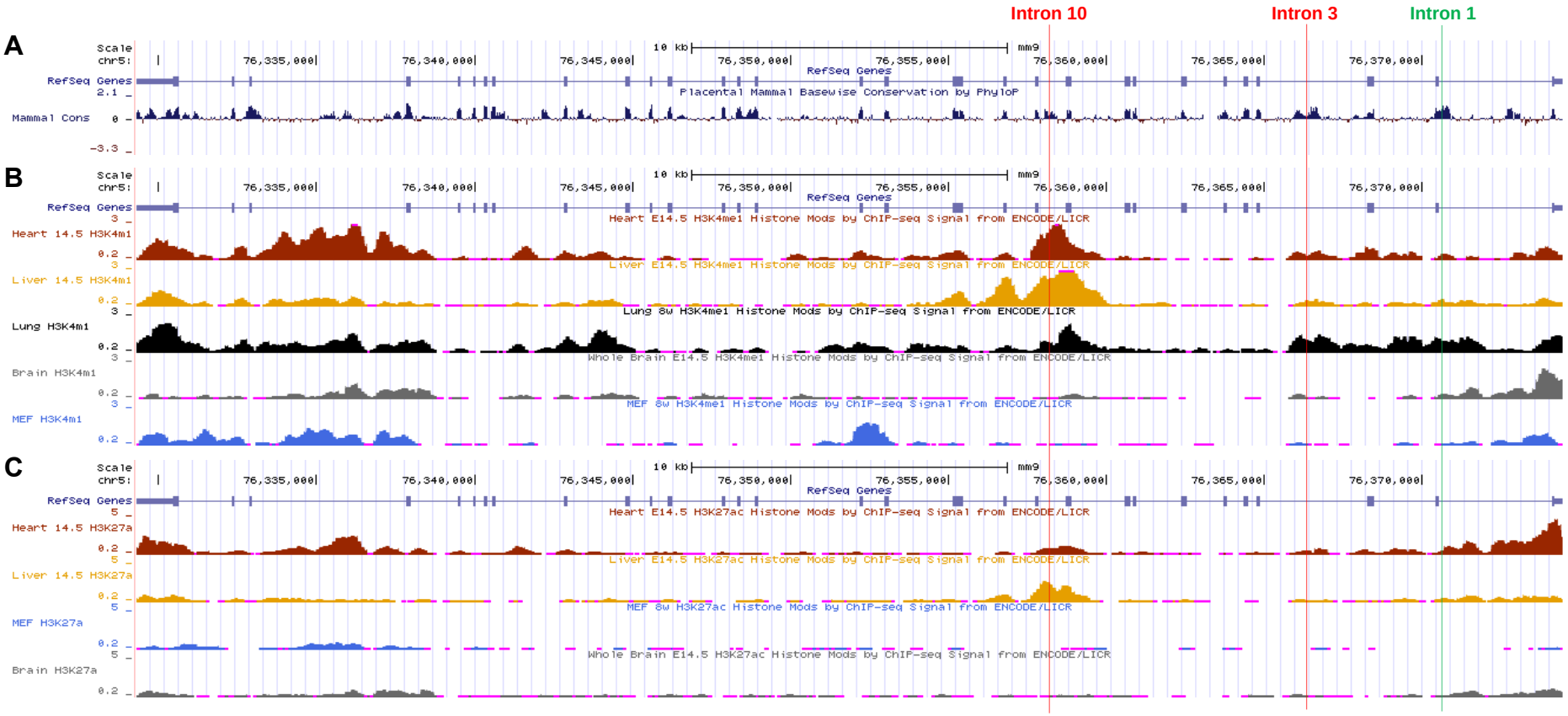


Figure 3.1: Analysis of the mouse Flk1 locus

The UCSC Genome Browser was used to visualize selected analyses of the murine Flk1 locus. Two regions in intron 3 and intron 10 are marked by a vertical red line, while the validated endothelial enhancer in the first intron is highlighted in green.
A: Levels of evolutionary conservation of the Flk1 gene among mammals is shown in the lower track. The upper track shows the reference sequence with boxes indicating exons while lines indicate introns. Both intron 3 and intron 10, as well as intron 1, show peaks of conservation.
B: ChIP for H3K4me1 histone modification which is associated with regulatory elements is shown for embryonic mouse heart (dark red track), liver (yellow), lung (black), brain (grey) and MEFs (blue). Intron 10 is marked by strong peaks in heart lung and liver, whereas introns 3 and 1 are marked by shallower peaks.
C: ChIP for H3K27ac associated with active enhancers is shown for the same organs (except lung). Intron 10 is marked by peaks in heart and liver tissue, smaller peaks are also seen in intron 1, while intron 3 and does not show significant H3K27ac enrichment.

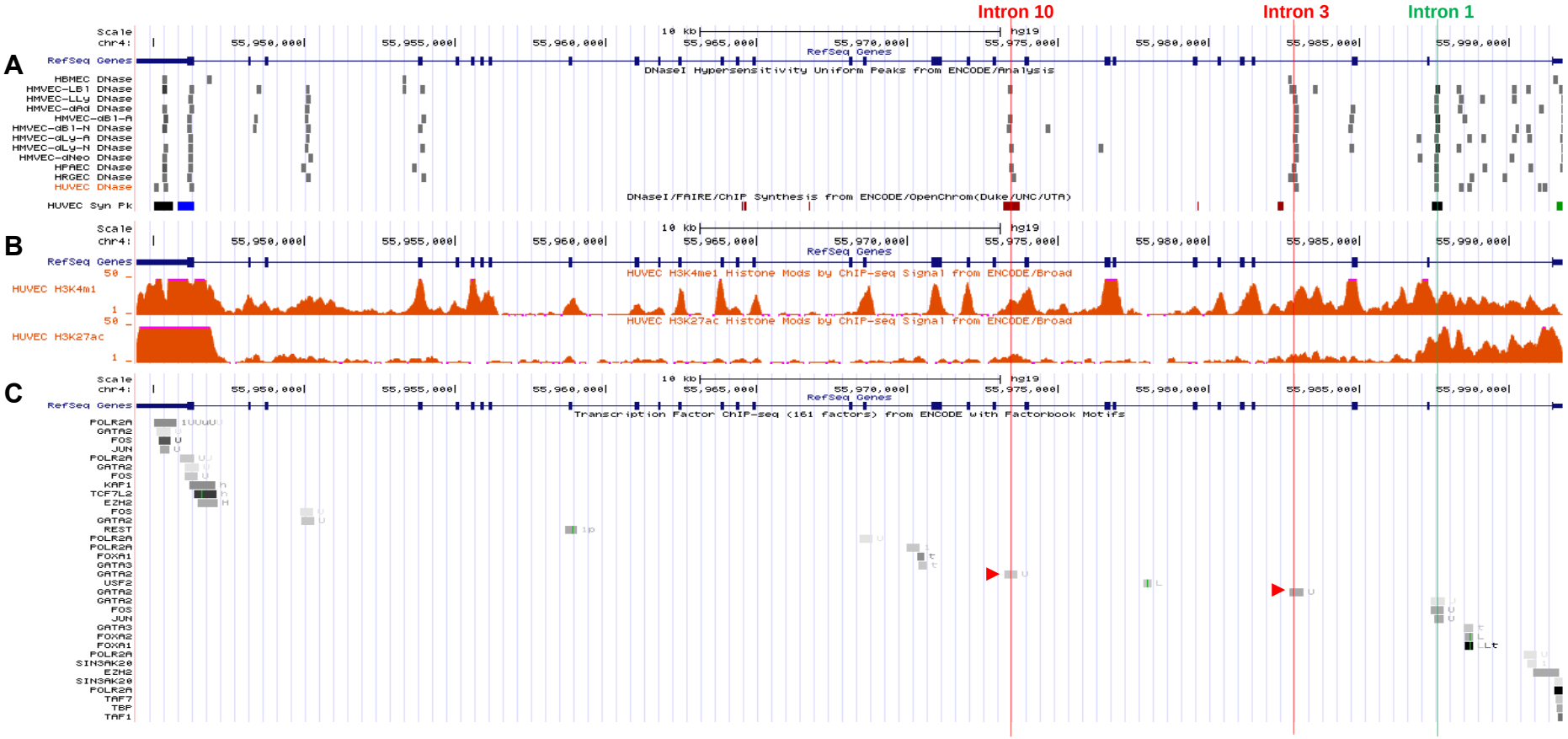


Figure 3.2: Analysis of the human Flk1 locus

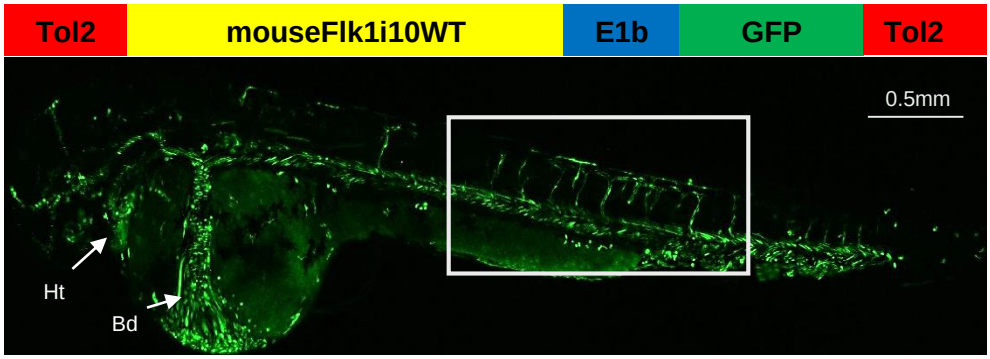
The UCSC Genome Browser was used to visualize selected analyses of the human Flk1 locus. Two regions in intron 3 and intron 10 are marked by a vertical red line, while the validated endothelial enhancer in the first intron is highlighted in green.
A: Regions of open chromatin (identified by FAIRE or DNase1 treatment) are shown for a panel of different human endothelial cell lines (open regions indicated by grey boxes). Both intron 3 and 10, as well as intron 1, are marked by regions of open chromatin in endothelial cell lines.
B: H3K4me1 and H3K27ac histone modifications associated with regulatory elements are shown for human umbilical vain endothelial cells (orange tracks). Peaks for H3K4me1 are observed for both intron 3 and intron 10. Significant H3K27ac is not apparent at either intron, whereas the first intron enhancer shows enrichment for both histone marks.
C: A combined track for transcription factor ChIP for 161 TFs in 91 human cell lines shows binding of GATA2 at intron 10 and 3 (red arrowheads), as well as intron 1.

3.2.2 Transgenic enhancer-reporter assay identifies Flk1i10 as a functional endothelial enhancer in zebrafish

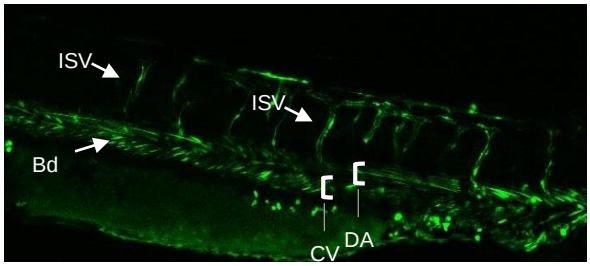
Sequence analysis of the *Flk1* locus identified two regions in the third and tenth intron of the *Flk1* gene with high potential to be functional regulatory elements in endothelial cells. To test the ability of these candidate enhancers to direct endothelial-specific transcription, the mouse sequences of the conserved regions in the third and tenth intron were amplified from genomic DNA and cloned using GateWay technology into a Tol2 transposon vector containing the silent E1b minimal promoter and green-fluorescent protein (GFP) reporter gene sequence (Birnbaum et al 2012). The Tol2 vector further contains short Tol2 recognition sequences flanking the enhancer-reporter construct. This work was performed by M. Thomas, a FHS summer student. The Tol2 enhancer-reporter plasmids were used to generate transient transgenic zebrafish with the Tol2 transposon system (Kawakami 2005). For this, the vector was injected together with Tol2 transposase *mRNA* into fertilized fish eggs. Once active Tol2 transposase has been made by the injected cells, the protein is able to recognize the Tol2 recognition sequences flanking the enhancer-reporter construct. Subsequently, Tol2 transposase mediates the ex*cis*ion of the construct and its integration into the host genome at random locations. In transgenic zebrafish created in this way, the expression of the reporter GFP is dependent on the ability of the candidate enhancer to activate transcription, as the minimal promoter alone is insufficient to drive transcription. Consequently, GFP expression becomes an easily detectable readout for enhancer activity *in vivo*. As injection occurs at a multicellular blastomere stage and not all cells experience integration events, mosaic transgenics are generated which only express the transgene in a subset of cells. Moreover, as integration occurs at random locations in the fish genome, GFP expression levels can

vary among individual transgenics. However, as Tol2 mediated transgenesis allows rapid generation and screening of high numbers of transgenic zebrafish, a comprehensive picture of enhancer activity can be gained in a single experiment. Injection of zebrafish embryos throughout this project was carried out by N. Sacilotto and A. Neal. Screening of mosaic transgenic zebrafish for reporter gene activity between 24h and 72h post fertilization revealed vascular expression of GFP was driven by the Flk1i10 in over 60% of transgenic embryos whereas no endothelial expression was observed for the Flk1i3 candidate enhancer (Fig. 3.3). Furthermore, the Flk1i10 enhancer also drives GFP expression in the heart musculature and in blood cells. These experiments identify the Flk1i10 candidate enhancer as a functional enhancer which, in combination with a silent minimal promoter, drives expression in endothelial cells in zebrafish.

A



B



C

Transient transgenic zebrafish	
Construct	**% of fish with vascular expression at 48hpf (total analysed)**
Flk1i3	0 (67)
Flk1i10	64 (121)

Figure 3.3: Flk1 intron 10 enhancer directs vascular expression

A: Transient transgenic zebrafish carrying 826bp Flk1i10 enhancer in front of E1b promoter and GFP reporter. Reporter expression is detected in intersegmental vessels (ISV) and the dorsal aorta (DA). Expression in the cardinal vein (CV) is obscured by GFP-positive blood cells (Bd). GFP expression is also seen in the heart (Ht).
B: Zoom-in (indicated by white box in A)
C: Vascular expression in transient transgenic embryos with either Flk1 intron 3 or intron 10 enhancer reporter construct

3.2.3 The Flk1i10 enhancer drives expression in arterial endothelial cells in stable transgenic zebrafish

For a detailed description of the vascular expression pattern a stable transgenic zebrafish line, *tg(Flk1i10:GFP),* was generated by breeding mosaic transient transgenics and screening for offspring which had inherited the transgene. In contrast to mosaic transient transgenics, the *tg(Flk1i10:GFP)* line allows assessment of enhancer activity in all cells of individual fish. Enhancer activity was first detected at 24hpf, with GFP expression clearly visible in the aorta and, to a lesser extent, in the cardinal vein as well as in the intersegmental vessels (ISV) which arise bilaterally from the dorsal aorta near the somite boundaries (Fig. 3.4, 24hpf) (Isogai et al 2001). By 36hpf, enhancer activity expanded to the dorsal longitudinal anastomotic vessels (DLAV) (Fig. 3.4, 36hpf). The DLAV form as two continuous, independent vessels following bifurcation and anastomosis of ISV with their ipsilateral neighbours near the dorsal side of the neural tube at around 32hpf (Childs et al 2002). Shortly after, at around 36–38hpf, interconnections between the two DLAVs begin to form as angiogenic sprouts extend from the dorsal surface of the DLAV and connect either with their closest contra-lateral neighbours, or to the opposite DLAV to form a plexus. By 48hpf, enhancer activity was no longer clear in the cardinal vein, while GFP expression remained strong in the aorta, the ISV and the DLAV plexus, suggesting that, at this stage, Flk1i10 enhancer activity is mainly restricted to mature arteries and newly formed vessels whereas the enhancer is not active in mature venous vessels (Fig. 3.4, 48hpf). At 72hpf, GFP expression was reduced in the dorsal aorta while maintained strongly in the DLAV plexus (Fig. 3.4, 72hpf). The DLAV plexus has been shown to be a site of active Vegf-dependent endothelial cell proliferation (Zygmunt et al 2012). This may indicate that the Flk1i10 enhancer is active in endothelial cells

during Vegf-induced proliferation and thus downstream of Vegf signalling, which would further explain why expression fades from the dorsal aorta as it enters a Vegf independent remodelling stage. At 72hpf there was also strong expression of GFP in some, but not all, ISVs. A similar pattern of enhancer activity has been observed for the arterial Dll4 enhancer and likely reflects the mixed arterial and venous identities of the ISVs (Sacilotto et al 2013).

In addition to the endothelium, the Flk1i10 enhancer was also active in blood cells and in the zebrafish heart. Expression in the blood was somewhat weaker during later stages of development, whereas GFP was highly expressed in the heart at all stages examined (Fig. 3.4).

These results indicate that the Flk1i10 enhancer can drive reporter gene expression in the entire endothelium at early developmental stages (24 – 36hpf). In later stages (48 – 72hpf) vascular expression becomes increasingly specified to the arterial endothelium, as expression is observed in the dorsal aorta but not the cardinal vein, and in arterial ISVs. Moreover, Flk1i10 enhancer activity appears to be strongest at sites of active vascular growth suggesting that its activity may be regulated by pro-angiogenic signalling.

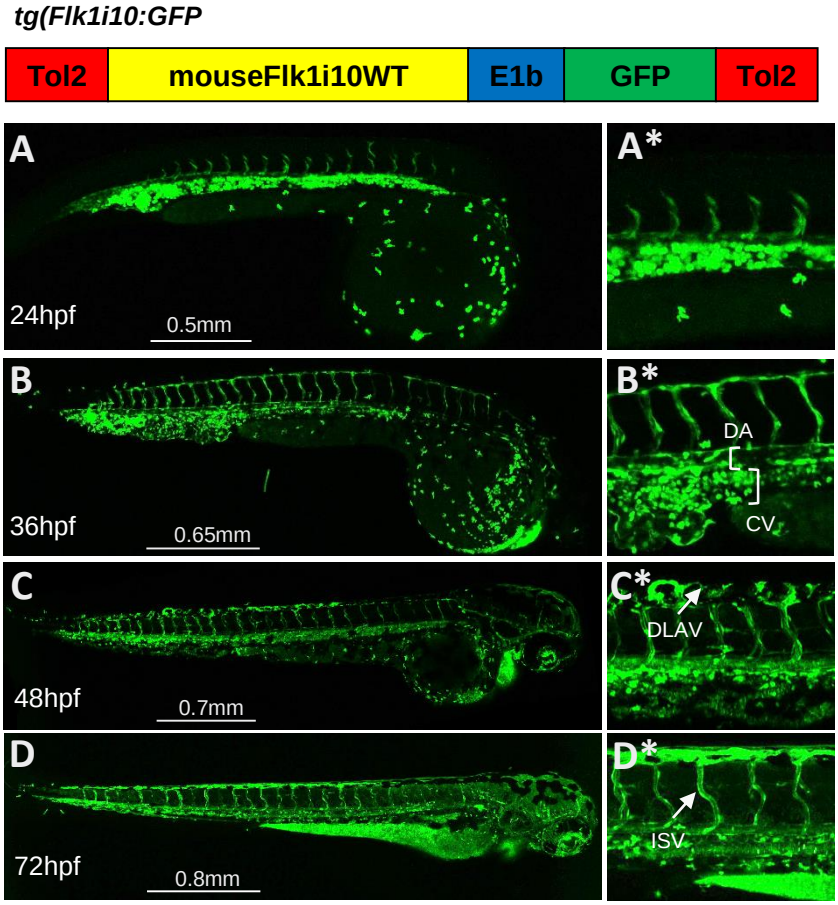


Figure 3.4: Time course of GFP expression in *tg(Flk1i10:GFP)* stable line

A: At 24hpf expression of GFP is detected in the axial and the sprouting intersegmental vessels (ISV).
B: At 36hpf, the Flk1i10 enhancer is active in the dorsal aorta (DA) the cardinal vein (CV) and the dorsal longitudinal anastomotic vessels (DLAV).
C: From 48hpf onwards, expression is stronger in the DA and fades from the CV.
D: At 72hpf expression is strongest in the DLAV and fades in more mature vessels.
Expression is also detected in blood cells and the heart at all stages.

3.2.4 Transgenic enhancer-reporter assay confirms arterial-specific expression pattern of Flk1i10 enhancer in mice

Experiments in zebrafish tell us that the mouse Flk1i10 enhancer is functional and drives expression in arteries, blood cells and the heart. However, while the transcription factors that bind enhancers are highly conserved among species, individual transcriptional networks governing tissue development may differ between fish and mice (Wittkopp and Kalay 2011). Therefore it was crucial to verify the observed expression pattern in transgenic mice. To this end, the 826-bp fragment containing the mouse Flk1i10 enhancer was cloned upstream of the silent hsp68 minimal promoter-βGalactosidase (*LacZ)* reporter gene and used to generate transient transgenic mice (Kothary et al 1989, Pennacchio et al 2006). This was achieved by pronuclear injection of the linearized vector DNA into mouse zygotes, which results in integration of the vector DNA at random locations in the host genome. After injection the zygotes were re-implanted into pseudo-pregnant foster mothers where they resume embryonic development (Ittner and Götz 2007). Generation of transgenic mice was performed by K. Liu. Foster mothers were sacrificed at E12.5 as arterial and venous structures are easily identifiable at this stage of development. Transgene expression was visualized by X-Gal staining, which results in the deposition of a blue precipitate in tissues where the enhancer drives expression of the *LacZ* reporter gene, thus providing an easily detectable readout for enhancer activity. Analysis of three individual E12.5 transgenic mouse embryos revealed that the Flk1i10 enhancer drives reporter gene expression in the vasculature of both the embryo proper and the embryonic yolk sac in all transgenic embryos analysed, although with varying intensity (Fig. 3.5). Such differences in intensity between independent transgenic embryos is a common occurrence in transient transgenics and depend on the

chromatin environment at the location of the integration event, the orientation of the construct, and the number of integration events per zygote. To more closely investigate the activity of the Flk1i10 enhancer within the vasculature, transgenic embryos were sectioned and counterstained with Nuclear Fast Red to enhance tissue contrast. Microscopy of reliably identifiable vessels in these sections, such as the dorsal aorta, the cardinal vein, the tail artery and tail veins, clearly demonstrates that X-Gal staining is predominantly observed in arterial vessels and in the microvasculature but not the venous, or lymphatic vasculature (Fig. 3.6). This suggests that the Flk1i10 enhancer directs expression specifically to arterial endothelial cells in mice. Histological analysis further showed Flk1i10 enhancer activity in both ventricles of the developing heart, which is consistent with the observations in zebrafish. The left and right atria, however, remained negative for X-Gal staining (Fig. 3.6).

Overall, the analysis of transgenic enhancer-reporter mouse embryos demonstrates that Flk1i10 is a functional endothelial enhancer also in its native species. As observed in *tg(Flk1i10:GFP)* zebrafish, the Flk1i10 enhancer drives arterial-specific expression in E12.5 mouse embryos as well as in the ventricles of the heart.

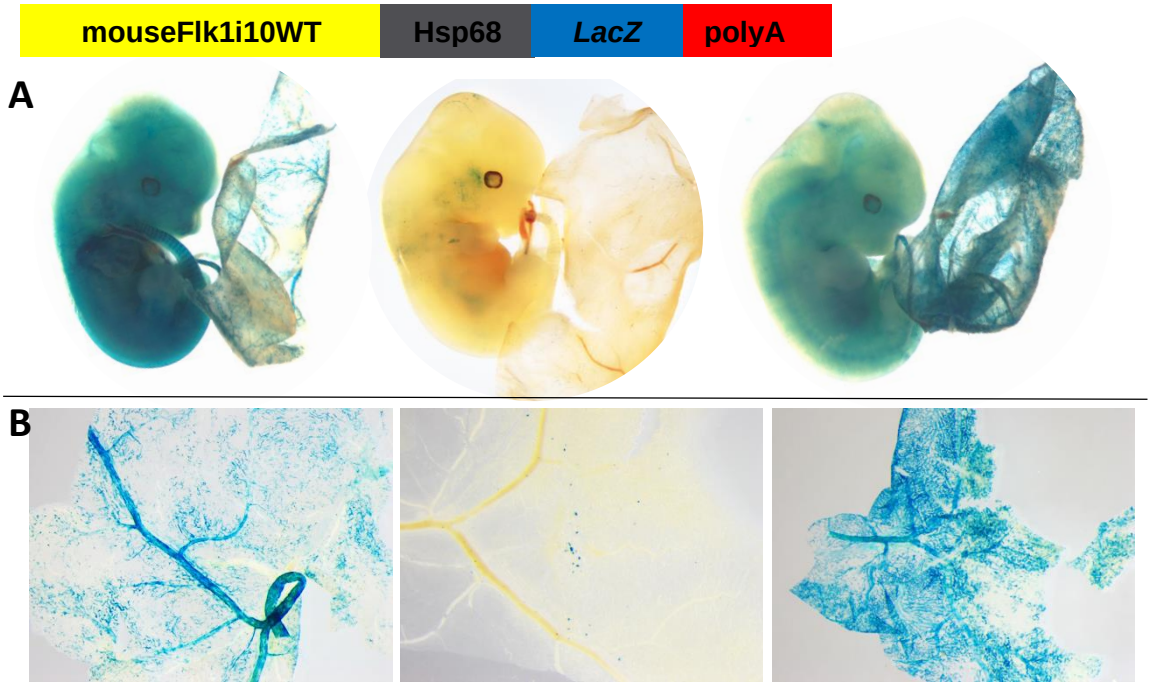


Figure 3.5: Flk1i 10 enhancer directs arterial expression

Transient transgenic E12.5 mouse embryos carrying 826bp Flk1i10 enhancer in front of Hsp68 promoter and *LacZ* reporter. Reporter expression is visualized by blue XGal staining.
A: Vascular expression can be detected in the embryo proper with varying intensities.
B: XGal staining in the vasculature of the yolk sac (corresponding embryos in A).

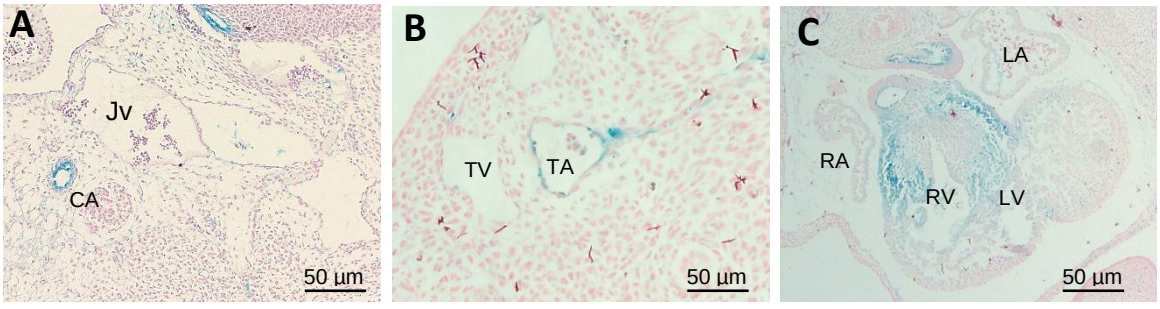


Figure 3.6: Flk1i10 enhancer directs arterial expression in transgenic mice

5µm paraffin sections of transient transgenic E12.5 mouse embryos carrying 826bp Flk1i10 enhancer in front of Hsp68 promoter and *LacZ* reporter. Reporter expression is visualized by blue X-Gal staining.
A: Section through the neck of the embryo shows staining in the carotid artery (CA) but not the jugular vein (JV).
B: Section of the embryonic tail shows staining in the single tail artery (TA) but not the paired tail veins (TV).
C: Section of the embryonic heart reveals X-Gal staining in the left and right ventricle (LV, RV) but not the atria (LA, RA).

3.2.5 The Flk1i10 enhancer becomes restricted to arteries during embryonic development in a mouse transgenic line

For a more thorough investigation of enhancer activity throughout development, transgenic mice generated by pronuclear injection were used to establish a transgenic line, *Flk1i10LacZ*, allowing analysis of *LacZ* expression at different stages of embryonic development (Fig. 3.7). X-Gal staining was detected from the initial establishment of the vasculature and continued throughout embryonic development. At E8.5, when the first vascular structures are visible, enhancer activity was clearly detected in both extra-embryonic yolk sac and intra-embryonic vascular structures (Fig. 3.7, E8.5). At this developmental stage, the paired dorsal aortae are newly formed, suggesting that the enhancer is active during vasculogenesis. By E9.5, enhancer activity was observed in both the dorsal aortae and the cardinal veins as well as in microvascular beds (Fig. 3.7 and 3.8, E9.5). The yolk sac vasculature of E9.5 embryos also exhibitied a uniform X-Gal staining. At E10.5 a similar pattern of expression was visible in the embryo proper, whereas individual arteries of the yolk sac became more clearly visible (Fig. 3.7 and 3.8, E10.5). At E11.5 a more restricted, arterial-specific *LacZ* expression pattern was seen in the embryo proper, and by E12.5 sections show that enhancer activity, while clearly detected in the dorsal aorta, was absent from the cardinal vein (Fig. 3.7, E11.5 and E12.5; Fig. 3.8, E12.5). The yolk sac vasculature of these embryos also shows robust reporter gene expression in arterial vessels, whereas adjacent veins remained negative. This was in agreement with the observations from transient transgenics, which also showed an arterial expression pattern at this stage in development (Fig. 3.5 and 3.6).

While in most cases arteries and veins can be identified based on their morphology, immunostaining was also performed for the endothelial marker Endomucin (Emcn), which is venous specific after E11.5, in combination with an antibody against the βGalactosidase reporter gene (Fig. 3.9) (Brachtendorf et al 2001). No overlap between Emcn and βGalactosidase immunostainings was detected in the vasculature, indicating that βGal expression driven by the Flk1i10 enhancer was not expressed in Emcn positive veins.

By late gestation, the Flk1i10 enhancer remained active in arterial vessels in various organs, including skin and the kidneys (Fig. 3.10). Interestingly, X-Gal staining seems to mark renal glomeruli, which, unlike most other capillary beds, are considered to be entirely arterial with both an afferent and efferent arteriole. Similar to *tg(Flk1i10:GFP)* zebrafish, *LacZ* expression was also observed in the ventricles of the developing mouse heart, although no staining was seen in the atria. Somewhat surprisingly, no staining was observed in the embryonic lungs, even though they are highly vascularized. However, it has to be taken into account that the lungs only become fully functional after birth, which may have an effect on enhancer activity. It also has to be considered that X-Gal staining may not be equally efficient in all tissues. Transgene expression was also highly arterial specific in the yolk sacs of E16 *Flk1i10LacZ* embryos.

To investigate the expression pattern of Flk1i10 post-natally, X-Gal staining was performed on retinas of new-born pups five and seven days after birth. The retinal vasculature is a popular model for the study of angiogenic sprouting as it develops only after birth, is easily accessible and initially forms a regular, 2 dimensional network which is convenient for imaging. Unfortunately, vascular expression of *LacZ* was not detected in the retinal vasculature of *Flk1i10LacZ*, although expression was observed

in non-vascular structures (not shown). Since endogenous Flk1 *mRNA* is highly expressed in the retina at these time points, it is likely that alternative enhancers are required for this expression, possibly downstream of different transcription factors.

In conclusion, the reporter gene expression patterns observed in the stable transgenic *Flk1i10LacZ* mouse line further substantiates the finding from transient transgenic mouse embryos, indicating that the Flk1i10 enhancer is specifically active in arterial endothelial cells at later stages of embryonic development. However, examination of early stage embryos revealed that arterial specificity is only acquired around E11.5 and that the enhancer is active throughout the vasculature at earlier time points, suggesting that expression becomes restricted throughout development. Further, analysis of the *Flk1i10LacZ* mouse line shows that the enhancer is active in several major organs, but switches off after birth.

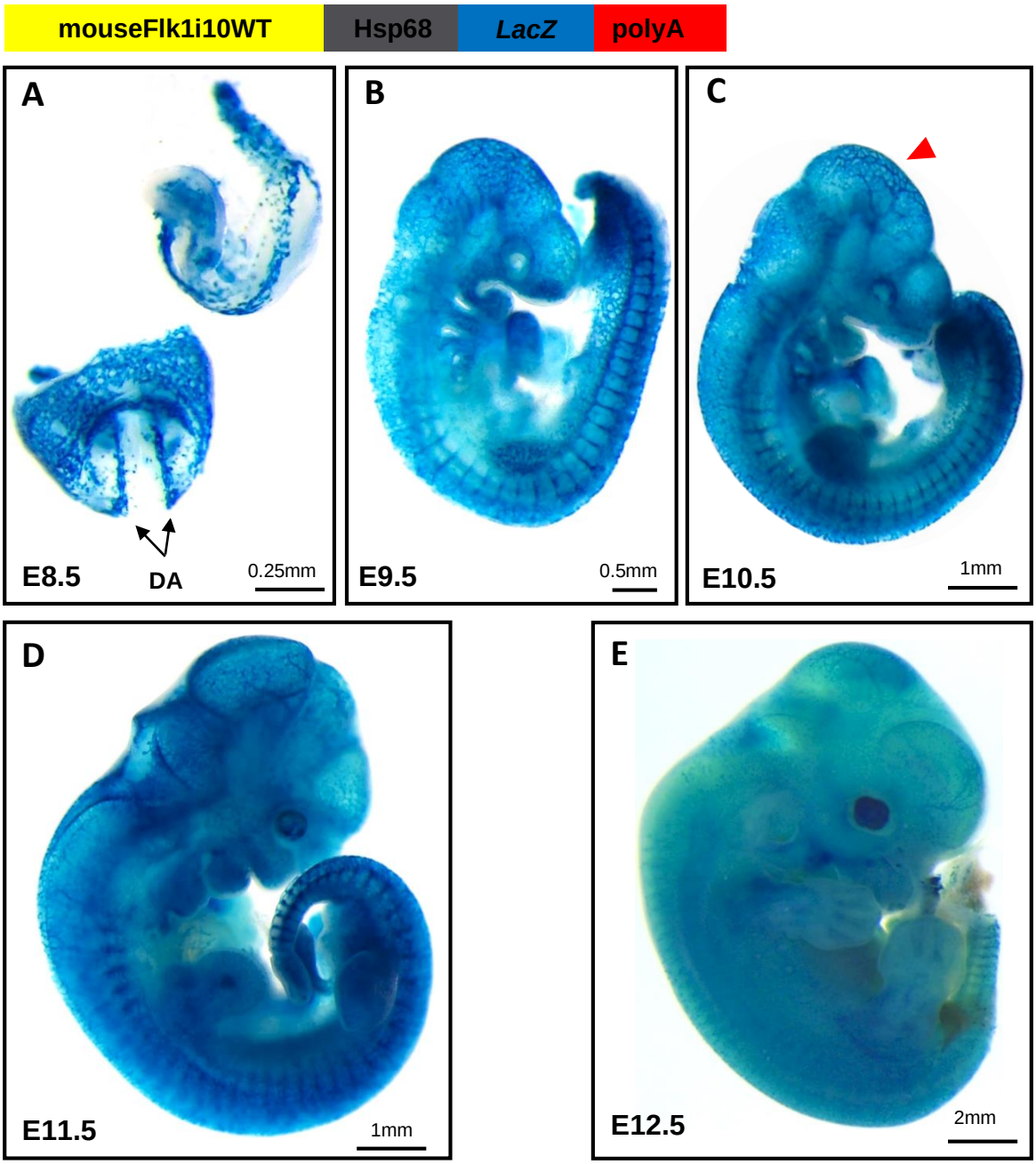


Figure 3.7: Expression pattern Flk1i10 enhancer at E8.5 – E12.5

X-gal staining of stable transgenic line of Flk1i10 enhancer driving β-galactosidase expression at various stages of embryonic development. The staining pattern at the various developmental stages indicates that the Flk1i10 enhancer becomes arterial specific around mid-gestation.
A: At E8.5, XGal staining can be detected in the newly formed paired dorsal aortae (DA) and angioblasts of the yolk sac.
B: Enahncer activity can be detected throughout the developing vasculature at E9.5.
C: The staining pattern of the head vasculature at E10.5 (red arrowhead) indicates increased enhancer activity in arterial vessels
D+E: At E11.5 and E12.5 the staining pattern suggests arterial specific enhancer activity.

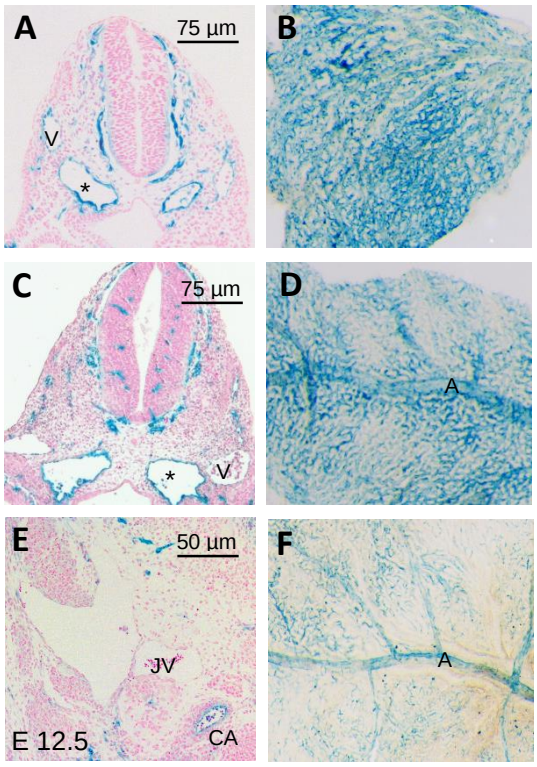


Figure 3.8: Flk1i10 enhancer is arterial specific from mid-gestation onwards

X-Gal staining of *Flk1i10LacZ* stable transgenic mice.
A+B: At E9.5 X-Gal staining is detected in both the dorsal aorta (*) and the cardinal veins (V). At this stage the yolk sac vasculature show a uniform staining pattern.
C+D: At E10.5 staining fades in the cardinal veins and individual arteries (A) become visible in the yolk sac.
E+F: At E12.5 staining is arterial specific in both the embryo proper as well as the yolk sac. JV = Jugular vein; CA = carotid artery

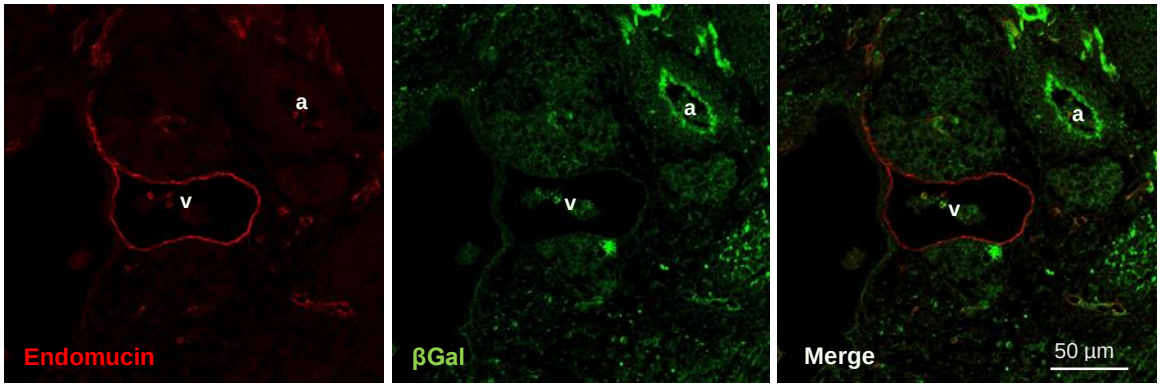


Figure 3.9: βGal immunostaining does not overlap with venous marker Endomucin

Double immunostaining for βGal (green) and the venous marker Endomucin (red) on 5µm sections of E12.5 of *Flk1i10LacZ* stable transgenic mice. *LacZ* reporter gene is expressed in arteries but not veins
v = cardinal vein, a = dorsal aorta

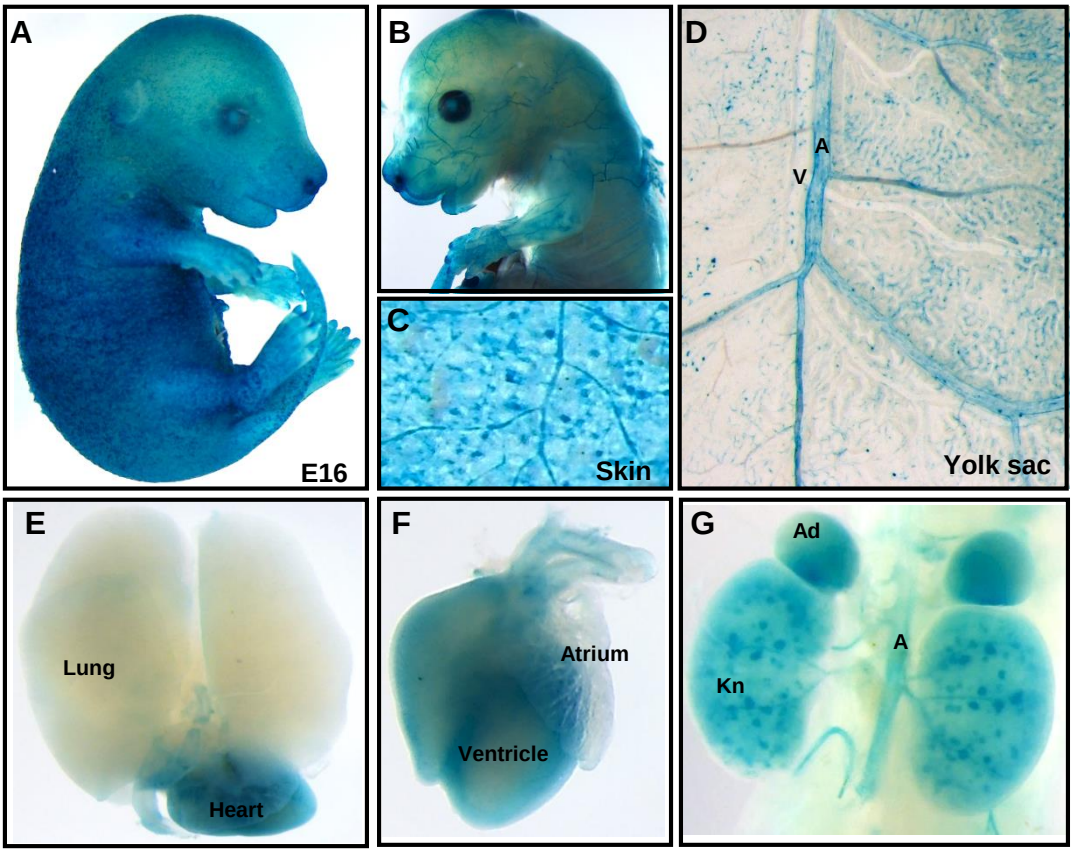


Figure 3.10: Arterial expression pattern Flk1i10 enhancer at E16

X-gal staining of stable transgenic line of Flk1i10 enhancer driving β-galactosidase expression at E16.
A: While XGal staining can be clearly observed vascular specific expression is difficult to detect in whole embryos at this stage.
B: Upper body of a skinned E16 embryo. Individual blue vessels are now clearly visible.
C: Despite non-vascular staining in the skin of an E16 embryo, an arterial tree structure is clearly offset.
D: In the yolk sac of an E16 embryo blue stained arteries (A) are distinct from unstained veins (V), indicating that the Flk1i10 enhancer is arterial specific.
E: Lungs and heart of an E16 embryo. While highly vascularized, the lung shows no XGal staining, whereas the heart shows robust staining.
F: Close inspection of the heart reveals that the atria show no enhancer activity as opposed to the ventricles which are clearly stained.
G: Both kidneys (Kn) and adrenal glands (Ad) show positive XGal staining. Granular staining within the kidneys may highlight glomeruli. The descending aorta (A) can also be seen in between the two kidneys.

3.2.6 The Flk1i10 enhancer is functionally conserved

The Flk1i10 enhancer is conserved throughout tetrapod evolution. Comparison of enhancer sequences to those of related species can identify conserved sequence motifs within the element and thus simplify the discovery of functional transcription factor biding sites (Nelson and Wardle 2013). However, sequence conservation of regulatory elements does not automatically imply conservation of regulatory function (Nelson and Wardle 2013). Therefore, it was investigated whether the activity pattern of the Flk1i10 enhancer was conserved in related species. For this, the orthologous chicken Flki10 enhancer was amplified from genomic DNA and used to generate transient transgenic zebrafish with the Tol2 system as described earlier. Zebrafish transgenic for the chicken Flk1i10 enhancer expressed the GFP reporter gene in the vasculature similar to the mouse Flk1i10 enhancer, indicating that Flki10 enhancer function is conserved among the two species (Fig. 3.11). This further suggests that the conserved sequence motifs within both enhancers play a key roles for their endothelial-specific activity.

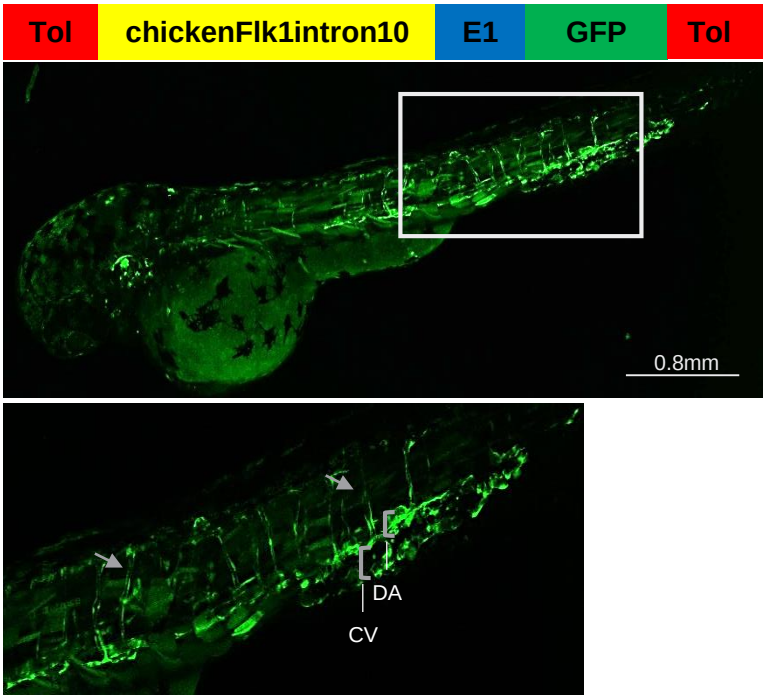


Figure 3.11: The chicken Flk1 intron 10 enhancer also directs vascular expression

Transient transgenic zebrafish carrying chicken 1011bp intron-10 enhancer in front of E1b promoter and GFP reporter. Reporter expression is detected in intersomitic vessel (arrows) and the dorsal aorta (DA). Expression in the cardinal vein (CV) is obscured by GFP-positive blood cells.

3.2.7 The Flk1i10 enhancer is bound by Rbpj, Gata, Sox, Tcf/Lef, Ets, and Fox

As Flk1i10 enhancer function was retained in chicken, it is likely that the element is also functionally conserved in more closely related species. Therefore, the sequences of human, mouse, opossum, and chicken were aligned using ClustalW2 and examined for the presence of conserved transcription factor binding motifs (Fig. 3.12) (Thompson et al 1994). This analysis revealed binding sites for the Notch downstream effector Rbpj, Gata, a Tcf/Lef, Ets, Pou, Sox, Fox transcription factors. Although, there are published consensus binding sites for all of these transcription factors, sequence information alone is not a reliable predictor of DNA binding. In fact, transcription factor binding is highly context dependent and can sometimes occur at sites that are not in perfect agreement with the consensus (Elnitski et al 2006). On the other hand, transcription factors are sometimes unable to bind apparently perfect consensus sites. Therefore, transcription factor binding to the conserved motifs was determined in a series of electrophoretic mobility shift assays (EMSAs) (Fig. 3.13 – 3.19). In this assay, a specific transcription factor is incubated with a radiolabeled DNA-oligo containing the binding motif of interest. Binding of the protein retains the labelled oligo during gel electrophoresis leading to a distinct shift of the signal on the gel.

Two highly conserved GATA motifs directly bound Gata2 (GATA-b and c) (Fig. 3.13). All three putative SOX motifs directly bound Sox7 (SOX-a, b and c) and both putative RBPJ motifs directly bound Rbpj protein, although binding at the less conserved RBPJ-b site was more robust than the RBPJ-a site (Figs. 3.14-16). A

putative Forkhead binding site (FOX-b) was directly adjacent to an ETS motif, forming a FOX:ETS motif (Fig. 3.12). However, although a composite oligo containing both FOX-a and b motifs robustly bound FoxC2, mutations to the FOX-b site did not ablate this binding, suggesting that only the distal FOX-a site is required for FoxC2 binding and that the putative FOX:ETS motif may not be functional (Fig. 3.17). Binding of Lef1 to the TCF/LEF motif was also demonstrated, while only very faint binding was observed for Brn1 to the POU domain site (Fig. 3.18).

This demonstrates that the Flk1i10 enhancer contains functional binding sites for RBPJ, GATA, SOX, TCF/LEF, and FOX transcription factors.

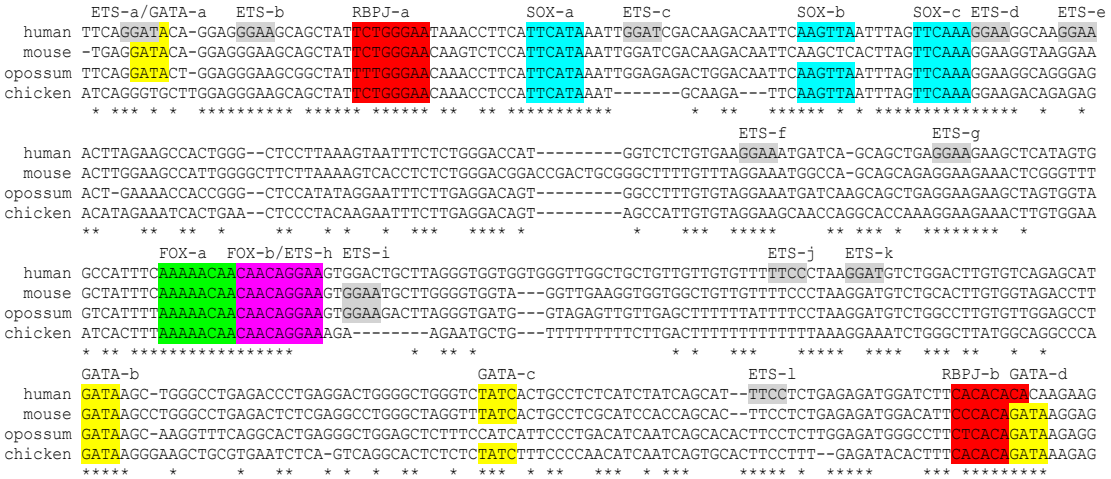


Figure 3.12: Validated binding sites in the Flk1i10 enhancer

Sequence alignment with ClustalW2 of human, mouse, opossum, and chicken sequence of the Flk1i10 enhancer. Binding sites validated by EMSA are highlighted in different colours. Multiple ETS binding sites characteristic of vascular enhances are also marked in grey.

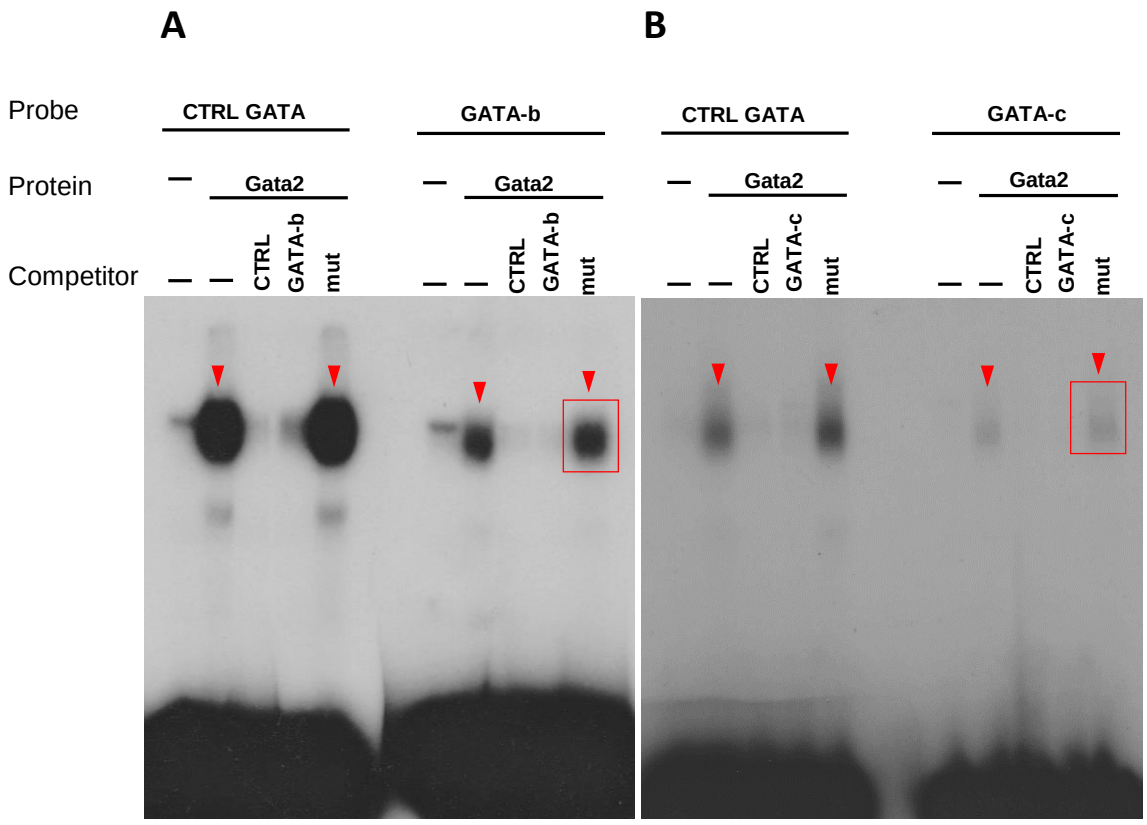


Figure 3.13: The conserved GATA sites b and c bind Gata2

Both labelled conserved GATA motifs b and c, as well as a labelled positive control motif (CRTL) bind Gata2. Binding can be outcompeted with non-labeled oligos for GATA b and c as well as CRTL. Binding is restored upon mutation (mut) of GATA motif in both GATA b and c oligos (red boxes) indicating that binding is specific to these sites.
The reduced signal observed for the GATA-c site (gel **B**) is caused by a lower amount of radiation used in this particular experiment.
Red arrowheads indicate transcription factor binding.

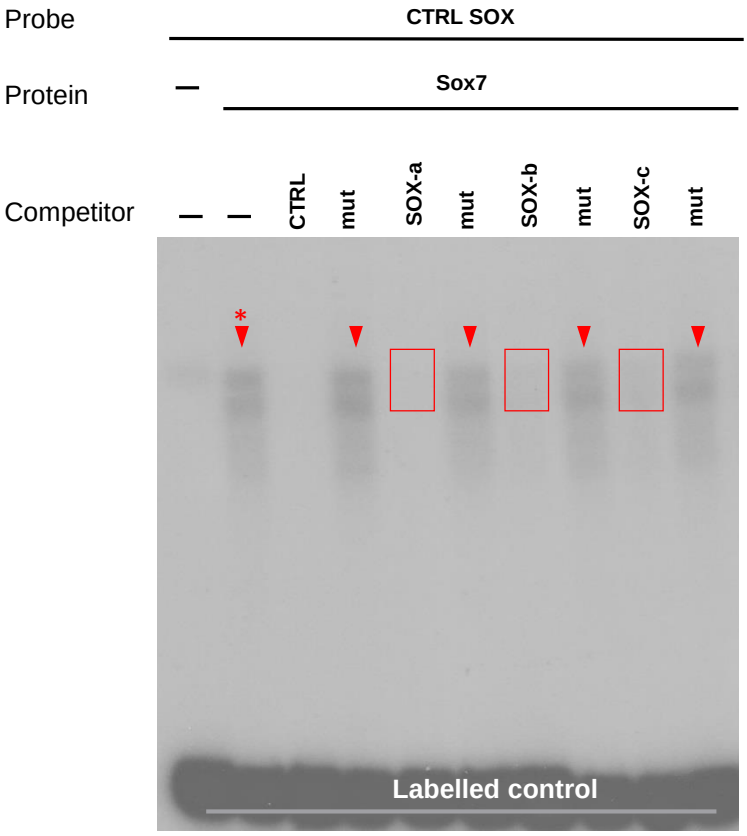


Figure 3.14: All three SOX binding sites a, b, c specifically compete with binding of SOX7 to labelled control

Sox7 binds to labelled control (arrowhead with star). All three non-labelled SOX sites a, b, and c can compete with this binding (red boxes). Disruption of the motifs re-establishes Sox7 binding implicating that this interaction is specific to the motif (remaining red arrowheads).
CTRL = positive control

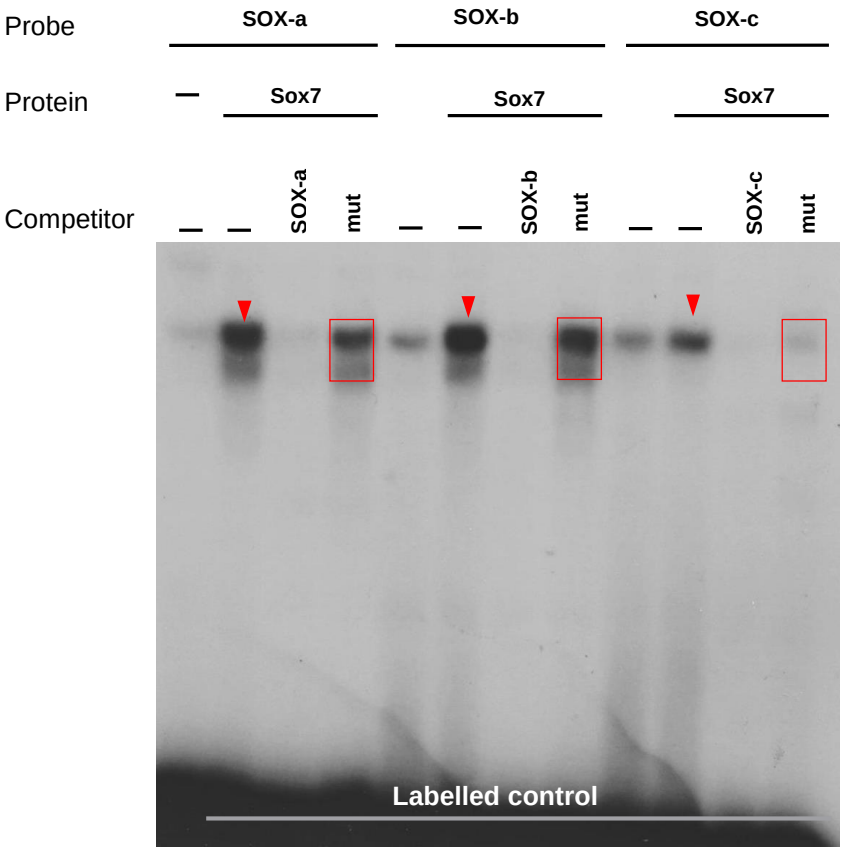


Figure 3.15: SOX sites a, b, c directly bind Sox7

Sox7 binds to SOX sites A, B, and C of the Flk1i10 enhancer (red arrows). Unlabelled oligos compete with the binding of Sox7 to the labelled oligos whereas mutated competitor oligos (mut) do not abolish Sox7 binding to the labelled oligo (red boxes).

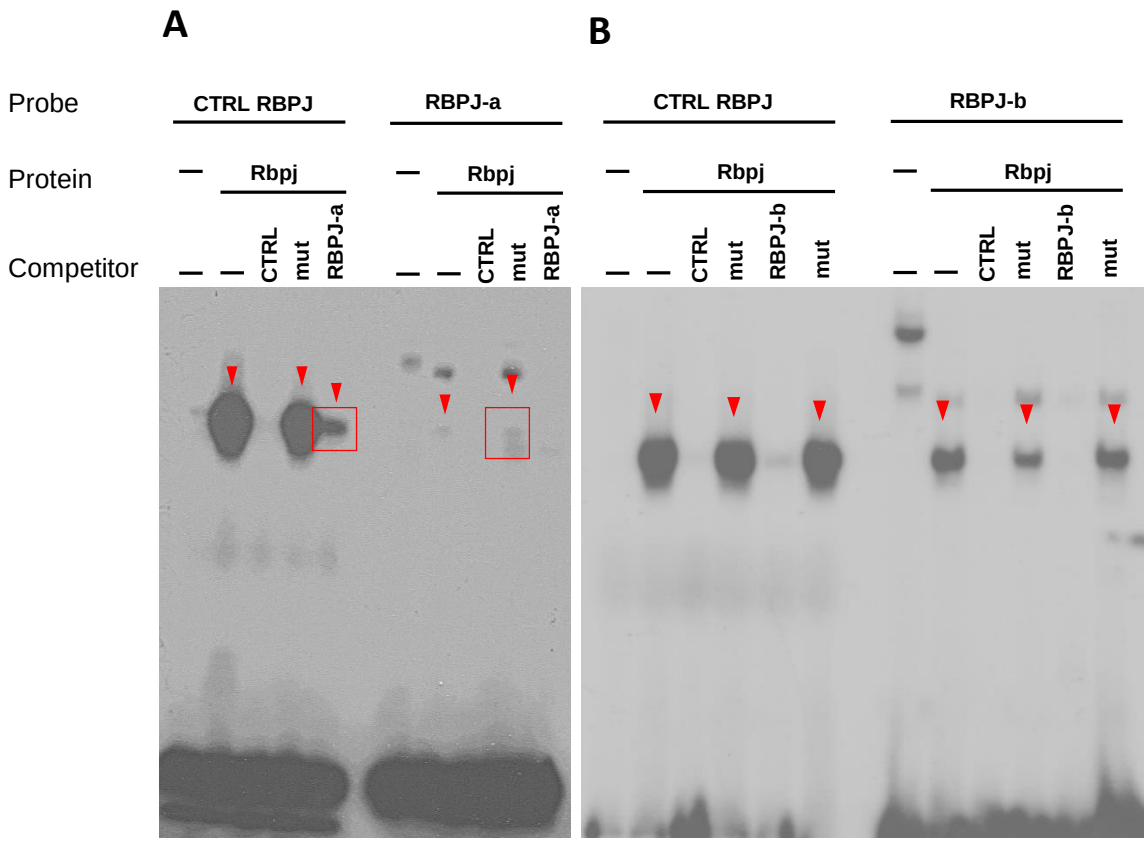


Figure 3.16: Rbpj weakly binds to binding site a and robustly to binding site b

Rbpj binds to labelled control oligos (left side of both gels). RBPJ-a site only weakly competes for binding to control (red box left side of gel **A**) and only faint binding to labelled Flk1i10 site is observed (right side of gel **A**). However, mutation of the RBPJ-a site does restore bonding (red box right side of gel **A**) indicating that binding is weak but specific. Robust, specific binding is detected for the RBPJ-b site (gel **B)** Red arrowheads indicate bound Rbpj. CTRL = positive control oligo

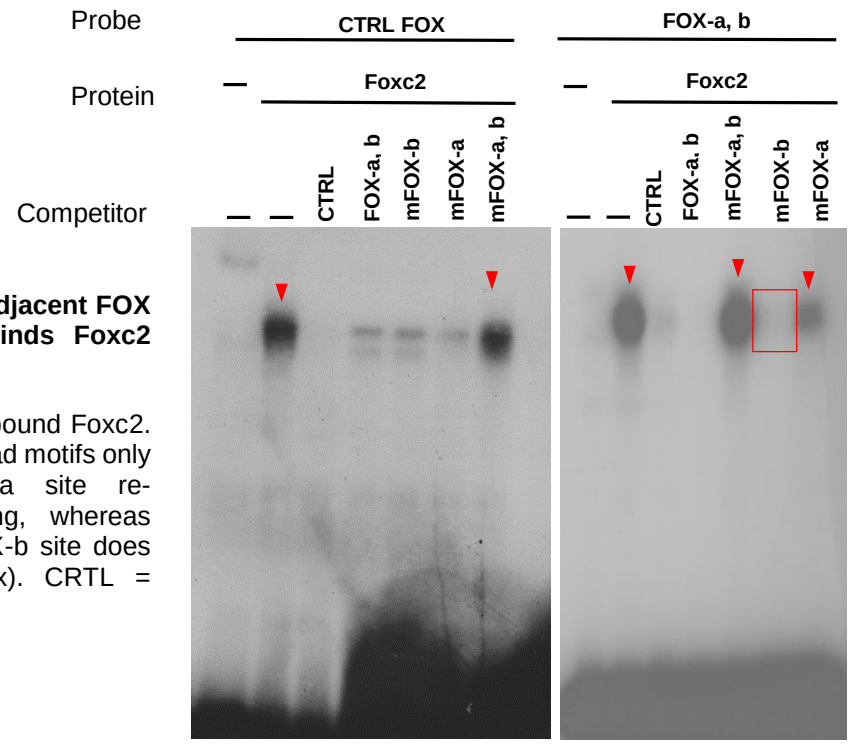


Figure 3.17: Of the two adjacent FOX sites only the Fox-a binds Foxc2 protein

Red arrowheads indicate bound Foxc2. Of the two adjacent forkhead motifs only mutation of the FOX-a site re-establishes Foxc2 binding, whereas mutation (mut) of the FOX-b site does not (marked by red box). CRTL = positive control oligo

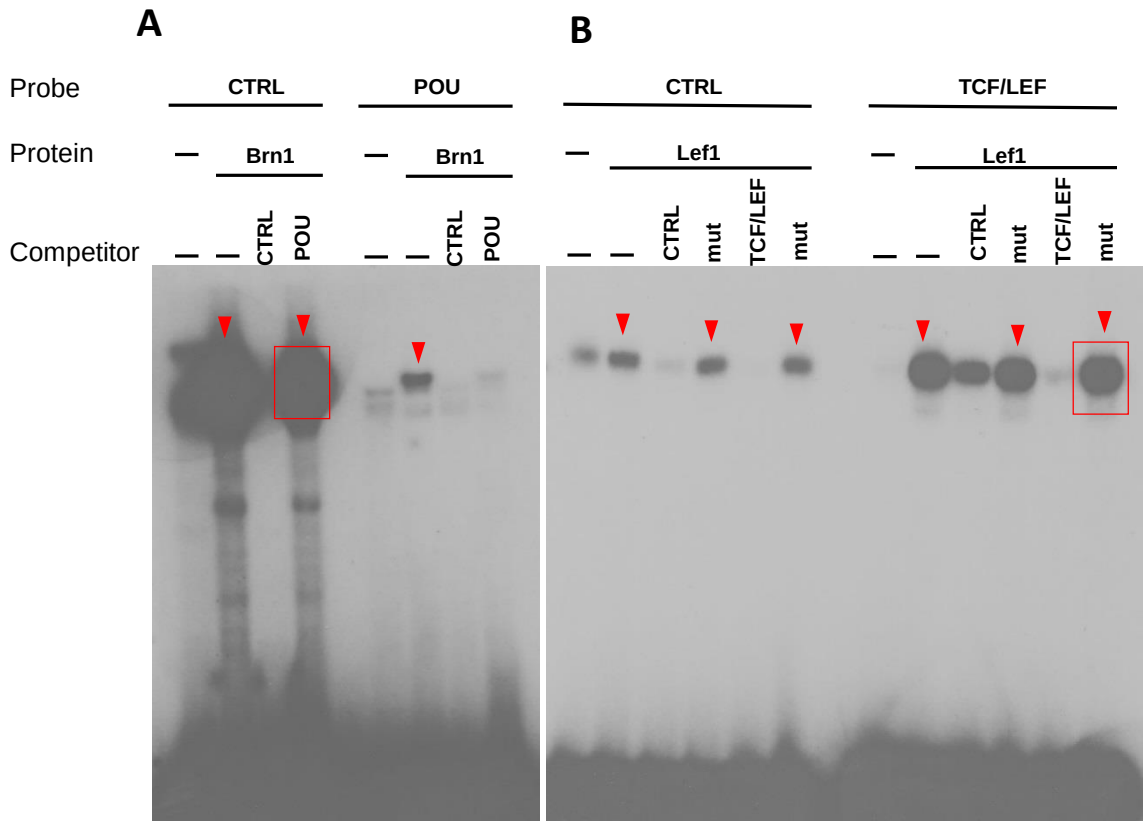


Figure 3.18: The POU domain binding site weakly binds Brn1 while the TCF/LEF motif robustly binds Lef1

A: Brn1 strongly binds the labelled control oligo (CRTL) (left side of gel **A**) but only weakly binds the labelled POU (right side of gel **A**, red arrowhead). Moreover, the unlabelled POU oligo is not able to compete with Brn1 binding to the control oligo (red box) indicating that it is not a good binding site.
B: Lef1 binds to the labelled TCF/LEF site (right side of gel **B**) more strongly than to the labelled control (left side of gel **B**). Mutation of the TCF/LEF binding site restores binding of Lef1 (red box) indicating that the binding is specific for this motif. Red arrowheads indicate Lef1 binding. CTRL = positive control oligo

3.2.8 Targeted mutagenesis in transgenic zebrafish suggests roles for GATA and RBPJ binding motifs in Flk1i10 enhancer function

While EMSA experiments confirmed that Rbpj, Gata, Sox, Tcf/Lef, Ets, and Fox factors interact with functional binding sites within the Flk1i10 enhancer, they do not suffice to establish whether binding events as such are critical for enhancer function (Farnham 2009). To assess the importance of the validated binding sites for enhancer activity *in vivo*, targeted mutations were introduced to individual or combinations of binding sites to preclude transcription factor binding. Mutated Flk1i10 enhancer sequences were then used to generate transient transgenic zebrafish, which were analysed for vascular reporter GFP expression at 48 – 52hpf (Table 3.1).

Comprehensive obliteration of all validated binding sites (mutAll) resulted in the complete loss of enhancer activity in transgenic zebrafish, indicating that the conserved binding motifs are indeed critical determinants of enhancer function. Based on this, a more thorough investigation of the contributions of individual sites or TFs was carried out.

Disruption of both conserved GATA motifs (mutGATA-b, c) reduced GFP expression by more than half, implicating that Gata provides an important input for enhancer activity. As the Flk1i10 enhancer contains two additional, weakly conserved GATA binding sites (a and d Fig. 3.12), it is possible that redundancy among these sites may lead to an underestimation of Gata factors for enhancer activity. Therefore a disruption of all four GATA binding sites (mutGATA-All) was tested next. Loss of all Gata binding resulted in almost complete loss of reporter gene expression in transgenic zebrafish identifying Gata binding as key for Flk1i10 enhancer function and suggesting that the non-conserved GATA sites are also functional. However, the

combined mutation of all conserved binding motifs also lead to a complete loss of enhancer activity, while leaving the non-conserved GATA sites intact. It appears, therefore, that, while functional GATA sites are absolutely necessary for Flk1i10 enhancer activity, other binding sites besides GATA motifs are also required for correct Flk1i10 enhancer function.

Mutation of the TCF/LEF site (mutTCF/LEF) had no effect on vascular GFP expression in transgenic zebrafish at 48-52hpf indicating that transcription factor binding to this site is dispensable for Flk1i10 enhancer function. Similarly, mutation of only the conserved RBPJ-a site (mutRBPJ-a) did not reduce reporter gene expression. However, combined mutation of both conserved RBPJ sites (mutRBPJ-a, b) reduced endothelial expression roughly by half. When additional mutations were introduced for the SOX sites (mutRBPJ-a, b mutSOX-a, b, c) or for the SOX sites and the FOX site (mutRBPJ-a, b mutSOX-a, b, c mutFOX), along with mutations of the conserved RBPJ sites, a further reduction of reporter gene expression was not apparent. This suggests that binding of Rbpj has an important role for enhancer function while a significant contribution of Sox, or Fox binding to enhancer activity could not be demonstrated.

Combined mutation of the conserved GATA and RBPJ sites (mutGATA-b, c mutRBPJ-a, b) lead to a much more drastic reduction in endothelial GFP expression than the mutation of conserved binding sites for either factor alone. This suggests that Gata and Rbpj are involved in a synergistic, combinatorial regulation of Flk1i10 enhancer activity. However, deletion of all validated binding sites did have a slightly stronger effect on GFP expression than combined loss of conserved GATA/RBPJ sites alone. Therefore, while Gata and Rbpj factors appear to provide the main inputs for regulatory activity, other conserved binding sites, such as SOX may contribute smaller effects that were not detectable with mutational analysis.

A

Transient transgenic zebrafish	
Construct	% of embryos with vascular expression at 48hpf (total analysed)
WT	64 (121)
mutTCF/LEF	59 (81)
mutRBPJ-a	67 (66)
mutRBPJ-a, b	33 (45)
mutRBPJ-a, b mutSOX-a, b, c	35 (99)
mutRBPJ-a, b mutSOX-a, b, c mutFOX	44 (87)
mutGATA-b, c	28 (72)
mutGATA-All	9 (106)
mutGATA-b, c mutRBPJ-a, b	10 (50)
mutAll	1 (69)

B

```
          ETS-a/GATA-a    ETS-b       RBPJ-a            SOX-a     ETS-c             SOX-b       SOX-c ETS-d    ETS-e
  human TTCAGGATACA-GGAGGGAAGCAGCTATTCTGGGAATAAACCTTCATTCATAAATTGGATCGACAAGACAATTCAAGTTAATTTAGTTCAAAGGAAGGCAAGGAA
  mouse -TGAGGATACA-GGAGGGAAGCAGCTATTCTGGGAACAAGTCTCCATTCATAAATTGGATCGACAAGACAATTCAAGCTCACTTAGTTCAAAGGAAGGTAAGGAA
opossum TTCAGGATACT-GGAGGGAAGCGGCTATTTGGGAACAAACCTTCATTCATAAATTGGAGAGACTGGACAATTCAAGTTAATTTAGTTCAAAGGAAGGCAGGGAG
chicken ATCAGGGTGCTTGGAGGGAAGCAGCTATTCTGGGAACAAACCTCCATTCATAAAT-------GCAAGA---TTCAAGTTAATTTAGTTCAAAGGAAGACAGAGAG
          * *** * *  ********** ****** ****** **  ** **********       *  **   ****** * * ***************  *   *
                                                                                    ETS-f              ETS-g
  human ACTTAGAAGCCACTGGG--CTCCTTAAAGTAATTTCTCTGGGACCAT---------GGTCTCTGTGAAGGAAATGATCA-GCAGCTGAGGAAGAAGCTCATAGTG
  mouse ACTTGGAAGCCATTGGGGCTTCTTAAAAGTCACCTCTCTGGGACGGACCGACTGCGGGCTTTTGTTTAGGAAATGGCCA-GCAGCAGAGGAAGAAACTCGGGTTT
opossum ACT-GAAAACCACCGGG--CTCCATATAGGAATTTCTTGAGGACAGT---------GGCCTTTGTGTAGGAAATGATCAAGCAGCTGAGGAAGAAGCTAGTGGTA
chicken ACATAGAAATCACTGAA--CTCCCTACAAGAATTTCTTGAGGACAGT---------AGCCATTGTGTAGGAAGCAACCAGGCACCAAAGGAAGAAACTTGTGGAA
          **    **  **  *     **   * *   *  ***   **** *          *    ***  *****     ** *** *  ********  *
                FOX-a  FOX-b/ETS-h ETS-i                                          ETS-j   ETS-k
  human GCCATTTCAAAAACAACAACAGGAAGTGGACTGCTTAGGGTGGTGGTGGGTTGGCTGCTGTTGTTGTGTTTTTCCCTAAGGATGTCTGGACTTGTGTCAGAGCAT
  mouse GCTATTTCAAAAACAACAACAGGAAGTGGAATGCTTGGGGTGGTA---GGTTGAAGGTGGTGGCTGTTGTTTTCCCTAAGGATGTCTGCACTTGTGGTAGACCTT
opossum GTCATTTTAAAAACAACAACAGGAAGTGGAAGACTTAGGGTGATG---GTAGAGTTGTTGAGCTTTTTTATTTTCCTAAGGATGTCTGGCCTTGTGTTGGAGCCT
chicken ATCACTTTAAAAACAACAACAGGAAAGA--------AGAATGCTG---TTTTTTTTTCTTGACTTTTTTTTTTTTTAAAGGAAATCTGGGCTTATGGCAGGCCCA
          * ** *****************          *  ** *                  * *   ***    *****  ****  *** **   *  *
          GATA-b                              GATA-c                    ETS-l              RBPJ-b GATA-d
  human GATAAGC-TGGGCCTGAGACCCTGAGGACTGGGGCTGGGTCTATCACTGCCTCTCATCTATCAGCAT--TTCCTCTGAGAGATGGATCTTCACACACACACAAGAAG
  mouse GATAAGCCTGGGCCTGAGACTCTCGAGGCCTGGGCTAGGTTTATCACTGCCTCGCATCCACCAGCAC--TTCCTCTGAGAGATGGACATTCCCACAGATAAGGAG
opossum GATAAGC-AAGGTTTCAGGCACTGAGGGCTGGAGCTCTTTCCATCATTCCCTGACATCAATCAGCACACTTCCTCTTGGAGATGGGCCTTCTCACAGATAAGAGG
chicken GATAAGGGAAGCTGCGTGAATCTCA-GTCAGGCACTCTCTCTATCTTTCCCCAACATCAATCAGTGCACTTCCTTT--GAGATACACTTTCACACAGATAAAGAG
          *****    *      *   **   * *  *  **   *  ***  * **   ***  * ***     ***** *  *****     *** *********
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C

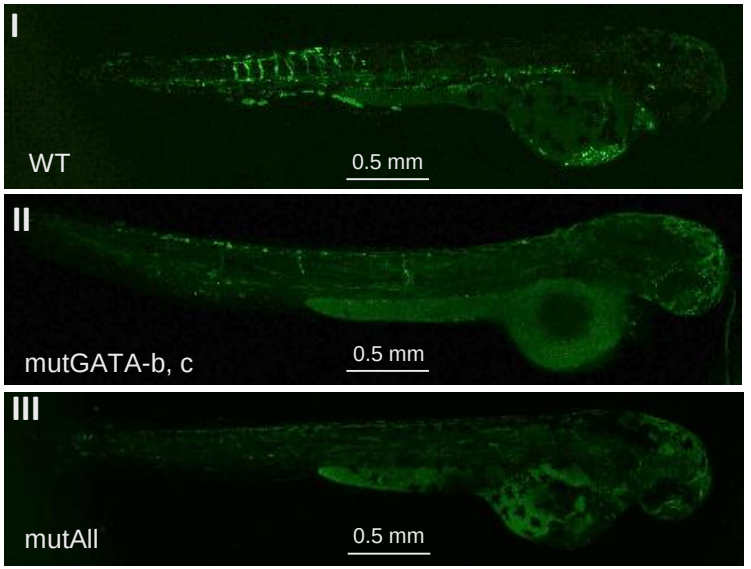


Table 3.19: Mutational analysis of Flk1i 10 enhancer binding motifs in transient transgenic zebrafish

A: Summary of reporter gene expression detected at 48hpf in Tol2-mediated mosaic transient transgenic zebrafish embryos.
B: Flk1i10 enhancer sequence with binding motifs highlighted in colour.
C: GFP expression was scored as positive in the vasculature when at least one intersegmental vessel, or equivalent expression in axial or head vessels, was observed. Representative examples are shown for high vascular expression in wild-type Flk1i10 transgenic fish(I), low vascular expression in mutGATA-b, c transgenics (II), and no vascular expression in mutAll transgenics (III).

3.2.9 Mutagenesis in transgenic mice indicates RBPJ binding motifs are required for arterial specificity of the Flk1i10 enhancer

Mutational analysis in zebrafish strongly suggests that both GATA and RBPJ binding motifs are critical for Flk1i10 enhancer function. However, there was also some evidence suggesting that the enhancer receives additional inputs from other binding sites. Additionally, while analysis of enhancers in zebrafish enabled the quick analysis of multiple mutant constructs, there were a number of draw-backs to this approach. The Flk1i10 enhancer is mammalian, and it is possible that the regulatory pathways controlling the enhancer are not conserved to fish. Additionally, the mosaic nature of the zebrafish transgenics made precise analysis of expression patterns within the vasculature challenging, which was further compounded by reporter gene expression in blood cells. Therefore, in order to validate and further examine the role of different binding motifs in the regulation of the Flk1i10 enhancer, mutational analysis of the Flk1i10 enhancer was also performed in a transient transgenic mouse model.

Mutated versions of the mouse Flk1i10 enhancer were generated either by site-directed mutagenesis or by commercial DNA synthesis, and were cloned upstream of the silent hsp68 minimal promoter to drive expression of the *LacZ* reporter gene. As before, transgene expression was visualized by X-Gal staining at E12.5. As in the zebrafish analysis, first all conserved, validated binding sites were mutated in combination (mutAll) to ensure that they are indeed critical for enhancer function. As observed in zebrafish, comprehensive disruption of all conserved motifs resulted in a complete absence of endothelial staining in all embryos analysed (Fig. 3.19, A). In one case, non-specific staining was detected throughout the entire embryo. This is a known phenomenon which results when the integration event occurs in a genetic locus

generally permissive of transcriptional activity and is also often observed as staining of the neural tube. A second construct, containing different, less aggressive mutations of the binding sites (mutsmallAll), was also used to generate transgenic mice to exclude the possibility that the initial sequent mutations had created novel, repressive binding motifs. Satisfyingly, no endothelial-specific reporter gene expression was observed in any transgenic embryos, (Fig. 3.19, B) confirming that transcription factor binding at the conserved binding motifs sites was required for enhancer activity.

Next, individual or combinations of binding sites were mutated to address their specific role in enhancer activity. Experiments in zebrafish did not indicate a critical role for a conserved TCF/Lef binding motif. However, as canonical Wnt signalling has been implicated in arterial differentiation, disruption of this binding motif was re-assessed in transgenic mice (mutTCF/LEF) (Morini and Dejana 2014). Similar to the previous observation in fish, mutation of the TCF/LEF motif had no discernible effect on vascular transgene expression or arterial expression pattern, suggesting that this site is not critical for Flk1i10 enhancer function (Fig. 3.20).

Zebrafish analysis indicated a role for the two conserved RBPJ motifs in Flk1i10 enhancer function, with disruption of these sites resulting in reduced reporter gene expression. Unlike in fish, however, disruption of RBPJ binding sites (mutRBPJ-a, b) did not appear to reduce vascular enhancer activity in E12.5 transient transgenic mouse embryos (Fig. 3.21, A). However, histological sectioning of the embryos revealed that endothelial transgene expression had expanded into the venous circulation (Fig. 3.22). This surprising results indicates that the RBPJ binding motifs in Flk1i10 are involved in the repression of enhancer activity in the venous endothelium. Since another arterial specific *cis*-regulatory element has previously been demonstrated to be regulated by a combination of Notch signalling and SOXF binding

(Sacilotto et al 2013), we tested whether SOX sites also played a role in Fl1i0 activity by introducing additional mutations to all three SOX sites in combination with mutation of the RBPJ sites (mutRBPJ-a, b mutSOX-a, b, c). However, reporter gene expression showed a similar pattern as observed after disruption of the RBPJ sites alone (Fig. 3.21, B and Fig. 3.22). Consequently, these results suggest a role for RBPJ in repression of Flk1i10 in venous endothelial cells independent of Sox binding.

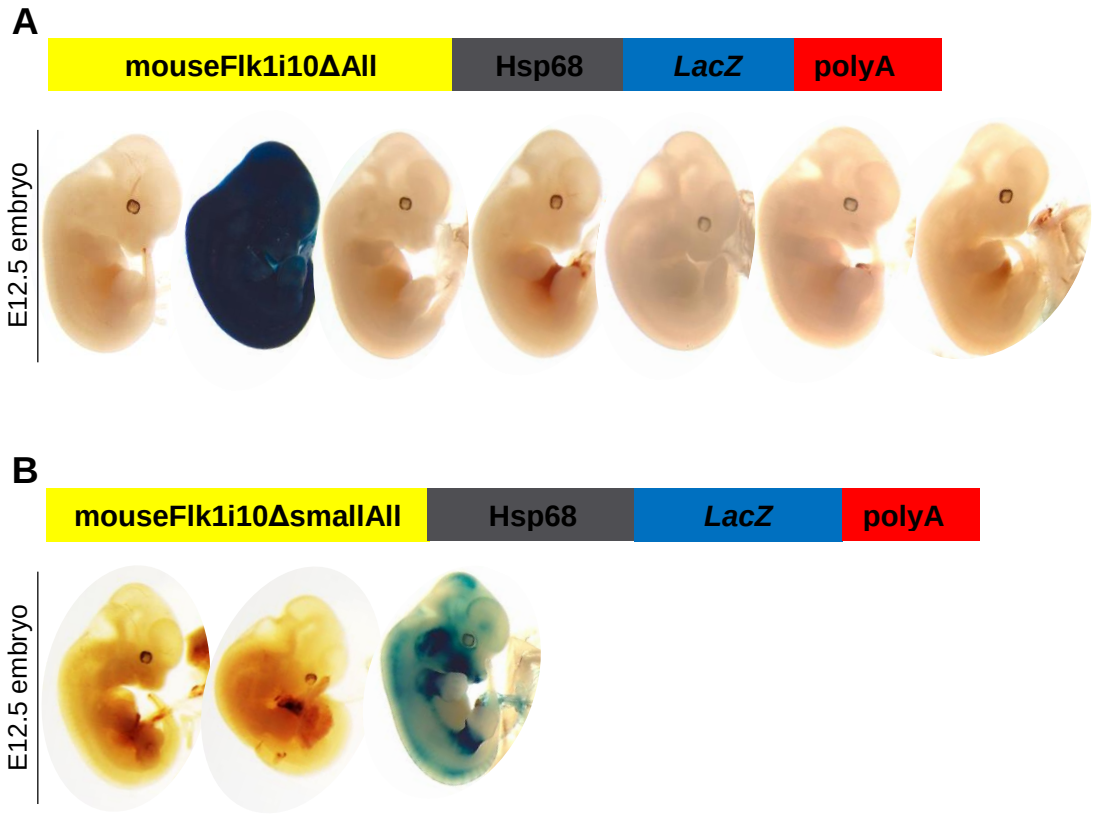


Figure 3.20: Mutation of all binding motifs abolishes vascular expression of Flk1i10 enhancer

Transient transgenic E12.5 mouse embryos carrying a mutated version of the 826bp Flk1i10 enhancer in front of Hsp68 promoter and *LacZ* reporter. Reporter expression is visualized by blue X-Gal staining.
A: All tested binding sites were deleted. Second embryo from the left shows typical “blueberry” staining resulting from random integration of the *LacZ* construct into a site with constitutive transcriptional activity. No vascular staining can be detected in the other embryos.
B: More precise mutation of the binding sites were performed. First embryo from the right shows only non-vascular staining. No reporter activity is detected in the remaining specimen.

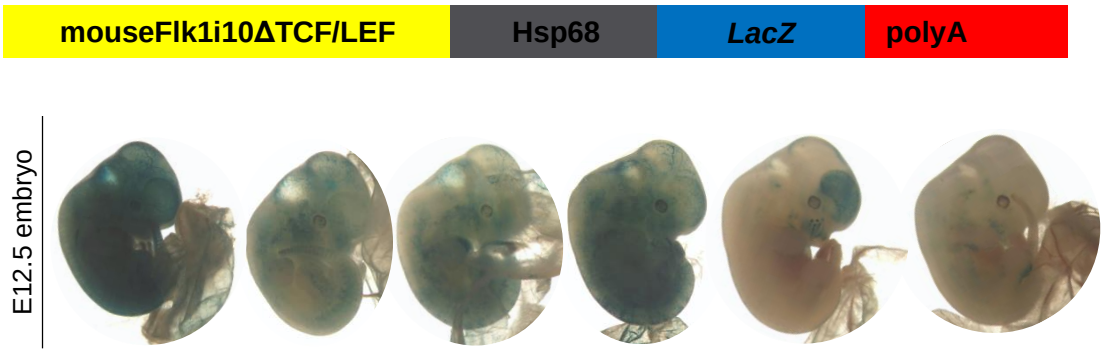


Figure 3.21: Mutation of TCF/LEF binding motif does not alter vascular expression pattern of Flk1i10 enhancer

Transient transgenic E12.5 mouse embryos carrying a version of the 826bp Flk1i10 enhancer in which the TCF/LEF motif was mutated to prevent binding. Reporter expression was visualized by blue X-Gal staining.

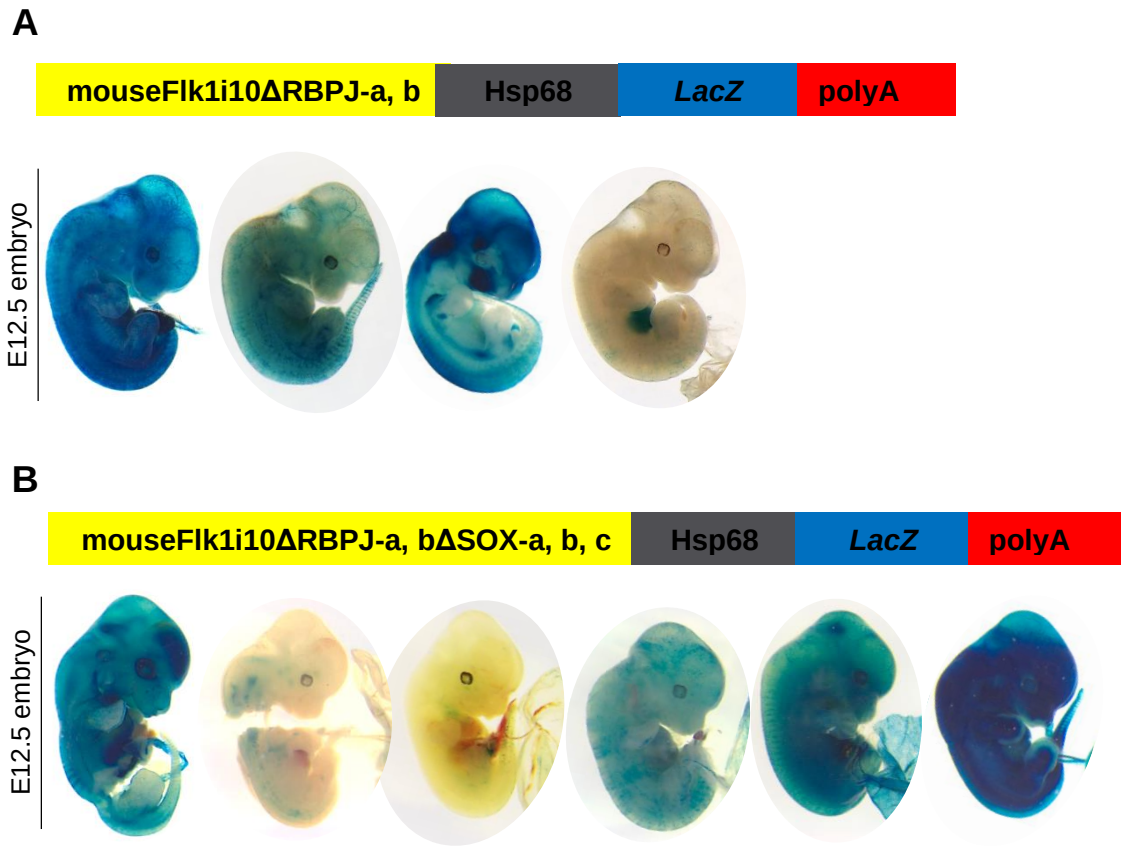


Figure 3.22: Mutation of the RBPJ binding sites either alone or in combination with mutation of the SOX motifs leads to expansion of enhancer activity into the venous endothelium

Transient transgenic E12.5 mouse embryos carrying a mutated version of the 826bp Flk1i10 enhancer in front of Hsp68 promoter and *LacZ* reporter. Reporter expression is visualized by blue X-Gal staining.
A: Combined mutations of both conserved RBPJ binding sites. Robust vascular expression is observed in two embryos on the left, whereas the third embryo shows ectopic expression of *LacZ*. The fourth embryo also shows weak but obvious vascular expression.
B: Combined mutations for conserved RBPJ and SOX binding sites. Vascular expression is seen at varying intensities in all embryos.

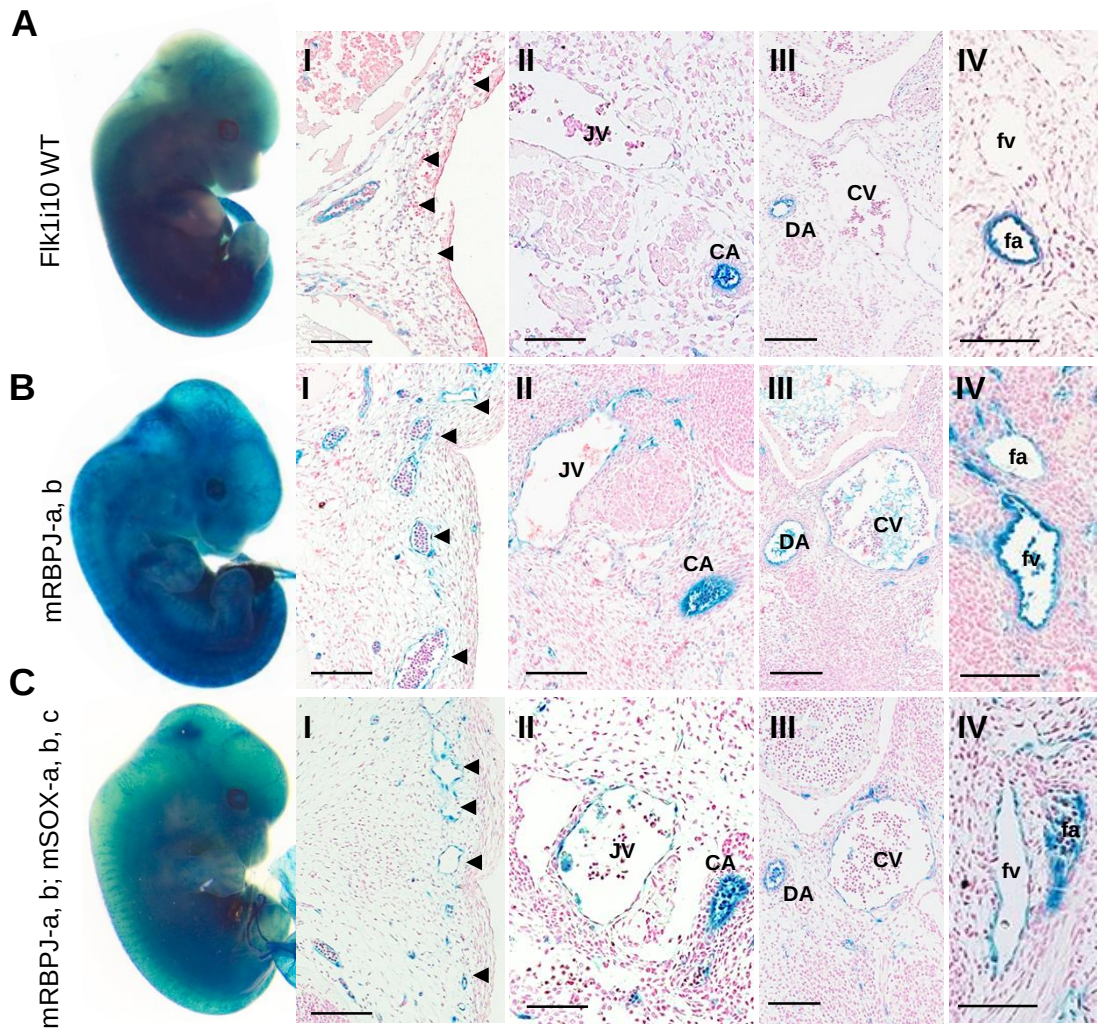


Figure 3.23: A loss of Rbpj binding causes expansion of Flk1i10 enhancer activity into the venous circulation

Transient transgenic E12.5 mouse embryos carrying differernt versions of the Flk1i10 enhancer in front of Hsp68 promoter and *LacZ* reporter. Reporter expression is visualized by blue X-Gal staining.
A: WT Flk1i10 enhancer, wholemount and sections (I-IV). No staining is observed in the peripheral veins of the head (arrowheads in I). Reporter gene expression can be seen in the carotid artery (CA,II), the dorsa aorta (DA, III), and the femoral artery (fa, IV) but not in the jugular veins (JV, II), the cardinal vein (CV, III), or the femoral vein (fv, IV).
B+C: In mutRBPJ-b, c (B) and mutRBPJ-b, c; mutSOX-a, b, c (C) versions of the Flk1i10 enhancer, XGal staining is observed not only in arteries but also in veins of the head (I), neck (II), torso (III) and femur (IV).
Scalbar = 40µm

3.2.10 Mutagenesis in transgenic mice indicates Flk1i10 enhancer activity requires Gata factors

Since mutation of the conserved GATA sites markedly reduced vascular enhancer activity in zebrafish, the importance of Gata binding for Flk1i10 enhancer function was also assessed in mice. In E12.5 transgenic mouse embryos, the disruption of the two conserved GATA binding motifs (mutGATA-b, c) almost completely abrogated vascular reporter gene expression (Fig. 3.23). While some X-Gal staining was seen in individual vessels of the head in some transgenic embryos, no staining was noticeable in the major trunk vessels. Interestingly, noticeable ectopic expression was observed in some of the transgenics. This suggests that, similar to the observation in zebrafish, Gata binding is crucial for Flk1i10 enhancer activity in the vasculature. As the combined mutation of both conserved GATA and RBPJ motifs almost completely abrogated Flk1i10 enhancer activity in transient transgenic zebrafish, a combined mutation of these binding sites was also tested in transgenic mice (mutGATA-b, c; mutRBPJ-a, b). However, unlike in the fish model, additional mutation of the two RBPJ sites did not lead to an obvious further reduction of vascular reporter gene expression compared to disruption of the two GATA sites alone (Fig. 3.24). As disruption of the GATA motifs alone had a more dramatic effect on Flk1i10 enhancer expression in mice, an additional effect of a loss of RBPJ binding motifs may not have been as striking. On the other hand, the high number of transgenics and the simple vascular structure of the fish present an advantage in the detection of smaller differences in vascular expression which can be challenging to discover in the mouse model.

Overall, the results indicate that Gata binding is critical for Flk1i10 enhancer activity and that this activity is modulated and restricted by Notch signalling. No direct effects could be demonstrated for further inputs, such as SoxF and Tcf/Lef binding, although the fact that some residual vascular expression was present in both mutGATA-b, c and mutGATA-b, c; mutRBPJ-a, b mutants may suggest a role for these factors, as combined disruption of all binding motifs results in a total loss of enhancer activity.

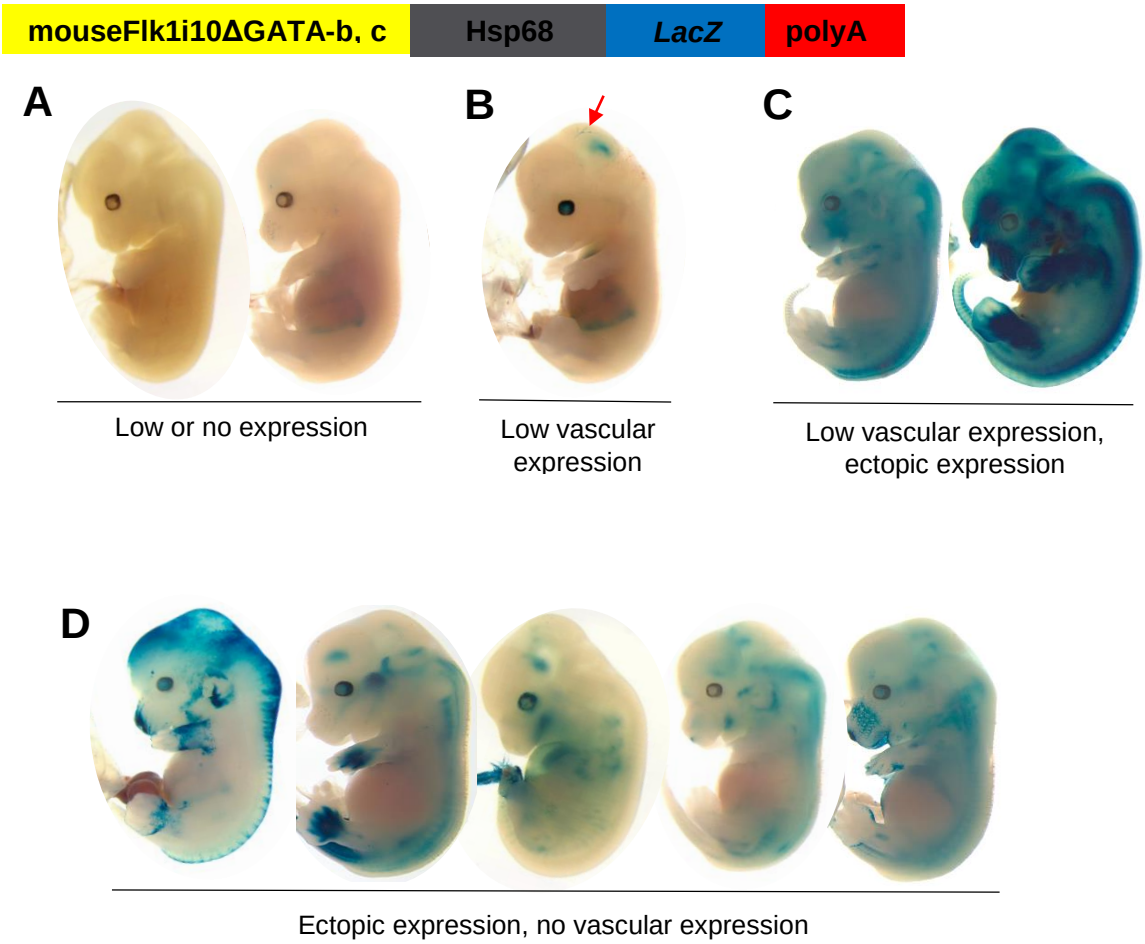


Figure 3.24: Mutation of the conserved GATA binding sites drastically reduces endothelial activity of Flk1i10 enhancer

Transient transgenic E12.5 mouse embryos carrying a mutated version of the 826bp Flk1i10 enhancer in front of Hsp68 promoter and *LacZ* reporter. Reporter expression is visualized by blue XGal staining. After mutation of both conserved GATA sites vascular reporter gene expression is greatly diminished.
A: XGal staining is absent in 2 out of 10 transgenic embryos.
B+C: Some vascular expression of the reporter gene is seen in the head vasculature (red arrow) of 3 out of 10 embryos. In 2 cases, vascular expression is accompanied by ectopic expression of *LacZ* in non-vascular tissues (C).
D: 5 out of 10 embryos show ectopic enhancer activity but no vascular expression of *LacZ.*

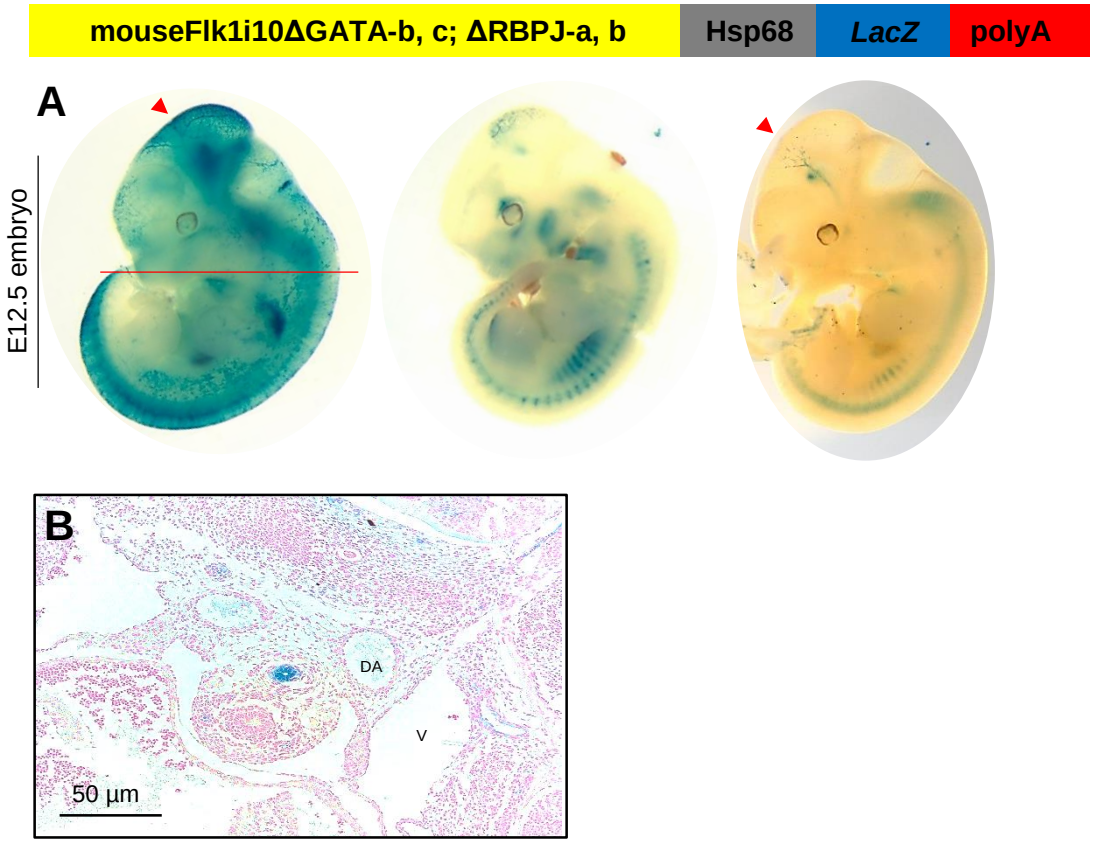


Figure 3.25: Combined mutation of conserved GATA and RBPJ motifs reduces endothelial Flk1i10 enhancer activity similarly to mutation of both conserved GATA sites alone

A: Transient transgenic E12.5 mouse embryos carrying a mutated version (ΔconGATA; ΔRBPJ) of the 826bp Flk1i10 enhancer in front of Hsp68 promoter and *LacZ* reporter. Reporter expression is visualized by blue X-Gal staining. After mutation of both conserved GATA sites and both RBPJ sites vascular reporter gene expression is diminished similarly as after mutation of conserved GATA sites alone. Again, some residual expression can be detected in vessels of the head (red arrowhead).
B: Histological sections (indicated by red line in A) show absence of X-Gal staining in both arteries and veins.

3.2.11 Inhibition of Vegfr2 signalling blocks Flk1i10 enhancer activity in *tg(Flk1i10:GFP)* zebrafish

The expression pattern of the Flk1i10 enhancer in stable transgenic *tg(Flk1i10:GFP)* zebrafish indicates that it drives expression in arterial endothelial cells that are proliferating in response to Vegf-signalling. Interestingly, it has been previously suggested that Flk1 expression is itself regulated by Vegf signalling (Chatterjee et al 2013, Chen et al 2014). Therefore, *tg(Flk1i10:GFP)* zebrafish were treated at 12hpf with the tyrosine kinase inhibitor Semaxanib (SU5416), which selectively blocks Vegfr2 signalling, to investigate the effects of a loss of Vegf signalling on Flk1i10 enhancer expression (Sakamoto 2001). As inhibition of Vegf-signalling interferes with arterial specification during vasculogenesis, proper segregation of the main axial vessels does not occur in the presence of SU5416. Furthermore, Vegf-dependent angiogenesis is entirely abrogated and angiogenic sprouting and formation of intersegmental vessels cannot be observed. At 24 and 36hpf endothelial reporter gene expression was not detected in the single axial vessel in *tg(Flk1i10:GFP)* embryos exposed to SU5416 (Fig 3.25). This indicates that enhancer activation may be dependent on Vegfr2 signalling, and suggests the presence of an auto-regulatory loop through which Vegfr2 reinforces its own expression.

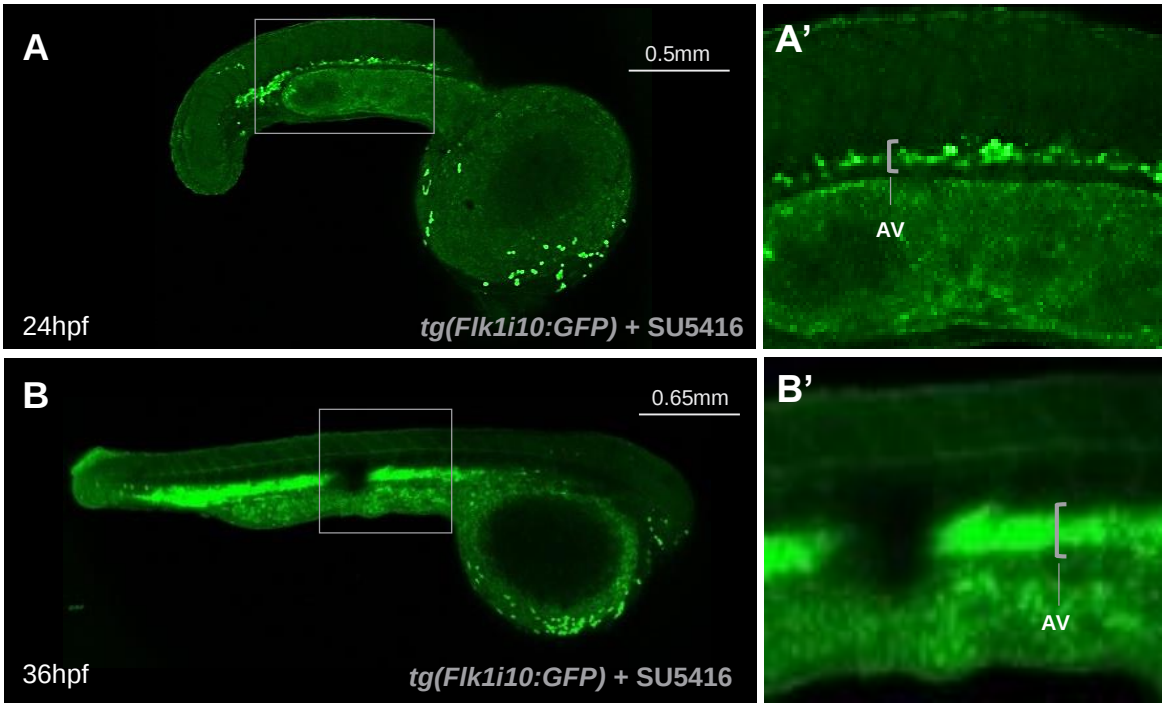


Figure 3.26: Chemical inhibition of Vegf signalling leads to a loss of enhancer activity in endothelial cells

Chemical inhibition of Vegf signalling in *tg(Flk1i10:GFP)* zebrafish embryos with 1µM SU5416 (added at 12hpf). Defective vasculogenesis results in the formation of only a single axial vessel (AV).
A: At 24hpf GFP expression is visible in blood cells which are pooled in the single axial vessel (AV), but no staining can be detected in endothelial cells.
B: Similarly, at 36hpf, endothelial GFP expression is not observed in the single axial vessel.

3.2.12 Inhibition of Notch signalling does not affect Flk1i10 enhancer activity in *tg(Flk1i10:GFP)* zebrafish

Mutation of RBPJ binding sites within the Flk1i10 enhancer leads to a reduction of reporter gene expression in transient transgenic zebrafish and to expansion of enhancer activity into the venous circulation in transgenic mouse embryos. As Rbpj has a well-defined role as a downstream effector of Notch signalling, this may suggest a role for Notch signalling in the regulation of Flk1i10 enhancer activity (Kopan and Ilagan 2009). To further examine the role of Notch signalling for Flk1i10 enhancer function, *tg(Flk1i10:GFP)* zebrafish were treated at 12hpf with DAPM, an inhibitor of NICD which blocks canonical Notch signalling (Walsh et al 2002). Since Notch signalling plays a role in vasculogenesis, segregation of the axial vessels is impaired following DAPM administration making it challenging to assess expansion of GFP expression into the vein. Notch inhibition further results in increased angiogenic sprouting and excessive branching of intersegmental vessels (Gridley 2010). Interestingly, analysis of GFP expression in DAPM treated *tg(Flk1i10:GFP)* zebrafish embryos at 24, 36, 48, and 72hpf shows that loss of Notch signalling does not lead to a reduction of enhancer activity in the endothelium, even though mutation of the RBPJ binding sites in transient transgenic zebrafish caused a moderate reduction in the GFP signal (Fig. 3.26, A). Robust expression of the reporter can be observed in the single axial vessel, the intersegmental vessel, and the DLAV. Moreover, a reduction in enhancer activity in the axial and intersegmental vessels at 72hpf is not observed to the same extend as in wild-type animals (Fig. 3.26, B). As Notch signalling has been reported to inhibit endothelial cell proliferation by repressing Vegfr2 in mature vessels, prolonged Flk1i10 enhancer activation may be the result of unchecked Vegf-induced

endothelial cell proliferation (Leslie et al 2007). Therefore, the enhancer activity observed in mutational analysis and chemical inhibition are difficult to compare

Overall, the results do not support a negative effect of Notch signalling blockade on Flk1i10 enhancer activity as suggested by mutational analysis in transient transgenic zebrafish. However, the results of the two experiments are not readily comparable. Chemical inhibition prevents binding of NICD to the downstream effector RBPJ, which can be bound to target sites even in the absence of Notch signalling. Binding motif mutation, on the other hand, prevents binding of RBPJ regardless of Notch signalling status. Furthermore, chemical inhibition has a global effect on embryonic development whereas mutational analysis only affects activity of the enhancer-reporter construct.

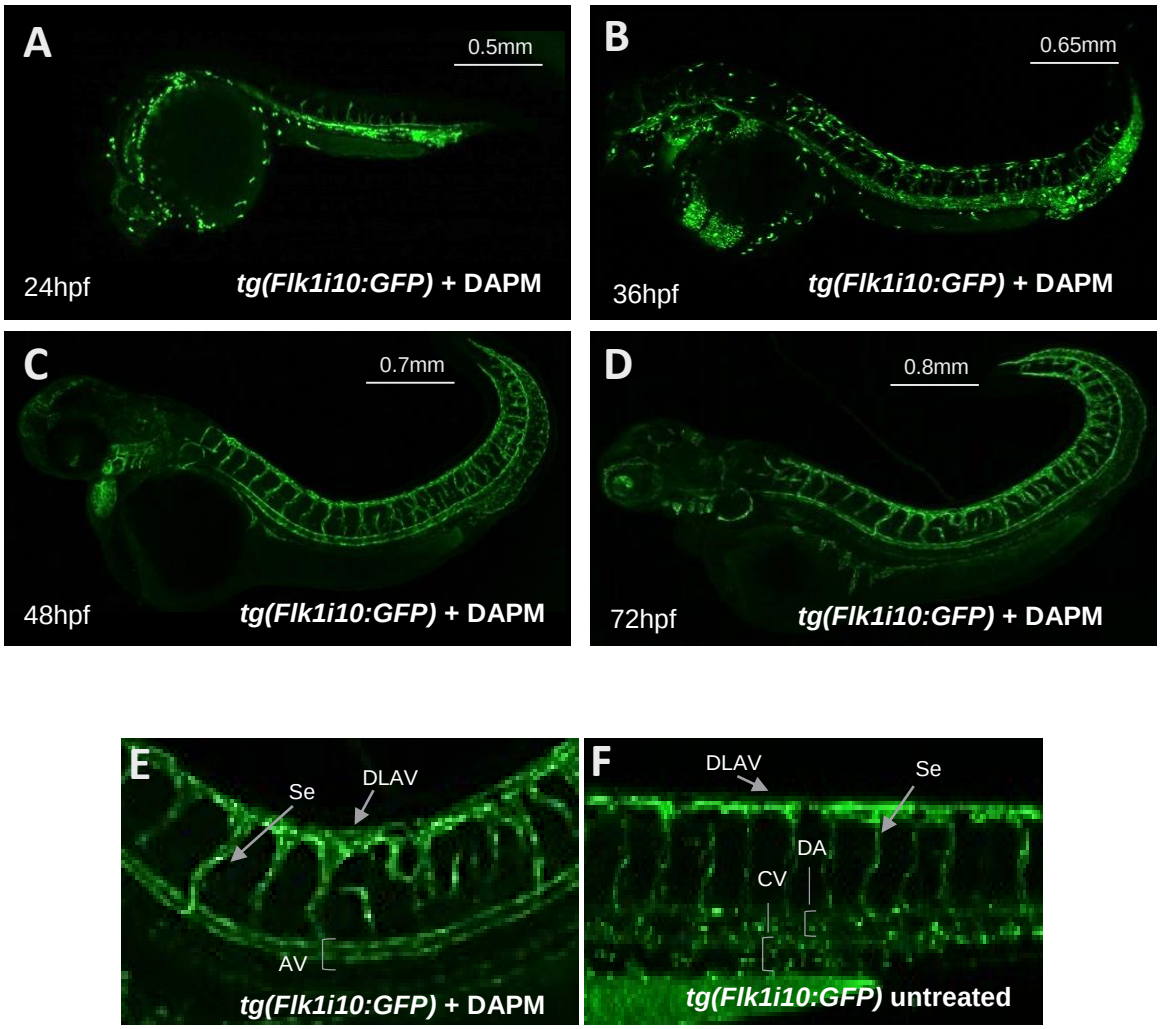


Figure 3.27: Chemical inhibition of Notch signalling with DAPM does not result in a loss of enhancer activity

A-D: Chemical inhibition of Notch signalling in *tg(Flk1i10:GFP)* zebrafish embryos with 100µM DAPM (added at 12hpf) at different developmental stages (24 – 72hpf). Defective vasculogenesis results in the formation of only a single axial vessel (AV). Notch inhibition leads to endothelial cell hyperproliferation and sprouting. Intersegmental vessels (Se) display irregular branching and the dorsal longitudinal anastomotic vessels (DLAV) are thickened. E+F: Vascular GFP expression in DAPM treated (E), and untreated fish (F) at 72hpf. While enhancer activity is reduced in the dorsal aorta (DA) and the intersegmentals of untreated *tg(Flk1i10:GFP)* zebrafish, GFP expression remains pronounced in the axial vessel in the presence of DAPM.

3.2.13 Morpholino knockdown in zebrafish suggests that inputs from *gata2* and *rbpj* regulate Flk1i10 enhancer activity

Results from mutational analyses in zebrafish and mice suggests a role for GATA and RBPJ binding sites in the regulation of the Flk1i10 enhancer. In order to address the question whether, reciprocally, a loss of the transcription factors that bind at the sites would also affect Flk1i10 enhancer activity, Morpholino knockdown was used to target transcription factor expression in *tg*(*Flk1i10:GFP*) zebrafish embryos. Morpholinos (MOs) are short oligomers of a nucleic acid analogue with the capacity to bind complementary RNA targets and in this way block interactions with cellular machinery. By targeting *mRNA*s their translation into protein can be prevented resulting in the knockdown of a specific gene product. MO injections for this project were performed by N. Sacilotto. *rbpj*, *gata2*, *gata1*, *sox7*, and *sox18* MOs were included in this experiment. While Gata2 is the most prominent Gata factor in endothelial cells, Gata1 has a critical function in blood development (Park et al 2013). Despite a lack of evidence for a direct effect of loss of Sox binding sites in mutational analysis, a role for SoxF in arterial specification has been reported previously and, therefore, MOs for *sox7* and *sox18* were included as well (Corada et al 2013, Sacilotto et al 2013).

As reported previously, MO-induced knockdown of *rbpj* resulted vascular hypersprouting as well as improper segregation of the axial vessels (Sacilotto et al 2013, Siekmann and Lawson 2007). In line with the observations after chemical Notch inhibition in *tg*(*Flk1i10:GFP*) zebrafish, knockdown of *rbpj* resulted in slightly enhanced reporter gene expression in the axial and intersegmental vessels (Fig 3.27). Therefore, both MO-induced knockdown of *rbpj* and chemical Notch inhibition do not support a

direct effect of Notch signalling, or Rbpj binding, for Flk1i10 enhancer activity, as suggested by mutational analysis in transient transgenic fish. However, as mentioned previously for chemical Notch inhibition, the global effects of *rbpj* knockdown on vascular development complicate the comparison to mutational analysis.

sox7 and *sox18* are both expressed in the developing vasculature in fish and have been suggested to play functionally redundant roles, as only the combined knockdown of both factor leads to an absence of trunk and tail circulation due to arteriovenous malformations (Cermenati et al 2007, Herpers et al 2008, Pendeville et al 2008). However, in line with the results from the mutational studies in transient transgenic zebrafish and mice, the combined knockdown of *sox7* and *sox18* did not reduce enhancer activity noticeably (Fig 3.27).

MO-induced knockdown of *gata1* has been shown to affect blood cell development leading to anaemia (Galloway et al 2005). Similarly, a loss of GFP positive blood cells was seen in *tg*(*Flk1i10:GFP*) zebrafish (Fig. 3.27). Studies suggest that a loss of *gata2* affects vascular development and leads to shortened and absent intersegmental vessels (Fiedler et al 2011). A drastic reduction of endothelial reporter gene expression was observed upon *gata2* knockdown in *tg*(*Flk1i10:GFP*) zebrafish, suggesting that *gata2* is required for Flk1i10 enhancer activity (Fig. 3.27). This result agrees with the previous observation that disruption of all GATA binding motifs almost completely abolishes endothelial reporter gene expression in transient transgenic fish and mice. Subsequently, *gata2* MO was titrated to determine a subcritical dose at which Flk1i10 enhancer activity is only slightly reduced (Fig. 3.28). This allowed us to examine the combined effects of *gata2* and *rbpj* on enhancer activity as experiments in both transgenic fish and mice indicate that the Flk1i10 enhancer receives inputs from both of these factors. Remarkably, the combined MO knockdown resulted in a

marked loss of enhancer activity in endothelial cells even with a sub-critical doses of *gata2*-MO (Fig. 3.29). This results suggest that the Flk1i10 enhancer receives activating inputs from both Rbpj and Gata2 binding and that these two factors combinatorially regulate enhancer activity.

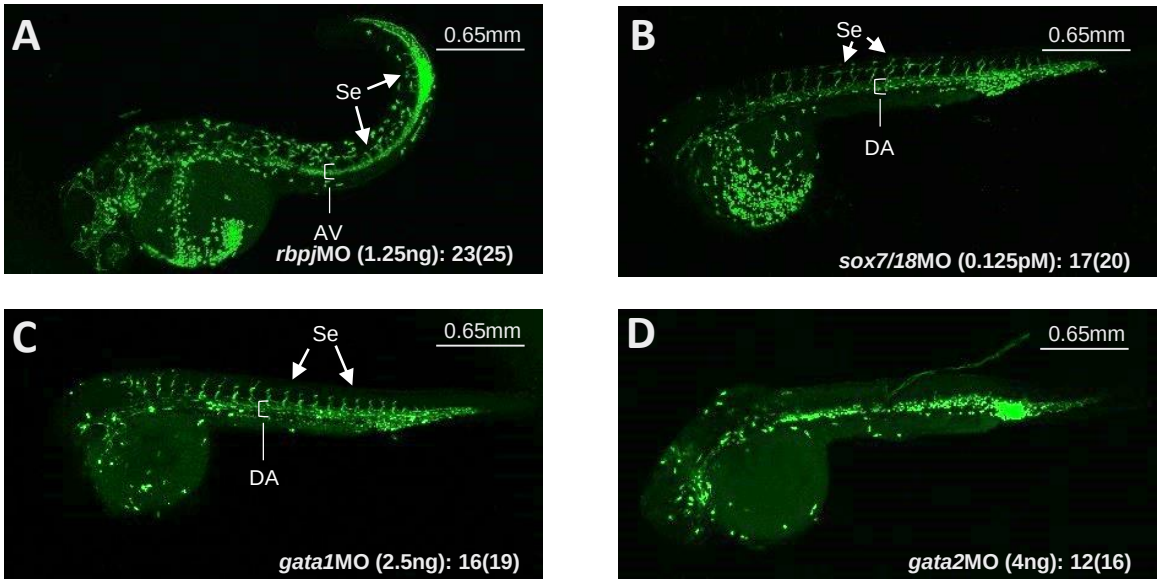


Figure 3. 28: Morpholino knockdown of *gata2* eliminates Flk1i10 enhancer activity in endothelial cells

Morpholino knockdown of genes *sox7*, sox*18*, *rbpj*, *gata1* and *gata2* in *tg*(*Flk1i10:GFP*) at 36hpf.
A-C: GFP expression is observed in dorsal aorta (DA) and intersegmental vessels (Se) after *sox7*/sox*18*, *rbpj*, and *gata1* knockdown.
D: Knockdown of *gata2* eliminates vascular expression of GFP.
Numbers after MO: indicate how many animals (out of total) displayed a phenotype, similar to the one depicted.

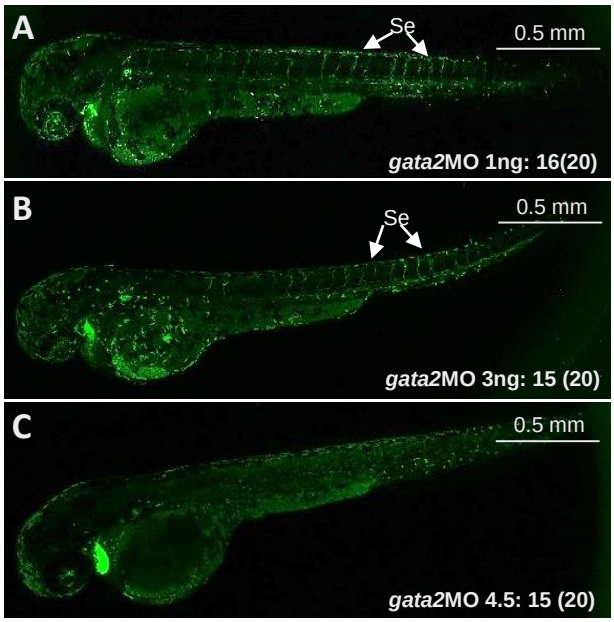


Figure 3.29: Titration of *Gata2* Morpholino

GFP expression in *tg(Flk1i10:GFP)* embryos at 48hpf after *gata2* Morpholino knockdown with 3 different amounts of Morpholino (1.5 – 4.5ng).
A, B: Expression is clearly visible in intersegmental vessels (Se) with sub-critical Morpholino amounts (1.5ng, 3ng).
C: No vascular enhancer signal is detected with the highest amount (4.5ng).
Numbers after MO: indicate how many animals (out of total) displayed a phenotype, similar to the one depicted.
MO = Morpholino

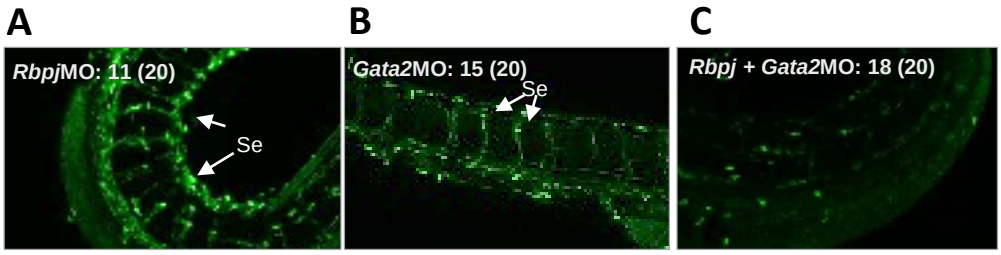


Figure 3.30: Combinatorial knockdown of *gata2* and *rbpj* results in loss of *Flk1*i10 enhancer activity

A, B: After individual knockdown with *rbpj* Morpholino (0.625ng) and sub-critical doses of *gata2*MO (3ng) GFP expression is observed in the vasculature of *tg(Flk1i10:GFP)* embryos at 48hpf.
C: Combined knockdown with both *rbpj* Morpholino (0.625ng) and sub-critical doses of *gata2* Morpholino (3ng) leads to a loss of vascular GFP expression.
Numbers after MO: indicate how many animals (out of total) displayed a phenotype, similar to the one depicted.
MO = Morpholino; Se = intersegmental vessels.

3.3. Discussion

3.3.1 Can enhancers in the Flk1 locus be accurately identified?

Vegfr2 is a key player in endothelial cell development and angiogenic signalling. Regulation of *Vegfr2* expression by promoter proximal and distal *cis*-regulatory elements has been extensively studied (Ema et al 2006, Ishitobi et al 2011, Kappel et al 1999, 2000, Kohler et al 2011, Patterson et al 1995, Rönicke et al 1996). However, our picture of the *cis*-regulatory landscape of the *Flk1* locus (which encodes Vegfr2 in mice) is incomplete, as studies suggest that additional regulatory elements are involved in the regulation of Vegfr2 expression. The present study identified an 826bp element within the tenth intron of the *Flk1* gene that directs reporter gene expression specifically to arterial endothelial cells in both zebrafish and mice. This element, termed the Flk1i10 enhancer, was conserved across mammalian evolution and showed biochemical activity associated with regulatory elements including histone modifications, chromatin accessibility, and transcription factor binding. Moreover, its DNA sequence contained clusters of transcription factor binding sites commonly found in endothelial enhancers. Remarkably, another element in the third intron of the *Flk1* gene did not direct reporter gene expression to the endothelium of transient transgenic zebrafish, despite displaying similar characteristics. On the one hand, this may suggest that even if both evolutionary and biochemical evidence support regulatory function, predictions based on such evidence are not always accurate (Kellis et al 2014). In fact, despite rapid progress in the genome-wide mapping of biochemical activity linked to regulatory function, our understanding of the function of this activity is rudimentary and false-positive rates in enhancer identification based on these

criteria remain high (Kellis et al 2014, Pennacchio et al 2013). On the other hand, even though transgenic enhancer assays, as used in this study, are considered a gold standard in enhancer identification, there are several reasons why this method may produce a false negative results. First, the activity of a regulatory element may not be replicated faithfully in a different species (Ritter et al 2010, Wittkopp and Kalay 2011). Therefore, the mouse Flk1i3 candidate enhancer may produce a different expression pattern in fish or may be inactive. Second, as zebrafish embryos were only screened between 24 – 72hpf, enhancer activity at earlier or later stages would not have been detected in this study. Finally, regulatory function is by no means limited to the direct activation of gene transcription. It has been shown previously that some conserved non-coding elements which lack noticeable enhancer activity can instead act to repress gene expression (Royo et al 2011). Further, conserved non-coding elements which bear regulatory signatures may also be involved in the three-dimensional organization of a genetic locus, as shown for the β-globin locus, without directly affecting transcription (Xu et al 2010). Therefore, the Flk1i3 element may well possess a regulatory function which could be revealed by a more in-depth study of its activity in transgenic mice, *in vitro* gene expression assays or chromosome conformation capture.

3.3.2 Which sequence motifs within the Flk1i10 enhancer are functional?

Non-coding regulatory elements are often enriched for evolutionary conservation, and conservation-based phylogenetic footprinting has been used successfully to identify regulatory elements (Pennacchio et al 2006). However,

enhancer sequences also show considerable degree of binding site turnover and, in some cases, conserved elements from different species have divergent activity patterns (Bullaughey 2011, Dermitzakis and Clark 2002, Wittkopp and Kalay 2011). Therefore, evolutionary conservation alone is not a reliable predictor of enhancer function. The orthologous Flk1i10 chicken enhancer directed endothelial expression of reporter genes in transient transgenic zebrafish, indicating that enhancer function is conserved among species. Based on this, examination of enhancer function was restricted on sequence motifs that were conserved between the mouse and the chicken sequence. While this greatly simplifies further analysis it does not necessarily indicate that the remaining sequence is not important for enhancer function. Studies have shown that transcription factor binding can be conserved even in the absence of noticeable sequence conservation, suggesting that, at regulatory regions, sequence conservation is not always required to retain function (He et al 2011). However, focusing on conserved sequence motifs is still a reasonable starting point in the identification of key transcription factor binding motifs.

As DNA sequence alone is not a reliable indicator of transcription factor binding, all conserved binding sites within the Flk1i10 enhancer were tested for their ability to bind transcription factors *in vitro* by EMSA. Although this is a widely accepted method for the investigation of DNA protein interaction, it has some limitations for the identification of binding motifs. As the assay tests interactions of an individual transcription factor with a potential binding site, it is not suitable to identify interactions that require the presence of a co-factor or co-dependent binding of neighbouring transcription factors as described, for example, in the enhanceosome model (Merika and Thanos 2001, Spitz and Furlong 2012). Likewise, transcription factor binding can be sensible to DNA methylation, and differential methylation is increasingly recognized

as an important feature of regulatory regions (Hon et al 2013, Jones 2012). As DNA in EMSA is usually non-methylated, factors that preferentially bind methylated DNA will not be identified. Binding motifs are often similar within transcription factor families, though differences in binding site preferences do exist. As comprehensive testing of all members of a transcription factor family was not practical in this study, which focused on transcription factors known to be expressed in endothelial cells, some interactions may not have been revealed. For these reasons, there is a possibility that sequences outside the conserved and validated binding motifs are involved in enhancer function. However, targeted mutation demonstrated that collective disruption of the conserved, EMSA-validated binding sites results in a complete loss of enhancer activity, which suggests that the conserved motifs are required for enhancer function and interact with upstream regulators.

3.3.3 What is the role of Gata for Flk1i10 enhancer activity?

As mentioned earlier, binding of a transcription factor alone also does not automatically imply regulatory function (Farnham 2009). To examine the contribution of individual, conserved and validated, binding motifs for Flk1i10 enhancer function, targeted mutations were introduced to disrupt discrete sites. This analysis revealed a critical role for GATA binding sites for Flk1i10 enhancer activation. While disruption of two conserved GATA sites only moderately lowered enhancer activity in transient transgenic zebrafish, additional mutation of two non-conserved GATA sites resulted in a drastic reduction in enhancer activity, suggesting that weakly or non-conserved sequences are indeed important for enhancer function. In mice, disruption of the

conserved GATA sites alone almost entirely abrogated Flk1i10 enhancer activity in the endothelium, confirming the importance of Gata binding for Flk1 enhancer function.

Given the role of Gata transcription factor in the regulation of endothelial gene expression it is perhaps not surprising that abrogation of Gata binding strongly effects Flk1i10 enhancer function (De Val and Black 2009). Moreover, Gata binding is also involved in the regulation of the *Flk1* promoter and both of the previously identified enhancers (Ishitobi et al 2011, Kappel et al 2000, Rönicke et al 1996). The fact that several *cis*-regulatory regions of a single gene are regulated by the same transcription factor may appear somewhat inexpedient. However, these elements, while also requiring input from Gata factors, do not drive expression in the same pattern as the Flk1i10 enhancer. This suggests that, in addition to a general requirement for Gata factors, other factors are involved in the tissue-specific regulation of *Flk1* expression.

3.3.4 What is the role of Notch signalling in Flk1i10 enhancer activity?

A central function of Rbpj is the repression of transcription in the absence of Notch activation. This involves the recruitment of an HDAC complex via co-repressors, resulting in transcriptional repression through chromatin remodelling (Kao et al 1998). For example, mutation of binding sites for the RBPJ homologue suppressor of hairless (Su(H)) in a socket cells-specific enhancer led to aberrant expression of the target gene in shaft cells of the external mechano-sensory organ in *Drosophila (Barolo et al 2000)*. In line with this view of Rbpj as a mediator of default repression in the absence of Notch signalling, deletion of Rbpj binding sites in the Flk1i10 enhancer in mice lead to mis-expression of the reporter gene in venous endothelial cells, suggesting that Rbpj suppresses activation of the enhancer in the absence of Notch signalling. This is

further consistent with reports that Notch signalling is repressed in the venous endothelium by the orphan receptor COUP-TFII (You et al 2005). Upon COUP-TFII deletion venous endothelial cells assume some characteristic of arterial endothelial cells, which the investigators attribute to active Notch signalling in the veins (You et al 2005). However, blocking of Notch signalling with DAPM did not result in repression of the Flk1i10 enhancer in arterial endothelial cells in fish, suggesting that default repression in the absence of active Notch signalling does not take place in this context. Similar results have previously been reported for the arterial-specific Dll4 enhancer, which is bound by Rbpj but not repressed upon abrogation of Notch signalling (Sacilotto et al 2013). Therefore, absence of Notch signalling alone does not seem to be sufficient to establish enhancer repression in arterial endothelial cells. It is possible that the distinct molecular identities of venous and arterial endothelial cells may only allow Rbpj-mediated repression in the former or selectively prohibit repression in the latter. For example, binding of other transcription factors, such as SoxF, to the enhancer could prevent formation of a repressive chromatin signature in arterial endothelial cells. Alternatively, not all factors required for a functional repressor complex might be present in arterial endothelial cells. In this hypothesis, Rbpj-mediated repression at the Flk1i10 enhancer would be independent of Notch signalling. A recent study using ChIP to investigate the effects of Notch signalling on Rbpj binding events confirms a role for Rbpj independent of Notch receptor activation in certain cases, and also challenges the view of Rbpj mediated default repression in the absence of Notch receptor activation (Castel et al 2013). Yet, it is clear that regulation of gene expression by Rbpj is more intricate than current models appreciate and the specific modalities of its complexity remain to be elucidated.

In zebrafish, expansion of expression into the venous endothelium after disruption of Rbpj binding could not be demonstrated convincingly. However, detailed investigation of vascular expression pattern in transient transgenic fish embryos was complicated by the presence of GFP expression in blood cells. Further, Morpholino-induced knockdown and DAPM treatment both have global effects on vascular and embryonic development and, in most cases, lead to incomplete or absent separation of the axial vessels. Therefore, these conditions are not well suited to observe changes in vascular expression patterns.

It is noteworthy that a moderate reduction in vascular reporter gene expression was observed after combined disruption of both Rbpj binding sites in transient transgenic zebrafish, which would suggest these sites actively enhance expression. Indeed, the NICD-Rbpj complex has previously been described as a weak activator of transcription (Tanigaki and Honjo 2010). However, MO-induced knockdown of *rbpj* or Notch inhibition with DAPM in *tg(Flk1i10:GFP)* fish embryos did not confirm diminished enhancer activity. Instead, an increase of reporter gene expression was observed in some parts of the vasculature, indicating that Notch signalling may mediate repression of the Flk1i10 enhancer.

Some models have indeed suggested that Notch signalling may negatively regulate Vegfr2 levels. This was first speculated after observations at the angiogenic front, where blockade of Notch signalling resulted in increased Vegf-dependent angiogenic sprouting (Duarte et al 2004, Hellström et al 2007, Lobov et al 2007). Furthermore, *in vitro* experiments demonstrated reduced levels of Vegfr2 upon activation of Notch signalling with the Notch ligand Dll4 (Williams et al 2006). However, it is now clear that the effect of Notch inhibition on Vegfr2 expression varies in different experimental contexts. For example, Benedito et al. found no significant alterations in

Vegfr2 expression after Notch inhibition during vascularization of the mouse retina which refutes a direct role for Notch in the activation of Vegfr2 expression (Benedito et al 2012). This is in line with our results, which suggest that Notch signalling itself does not directly increase Flk1i10 enhancer activity. It has also been shown that inhibition of Notch signalling augments expression of Vergfr3 which has recently been demonstrated to negatively regulate Vegfr2 levels (Tammela et al 2008, Zarkada et al 2015). Therefore, the observed effects of Notch inhibition can also be explained by an indirect mechanism involving Vegfr3 rather than a direct repression of Vegfr2 by Notch signalling.

As mentioned above, excessive sprouting and Vegf-dependent endothelial hyperproliferation in response to reduced Notch signalling in the vasculature is a well-documented phenomenon (Gale et al 2004, Hellström et al 2007, Leslie et al 2007, Lobov et al 2007, Suchting et al 2007, Thurston et al 2007). Therefore, the robust activity of the Flk1i10 enhancer seen after Rbpj knockdown and Notch inhibition can also be explained by increased activity of the enhancer upon Vegf-induced proliferation. This is supported by the observation that in wild-type *tg(Flk1i10:GFP)* fish, GFP expression is highest in proliferating cells of the sprouting intersegmental vessels and the expanding DLAV plexus, while it is decreased in mature vessels. As Gata2 has been shown to be up-regulated in angiogenesis, elevated levels of Gata2 in response to excessive angiogenic sprouting in the absence of Notch signalling may account for the increase in activity of the Flk1i10 enhancer (Chan et al 2012, Mammoto et al 2009).

Another important difference between chemical Notch inhibition and Rbpj binding site mutation is that Rbpj-mediated default repression is not affected by chemical treatment, whereas all Rbpj binding is abrogated after binding site mutation, making

direct comparisons between the two experiments difficult. Expansion of Flk1i10 enhancer activity into the venous endothelium could not be observed after DAPM treatment or MO knockdown of *rbpj*, as both affect arteriovenous differentiation during vasculogenesis and cause fusion of the axial vessels (Sacilotto et al 2013, Siekmann and Lawson 2007).

3.3.5 Do GATA and RBPJ combinatorially regulate the Flk1i10 enhancer?

When the RBPJ sites were mutated in combination with the conserved GATA motifs endothelial GFP expression in zebrafish was almost entirely lost, suggesting a synergistic role for Gata and Rbpj binding at the Flk1i10 enhancer. In mice, however, such a synergistic effect of combined mutation of GATA and RBPJ sites could not be demonstrated, as disruption of Gata binding alone resulted in a drastic reduction of reporter gene expression.

Additional evidence for a combinatorial regulation of the Flk1i10 enhancer by inputs from Rbpj and Gata also comes from Morpholino knockdown experiments in which subcritical levels of MO for *rbpj* and *gata2* that do not significantly reduce enhancer activity on their own, drastically diminish reporter gene expression when combined. While this particular experiment directly affects transcription factor levels and thus does not allow any conclusions about the contribution of individual binding motifs, it does demonstrate an interdependence for regulatory inputs from both Notch and Gata at the enhancer. In particular, the fact that even high doses of *rbpj* MO, or inhibition of Notch signalling upstream of *rbpj*, do not cause a reduction in GFP expression suggests that Notch signalling-mediated transcriptional activation via Rbpj,

whether Notch dependent or independent, relies on additional contributions from other activating factors to produce its full effect. Indeed, it has been previously reported that the NICD-Rbpj complex requires additional tissue specific transcriptional activators, such as Gata, to mediate strong target gene expression (Tanigaki and Honjo 2010). Experiments with artificial enhancer constructs containing multiple Rbpj binding sites have demonstrated that active Notch signalling alone is insufficient to drive reporter gene expression in transgenic mice (Hsieh et al 1996). On the other hand, local activators often have the capacity to drive target gene expression even in the absence of active Notch signalling which is in line with our observation that mutation of Rbpj binding sites does not result in a complete loss of enhancer activity, whereas disruption of all Gata binding causes a much more pronounced reduction in reporter gene expression (Barolo and Posakony 2002).

Interestingly, cooperative in vitro binding between the C. elegans homologue of Rbpj, Lag-1, and the Gata transcription factor Elt2 has previously been reported (Neves et al 2007). This raises the interesting question whether Gata and Rbpj interact with each other. This would present a possible explanation for the observed reduction of Flk1i10 enhancer activity after deletion of Rbpj binding sites in zebrafish as a loss of Rbpj binding would affect cooperative binding of Gata. As this effect was not observed in mice, or after knockdown of *rbpj* in fish a major effect of a potential direct Gata-Rbpj interaction does, however, not seem likely.

3.3.6 Is the Flk1i10 enhancer downstream of Vegf signalling?

Chemical inhibition of Vegf-signalling in *tg(Flk1i10:GFP)* leads to a complete loss of enhancer activity. It appears, therefore, that the Flk1i10 enhancer is downstream of

Vegf signalling in endothelial cells, pointing to the existence of an auto-regulatory mechanism for Vegfr2 expression. Interestingly, such feed-forward loops have been reported previously (Chatterjee et al 2013, Chen et al 2014). On the other hand, as Vegf signalling is also required for the establishment of arterial endothelial identity during vasculogenesis it is not surprising that Vegfr2 inhibition abolishes expression from an arterial specific enhancer. The arterial-specific enhancer for the Notch ligand Dll4, which is regulated by Notch and SoxF, also loses activity after Vegfr2 blockade (Sacilotto et al 2013). Therefore, a lack of GFP expression in SU5416 treated *tg(Flk1i10:GFP)* embryos may simply reflect an inability of the enhancer to function outside of a clearly established arterial environment. This, in turn, would suggest that the Flk1i10 enhancer is dispensable for initial arterial specification and instead regulates Vegfr2 expression in differentiated endothelial cells.

3.3.7 What is the role of the other Flk1i10 enhancers?

In addition to the Flk1i10 enhancer described here, there are two other enhancer elements previously identified in the *Flk1* locus. The DMME regulatory element 30kb upstream of the *Flk1* locus has been shown to direct expression in mesodermal cells during early embryonic development downstream of fibroblast growth factor (Fgf), but is not active in mature endothelial cells (Ishitobi et al 2011). Since Fgf signalling is required for angioblast formation, it is possible that the DMME and Flk1i10 enhancers are activated in a hierarchical fashion, with the DMME enhancer controlling Vegfr2 expression in response to Fgf during initial specification of the arterial phenotype, and the Flki10 enhancer regulating later expression of *Flk1* in established vascular beds. However, deletion of the +30kb upstream enhancer was only lethal after birth, arguing

against an essential function during vasculogenesis and arteriovenous specification. Similarly, the Flk1 intron1 enhancer, which is active in both venous and arterial endothelial cells, has also been shown to be dispensable for endothelial expression of Vegfr2, although the effects of a homozygous deletion of this element were not examined (Ema et al 2006, Kappel et al 1999). Both DMME and Flk1 intron 1 enhancers also receive positive inputs from Gata factors, suggesting that GATA factors are the general driver of transcription in all endothelial cells. Notably, however, although all three regulatory elements require Gata factors, they do not share expression patterns, particularly in later developmental stages. This suggests that alternative regulatory factors, binding other motifs within these enhancers, are responsible for the patterning of these elements in the vasculature. Since only the Flk1i10 enhancer is bound by Rbpj, this supports the idea that the arterial-restricted activity of the Flk1i10 enhancer is achieved by repression through RBPJ sites in veins. Together, the three enhancers allow differential regulation of Vegfr2 expression throughout different parts and developmental stages of the vasculature, thus providing an illustrated example how *cis*-regulatory diversity participates in the management of genetic information. Moreover, as the results demonstrate, integration of multiple signalling inputs at an individual enhancers permits further modulation of expression in specific environments or at certain points during the differentiation of a single cell type.

To some extent, the fact that heterozygous deletion of *Flk1* can be tolerated, at least in certain mouse models, may let such an elaborate *cis*-regulation of its expression seem disproportionate. And it has been shown that even strongly reduced levels of Vegfr2 are sufficient to sustain endothelial cell survival (Zarkada et al 2015). However, there is also evidence that relative, rather than absolute, levels of Vegfr2

expression are crucial in certain contexts such as for tip/stalk cell selection during angiogenic sprouting (Jakobsson et al 2010), suggesting that the complex patterns of *Flk1* expression directed by these multiple enhancers may be necessary to maintain the vasculature in the correct balance of angiogenic growth and quiescence.

Interestingly, another recently identified arterial-specific enhancer, for the Notch ligand Dll4, is cooperatively regulated by Notch signalling and SoxF (Sacilotto et al 2013). Given the importance of SoxF factors for the maintenance of arterial endothelial identity, it is somewhat surprising that, despite the presence of several conserved SOX binding sites in the Flk1i10 enhancer, SoxF does not appear to play a critical role in Flk1 regulation (Corada et al 2013, Pendeville et al 2008, Sacilotto et al 2013). Deletion of SOX binding sites, either alone or in combination with mutation of RBPJ sites, did not result in a marked reduction of GFP expression in transient transgenic zebrafish, and combined mutation of RBPJ and SOX binding sites in transgenic mice resulted not in reduction, but in expansion of enhancer activity into the venous endothelium, as observed for the disruption of the RBPJ binding sites alone. However, evidence indicates that Vegf signalling may be upstream of SoxF expression in arterial endothelial cells, suggesting that, the Flk1i10 enhancer may operate upstream of SoxF initially, while still receiving reinforcing inputs through the conserved SOX binding sites (Sacilotto et al 2013). Additionally, different aspects of a particular cellular phenotype may be regulated by interdependent, yet distinct, transcriptional networks or sub-networks, which may integrate both common and specific inputs. For example, Notch signalling is a common input for both Dll4 and the Flk1i10 enhancer, whereas SoxF and Gata2 have more specific roles. This would provide robustness to the system, as the regulation of all enhancer activity in a specific cell type by the same factors would dramatically reduce the flexibility of gene expression, and inhibit the ability of the

organism to sensitively adjust gene expression levels in response to multiple different inputs.

3.3.8 Are other transcription factors involved in Flk1i10 enhancer function?

Despite the presence of several conserved ETS binding motifs, the role of Ets factors in the regulation of the Flk1i10 enhancer was not a focus of investigation. Members of the Ets transcription factor family, which share a highly conserved Ets DNA-binding domain, have long been known to be crucially important for endothelial gene expression (De Val and Black 2009). All known endothelial promoter and enhancer regions contain essential clusters of ETS binding motifs, and multiple Ets family members can directly bind these regions (De Val 2011). Moreover, disruption of Ets binding at vascular enhancers invariably abrogates vascular-specific enhancer activity. One idea is that Ets factors are necessary for an initial priming of all vascular enhancers but that subsequent binding of other factors is required to activate transcription. Therefore, an essential role for Ets factors in the regulation of the Flk1i10 enhancer was taken for granted rather than ignored in this investigation.

Finally, although a role for Tcf/Lef and Sox transcription factors could not be demonstrated convincingly in this study, it is possible that they contribute to the regulation of the Flk1i10 enhancer at a level which is not detectable in our assays. Therefore, while we show that GATA and RBPJ sites are critical for Flk1i10 enhancer activity we do not provide evidence that they are exclusively responsible for its function.

3.4. Future work

3.4.1 Targeted deletion of endogenous enhancers and binding motifs

Targeted deletion of the Flk1i10 enhancer will be an informative experiment to demonstrate its role in vascular development. Although complete abrogation of Vegfr2 cannot be expected, as at least one other regulatory element drives its expression in the endothelium, disruption differential expression of Vegfr2 in veins and arteries may well result in an observable phenotype, which would therefore elucidate the specific role of the Flk1i10 enhancer. As the homozygous deletion of the endothelial enhancer in the first intron of the *Flk1* gene has not been examined, targeted deletion of this element will be equally valuable. Further, targeted deletion of individual binding sites of the endogenous Flk1i10 enhancer would be useful to confirm our conclusion that the enhancer is predominantly activated by Gata binding and repressed in the venous endothelium through Rbpj binding sites. Consequently, disruption of endogenous Rbpj sites should lead to ectopic Flki10 enhancer activity in veins and result in deregulation of Vegfr2 levels, the results of which would be interesting to study. Additionally, contributions of other binding sites such as SOX or TCF/LEF could be assessed. Currently, the best way to perform these deletions in mouse zygotes would utilize the CRISPR-Cas system (Sander and Joung 2014).

3.4.2 The Role of Notch signalling in Flk1i10 enhancer activation

Another questions which has not been definitely answered in this study is whether the RBPJ motifs within the Flk1i10 enhancer have a Notch-dependent or

independent function. This problem could be addressed by exposing cultured endothelial cells to both Notch activators and inhibitors and then performing ChIP with antibodies targeting Rbpj as well as P300 which is known to be part of the activating complex that forms together with Rbpj and NICD after Notch receptor activation (Castel et al 2013, Oswald et al 2001). If Rbpj binding is indeed Notch independent, neither activation nor repression should have an influence on the Rbpj binding profile and P300 binding should not be observed.

3.4.3 The Flk1i10 enhancer in sprouting angiogenesis

It is possible that the Flk1i10 enhancer has a role in regulating Vegfr2 expression during processes that are sensitive to relative Vegfr2 levels such as tip/stalk cell selection during sprouting angiogenesis. A common *in vivo* model for the study of endothelial cell sprouting is the mouse retina (Gerhardt et al 2003). Unfortunately, endothelial Flk1i10 expression was not detected in retina endothelial cells in either of the *Flk1i10LacZ* mouse lines investigated. However, other models could be investigated. For example, a useful *in vitro* model of tip/stalk cell selection is the embryoid body assay, which could be performed using embryonic stem cells transgenic for a Flk1i10 enhancer reporter construct.

3.4.4 Other regulatory elements of Flk1

As discussed earlier, identification of regulatory elements is not straightforward. In our analysis of the *Flk1* locus only the most promising candidate enhancers were tested in enhancer-reporter assays and one of them, the Flk1i3 enhancer, did not show

endothelial expression. It is possible that the Flk1i3 element acts as a repressor of Vegfr2 expression, which can be assessed in *in vitro* assays. As data sets for biochemical activity of non-coding regions are constantly refined and expanded, other promising candidate regions in the *Flk1* locus or in more distant parts of the genome may become apparent. Analysis of these elements would further contribute to our understanding of the regulation of Vegfr2 expression.

Chapter 4 – Tbx2 in vascular development

4.1 Introduction

4.1.1 T-Box transcription factors

T-box proteins are an evolutionary ancient family of transcription factors found in all metazoans. The 17 known mammalian T-box proteins are characterized by a common DNA-binding domain, the T-box, which can be located anywhere within the polypeptide chain (Abrahams et al 2010). T-box proteins can act as homodimers or heterodimers with other T-box proteins and also interact with other transcription factors and co-factors. Tbx20, for example, interacts with Gata5 but not the related Gata4, whereas Lmp4 binds both of the closely related Tbx4 and Tbx5, but interacts with each via a different LIM domain repeat (Krause et al 2004).

The T-box recognizes the T-box binding element (TBE) or T-site, a palindromic sequence which can be bound as a dimer. All T-box proteins are capable of binding one half of the TBE consensus motif as monomers. However, variations of the TBE are bound more specifically by individual T-Box family members (Naiche et al 2005). In general, T-box factors can act as either activators or repressors of transcription although some, like Tbx2, feature both activation and repression domains (Paxton et al 2002).

4.1.2 T-Box factors in vascular development

While T-box factors are known to have important functions during the development of various organs, embryonic patterning, and cell fate decisions, their role in the vasculature has not been studied in detail. There are, however, some descriptions of T-box factors involved in vascular development.

In mice, Tbx1 is required for the proper patterning of the pharyngeal arch arteries, aortic arch arteries and the proximal coronary arteries (Théveniau-Ruissy et al 2008, Vitelli et al 2002). Tbx1 has further been shown to activate the transcription of Vegfr3 in lymphatic endothelial cells through direct interaction with a cis-regulatory element (Chen et al 2010). Recently, loss of Tbx1 has been shown to result in brain vascular defects, including vessel hyperplasia, increased angiogenic sprouting and disorganized vascular patterning, which were linked to a cell autonomous role for Tbx1 in the regulation of Vegfr3 and Dll4 expression (Cioffi et al 2014).

In zebrafish, epicardial cells begin to express Tbx18 after myocardial injury concomitant with increased epicardial cell invasion into the wound and neovascularisation of the newly formed tissue, suggesting a potential role for Tbx18 in epicardial epithelial-to-mesenchymal transition or subsequent migration during coronary vasculogenesis (Lepilina et al 2006).

A role for Tbx5 has been proposed in the regulation of proepicardial cell migration, a critical event in the establishment of the epicardium and coronary vasculature, although Tbx5 has been reported to have a cytoplasmic localization during coronary vasculature development, suggesting that it may have a function unrelated to its role as a transcription factor (Bimber et al 2007, Hatcher et al 2004). Mutation of Tbx3 in mice results in reduced yolk sac vasculature and high levels of

apoptosis in the yolk sac endodermal layer (Davenport et al 2003). Loss of Tbx4 does not affect endothelial differentiation in the allantois, but has been shown to inhibit vascular plexus formation (Naiche and Papaioannou 2003).

4.1.3 Tbx2

During murine embryogenesis, Tbx2 plays an important role in the regulation of cell fate decisions, cell migration, and morphogenesis in a variety of organs including the limbs, heart, kidney, nervous system, and eyes, although the precise involvement of Tbx2 in these processes remains poorly defined on a mechanistic level (Abrahams et al 2010). Tbx2 is also implicated in cell cycle regulation, where it has been shown to both promote and inhibit proliferation in different models (Abrahams et al 2010). Inappropriate activation of Tbx2 in cancer has been implicated in tumour progression through its ability to override cellular senescence and therefore sustain tumour growth, involving both p53-dependent and p53–independent mechanisms (Abrahams et al 2010) (Jacobs et al 2000).

Tbx2 has predominantly been described as a repressor of transcription and contains two separate repressor domains (Paxton et al 2002). Tbx2 mediates repression at least in part by recruiting the histone deacetylase 1 (HDAC1) to regulatory elements (Vance et al 2005). However, Tbx2 can also weakly activate transcription through the T-Box domain, and in some studies Tbx2-dependent activation of transcription has been reported (Barron et al 2005, Paxton et al 2002). Tbx2 also forms heterodimers with other transcription factors such as Nkx2.5, MyoD, and Msx genes, and interactions with other T-Box factors have also been reported (Barron et al 2005, Boogerd et al 2008, Habets et al 2002, Zhu et al 2014).

In inbred mice with a mixed background, homozygous deletion of Tbx2 is lethal after E11.5 and leads to defects in the development of the atrioventricular canal and septation of the outflow tract, linked to a requirement for Tbx2 in the repression of chamber differentiation in the atrioventricular canal (Harrelson et al 2004). Further, Tbx2 nulls display defective hind-limb development. Heterozygous deletion of Tbx2, on the other hand, resulted in viable and fertile animals (Harrelson et al 2004). When Tbx2 was deleted in an outbred NMRI background, mice displayed less severe heart defects and survived until late gestation (Zirzow et al 2009).

4.1.4 A novel role for Tbx2 in arterial mural cell development.

The role of Tbx2 in the development of the vasculature has not been a focus of scientific investigation so far. Recently, however, Tbx2 expression in the vasculature has been reported for the first time but no role for Tbx2 in the vasculature has yet been described (Pontecorvi et al 2008). In this study, the expression pattern of Tbx2 in the vasculature of the developing embryo was investigated, revealing nuclear Tbx2 expression in vascular mural cells of arteries and microvessels after E9.5. This arterial-specific expression pattern may indicate a role for Tbx2 in the establishment of an arterial mural cell fate. *In vivo* deletion suggests Tbx2 may be important for mural cell recruitment, survival, proliferation and/or differentiation, and knockdown of Tbx2 *in vitro* points to a role of Tbx2 upstream of Notch3 and Pdgfrβ, both of which have important functions in these processes during mural cells development (Armulik et al 2011).

4.2 Results

4.2.1 Literature search

A literature search initially identified Tbx2 as a potential vascular transcription factor. Although a role for Tbx2 in the regulation of vascular gene expression has not been described, two recent publications, researching other roles of Tbx2, independently reported expression in blood vessels. Pontecorvi et al. used *in situ* hybridization (ISH) to study the expression pattern of Tbx2 and Tbx3 in the developing hypothalamic–pituitary axis, where they also noted expression of Tbx2 in the brain vasculature of E13.5 embryos. This was confirmed by co-staining with the basement membrane marker Collagen IV (Pontecorvi et al 2008). Similarly, Begum and Papaioannou, during an investigation of the expression patterns of Tbx2 and Tbx3 in the mouse pancreas, reported vascular expression of Tbx2 during embryonic development after E12.5, but not in new-born or adult mice (Begum and Papaioannou 2011). Neither study reported vascular expression for the closely related Tbx3.

Together with the previously described roles for other T-box factors in vascular development, these reports point to Tbx2 as an interesting candidate with a potential role in the transcriptional regulation of vascular gene expression. Therefore, a detailed investigation of Tbx2 expression and function in vascular development emerged as an interesting research question.

2.2 In situ analysis of Tbx2 expression in development

As a first step, ISH was performed to visualize Tbx2 *mRNA* on transversal sections of E13.5 mouse embryos in order to reproduce the previously reported observations of vascular expression of Tbx2 at this stage of development. Expression of Tbx2 was detected in the developing lung, eyes, the heart and hind-limbs, in line with published descriptions of Tbx2 expression patterns (Abrahams et al 2010). Further, in agreement with the observations of Pontecorvi et al. and Begum and Papaioannou, the results show robust expression of Tbx2 the developing vasculature (Fig. 4.1) (Begum and Papaioannou 2011, Pontecorvi et al 2008). However, detailed histological analysis of section *in situs* revealed that Tbx2 was expressed only in a subset of vessels. Morphologically, this subset appeared to represent the arterial compartment of the circulation. In order to verify this observation, ISH targeting the arterial marker Efnb2 was performed on adjacent tissue sections (Fig. 4.1, C,) (Gerety and Anderson 2002). The experiments revealed a consistent overlap of Tbx2 and Efnb2 expression in the vasculature suggesting that, within the vasculature, Tbx2 is localized specifically to arteries. There was, however, an exception to this finding with respect to the dorsal aorta, where Tbx2 expression was not detected in spite of strong Efnb2 expression (Fig. 4.1, D, E).

To address the possibility that Tbx2 is expressed in the aorta during earlier stages and is no longer present at E13.5, ISH analysis for Tbx2 and Efnb2 was performed on sections of E11.5 and E9.5 embryos. While Efnb2 is expressed in the dorsal aortae at E9.5, vascular expression of Tbx2 was not detected at this stage, although previously reported expression in the pharyngeal mesoderm was clearly visible, indicating the ISH had been successful (Fig. 4.2) (Mesbah et al 2012). At E11.5 Tbx2 was expressed in peripheral arteries similar to Efnb2, indicating that

Tbx2 is expressed in newly formed peripheral arteries from an early stage (Fig. 4.2). However, expression of Tbx2 was not be detected in the dorsal aorta at E11.5.

These results show that Tbx2 is not expressed in the dorsal aorta during murine development, whereas it is expressed in the remaining arterial circulation after E9.5, as demonstrated by comparison with the expression pattern of the arterial marker Efnb2. Although the dorsal aorta is considered an intergral part of the arterial circulation, it differes from peripheral arteries in that it is assembled *de novo* during vasculogeneis, whereas other vessels are thought to be primarily fromed by sprouting angiogenesis from preexisting vascular structures. This may point to a role for Tbx2 in a process specific to angiogenesis.

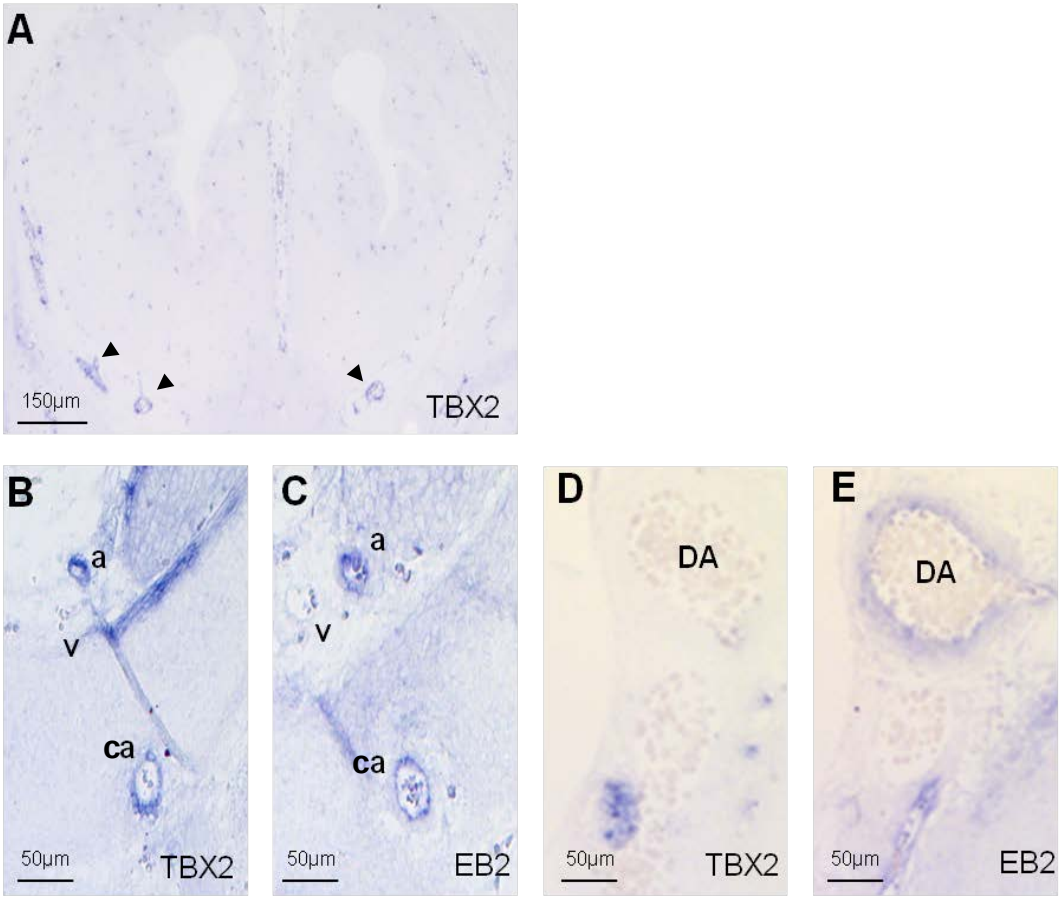


Figure 4.1: Tbx2 expression overlaps with the expression of Efnb2 in arteries but not in the dorsal aorta

5µm section of E13.5 paraffin embedded mouse embryo. In situ hybridization was performed for Tbx2 (A, B, D) and Efnb2 (C, E). a: artery; v: vein; DA: dorsal aorta; ca = carotid artery; cerebral arteries (black arrowheads) EB2 = Efnb2

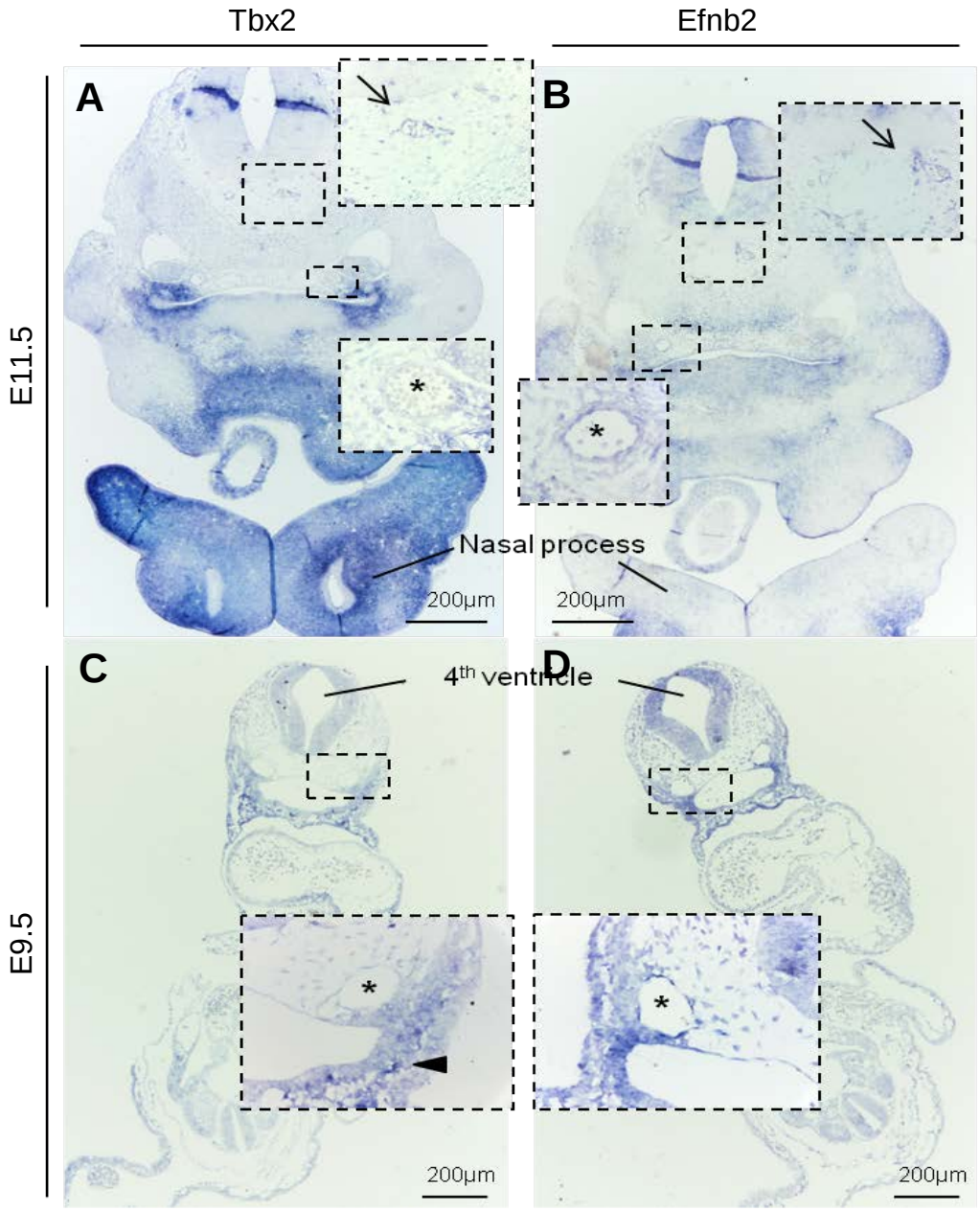


Figure 4.2: Tbx2 expression in the vasculature mouse embryos

A, B: At E11.5 Tbx2 is expressed in vessels which are also positive for Efnb2. Arrows in scale-up show the vertebral artery. Tbx2 expression cannot be detected in the dorsal aortae (*).
C, D: At E9.5 no expression of Tbx2 is seen in the dorsal aorta (*), whereas expression can be detected in the in the pharyngeal mesoderm (black arrowhead). Efnb2 expression is seen in the endothelium of the dorsal aorta at this stage.

4.2.3 Tbx1 and Tbx3 are not expressed in the embryonic vasculature

This study demonstrates that Tbx2 is expressed in peripheral arteries during mouse embryonic development. Tbx2 and Tbx3 are closely related and have been shown to have redundant functions during outflow tract development and have further been implicated in a regulatory pathway together with Tbx1 (Mesbah et al 2012). Further, Chen et al. noted faint expression of Tbx1 in arteries of Tbx1 reporter mice (Chen et al 2010). To examine whether a Tbx2/Tbx3 redundancy, and an interdependence of these two T-Box factors with Tbx1 also exists in the vasculature, expression of Tbx1, Tbx2, and Tbx3 was investigated by ISH on adjacent tissue sections of E13.5 and E15.5 mouse embryos. Staining indicated robust arterial expression of Tbx2 at both stages, while no expression of vascular Tbx3 could be detected in the same vessels at either time point (Fig. 4.3). At E13.5, faint staining for Tbx1 was detected in arteries, as previously observed by Chen et al. (Fig. 4.3) (Chen et al 2010). However, the staining intensity was much lower than for Tbx2 and was no longer detected at E15.5 (Fig. 4.3).

These results do not support a role for redundancy of Tbx2 and Tbx3 in the vasculature, and further do not argue in favour of a reciprocal Tbx1/Tbx2/Tbx3 axis as described for pharyngeal morphogenesis. This suggests that a potential role for Tbx2 in the development of the arterial vasculature is likely independent of Tbx1 and Tbx3.

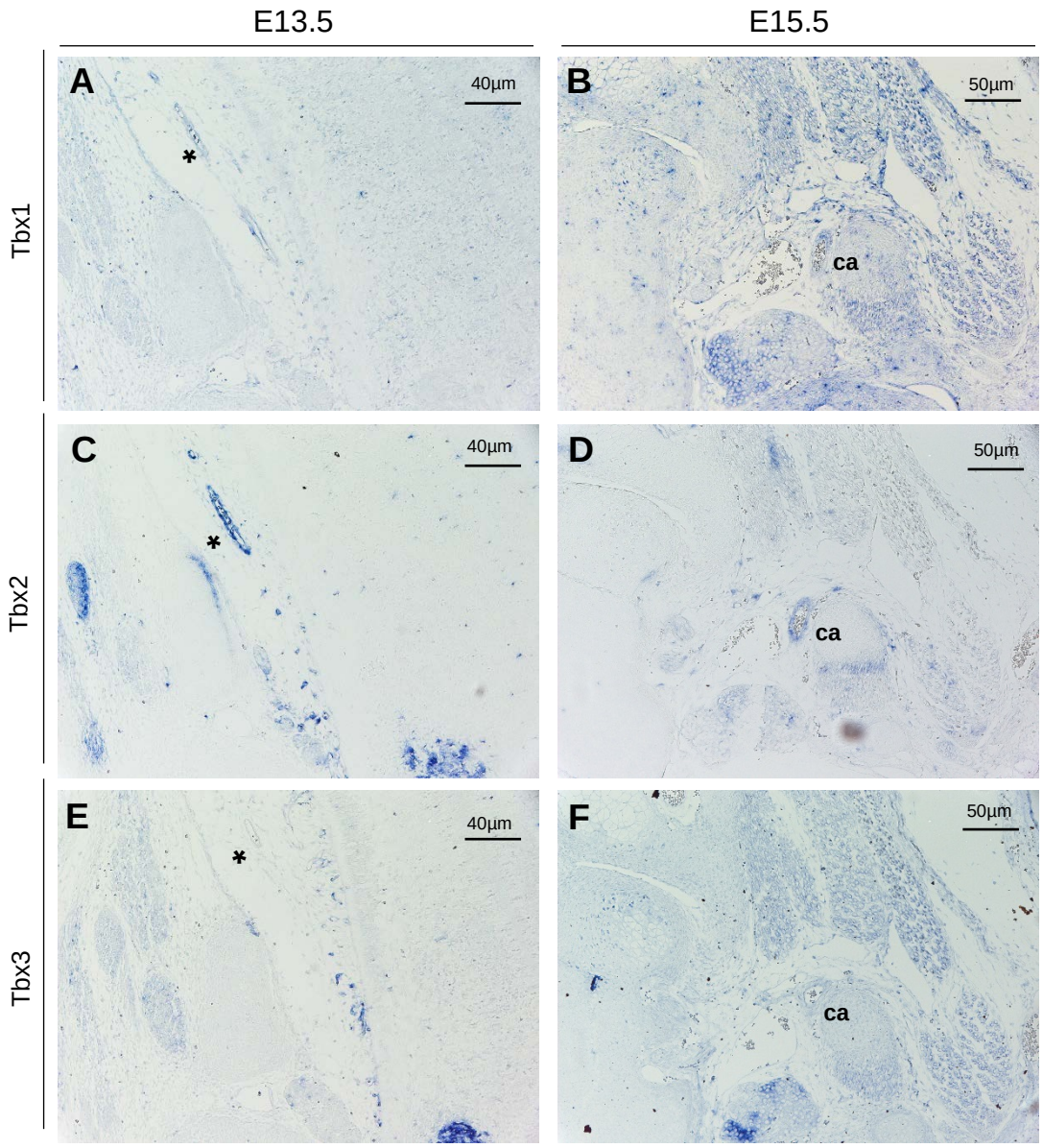


Figure 4.3: Tbx1 and Tbx3 are not expressed in the embryonic vasculature

5µm section of paraffin embedded mouse embryos. In situ hybridization was performed on adjacent sections for Tbx1 (A, B), Tbx2 (C, D), and Tbx3 (E, F) of E13.5 (A, C, D) and E15.5 (B, D, F) mouse embryos.
* = middle cerebral artery; ca = carotid artery

4.2.4 Analysis of the vasculature in Tbx2 null mice

The results from ISH demonstrate that Tbx2 is expressed in the vasculature after E9.5. Homozygous knockouts of Tbx2 die between E10.5 and E12.5 with defects in cardiac development, leaving a short window in which the consequences of a loss of Tbx2 can be studied in *Tbx2*$^{-/-}$ embryos (Harrelson et al 2004). To investigate a potential role for Tbx2 in vascular development, E11.5 *Tbx2*$^{-/-}$ mouse embryos (obtained from V. Papaioannou), alive but growth retarded at the time of harvesting, were examined for the expression of various vascular markers. Immunostaining for the early arterial marker Neuropilin1 (Nrp1) showed expression of the marker on sections of both mutant and wild-type control embryos, suggesting that both contain differentiated arteries (Fig. 4.4) (Herzog et al 2001). Even though Tbx2 expression was only observed in arterial vessels, antibody staining was also performed for the venous marker Ephb4 to ensure that development of the venous circulation was unaffected by Tbx2 deletion (Helbling et al 2000). Again, Ephb4 was expressed in a nearly identical pattern as seen in wild-type controls (Fig. 4.4). Together with the observation that Tbx2 is not expressed in the axial vessels during vasculogenesis, these findings suggests that a loss of Tbx2 does not influence acquisition or maintenance of arterial or venous identity.

Immunostaining for Endomucin (Emcn), which is expressed throughout the endothelium up to E11.5, revealed that the vasculature is similarly developed in knockout and wild-type mice suggesting that a loss of Tbx2 does not result in deficient angiogenesis (Brachtendorf et al 2001, Morgan et al 1999). However, in Tbx2 nulls Emcn staining revealed a disorganized and dilated vascular organisation compared to wild-type controls (Fig.4.5). This was especially apparent in mid-body sections of E11.5 embryos. These results potentially suggest a defect in vascular

remodelling caused by Tbx2 ablation. This impression was further supported by immunofluorescence staining against the mural cell marker α-smooth muscle actin (αSMA), which revealed a discontinuous smooth muscle cell layer in arteries of Tbx2 knockouts (Fig. 4.6). Mural cells are recruited to newly formed vessels during angiogenesis, where they are thought to play an important role in vascular remodelling and vessel stabilization (Armulik et al 2011, von Tell et al 2006). Therefore, these observations may indicate a defect in vascular remodelling caused by defective mural cell recruitment after loss of vascular Tbx2 expression. However, as the global deletion of Tbx2 results in severe cardiac defects, it is impossible to separate any potential primary vascular defects from secondary effects downstream of cardiac malfunction and lack of blood flow, both of which are essential for efficient vascular remodelling.

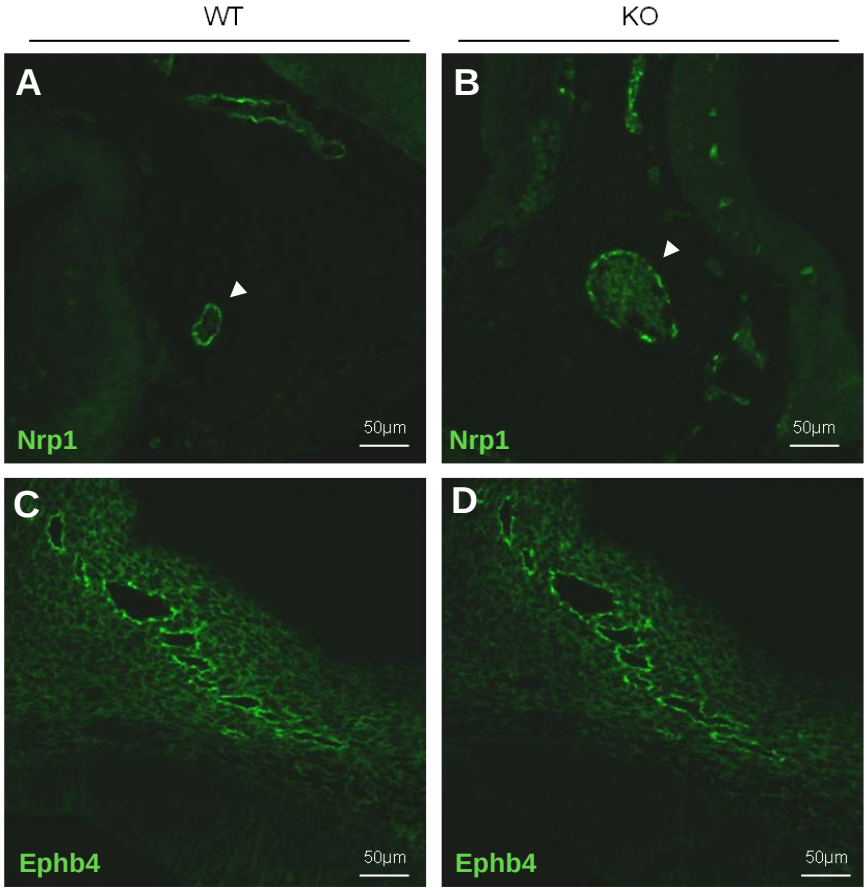


Figure 4.4: Deletion of Tbx2 does not affect arteriovenous differentiation

5um sections of E11.5, paraffin-embedded mouse embryos. Expression of arterial marker Nrp1 in the carotid arteries (arrowheads in A and B) and venous marker Ephb4 in the branches of the primary head veins (C, D) are shown for wild-type (A, C) and knockout (B, D) animals. Both markers are expressed in knockouts and controls.

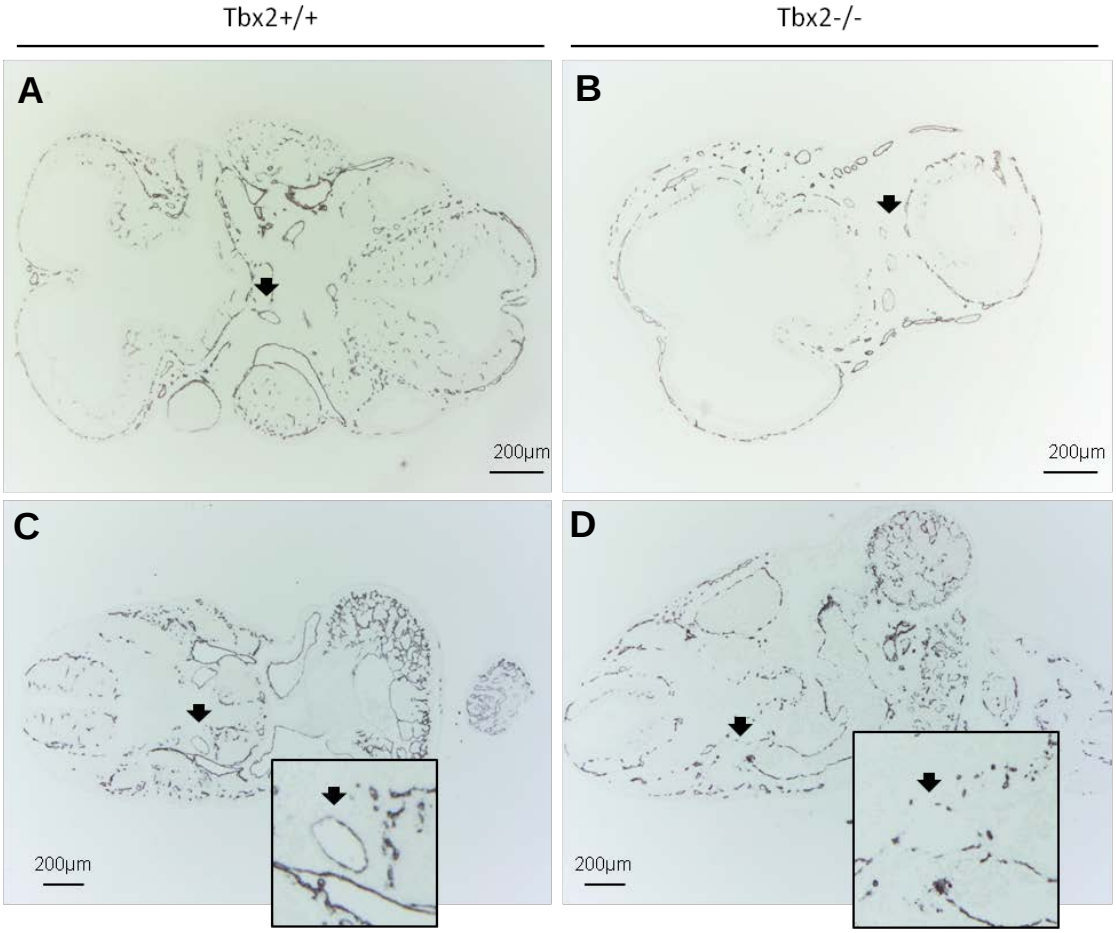


Figure 4.5: The vasculature of Tbx2 null mice is disorganized

Endomucin antibody staining of E11.5 wild-type (A, C) and knockout (B, D) mouse embryos. Different sectional planes are shown for head (A, B; black arrows indicate internal carotid arteries) and torso (C, D; black arrows indicate branchial arch artery). Staining appears discontinuous in vessels of Tbx2 null embryos and the vasculature appears disorganized and dilated in comparison with wild-type controls.

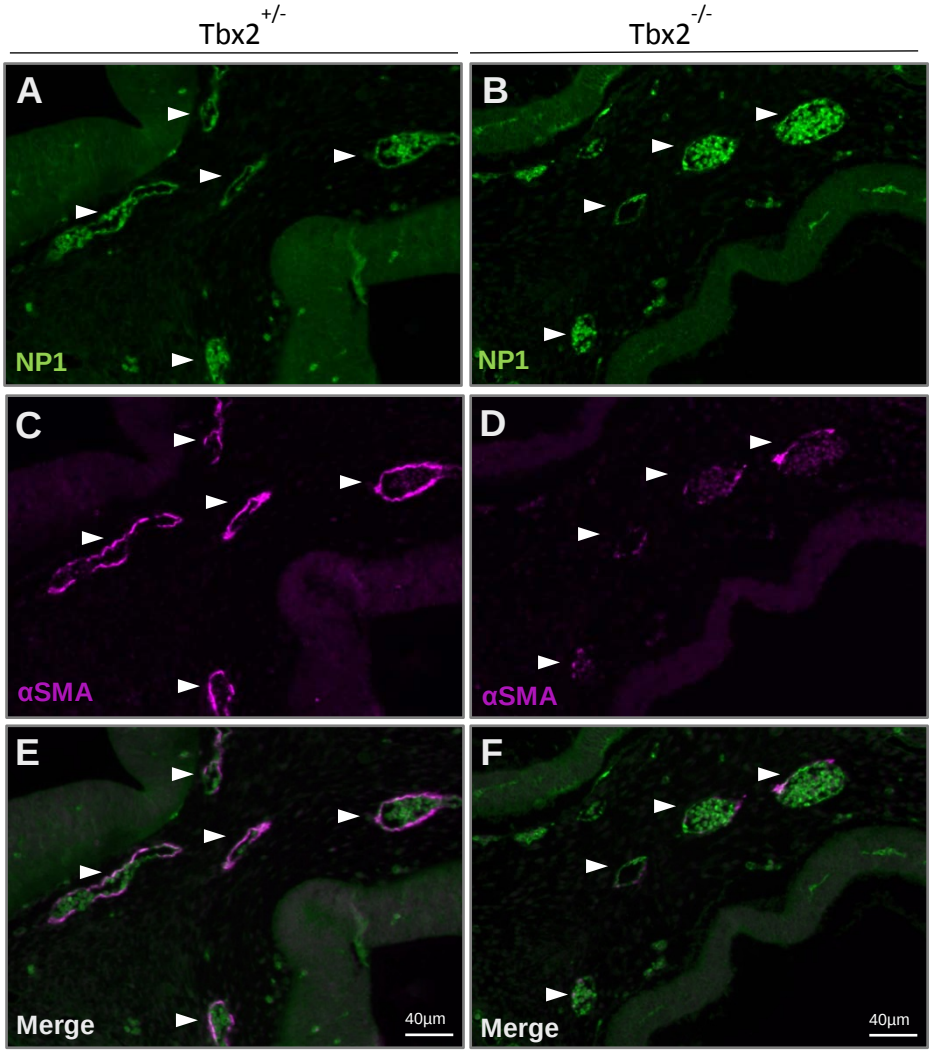


Figure 4.6: Mural cell coverage is reduced in Tbx2$^{-/-}$ mice

Antibody staining for arterial Neuropilin1 (NP1) and the mural cell marker αSMA on 5µm paraffin sections of wild-type (A, C, E) and knockout (B, D, F) E11.5 mouse embryos. White arrowheads marks cerebral and carotid arteries.

4.2.5 Generation of an endothelial-specific deletion of Tbx2

Investigation of Tbx2 knockout mice suggest that a loss of Tbx2 may affect vascular remodelling and mural cell recruitment. Proper recruitment of mural cells is dependent on efficient cross-talk between vascular endothelial cells and mural cell precursors in the vascular periphery (Armulik et al 2005, Gaengel et al 2009). Therefore, a role for Tbx2 in either endothelial or mural cells, or potentially both cell types, may be the basis of the observed remodelling defect. As a detailed analysis of the global Tbx2 knockout is complicated by the early mortality and confounding effects of cardiac malfunction on vascular development an endothelial-specific knockout of Tbx2 was generated to investigate a potential role for Tbx2 in the endothelium.

To this end, transgenic mice expressing Cre-recombinase under the control of the endothelial-specific Tie2 promoter were crossed with conditional Tbx2 nulls which carry *Lox* sites for the Cre-mediated removal of the second exon of *Tbx2* to generate an early stop-codon in the mutant *mRNA* (Kisanuki et al 2001, Wakker et al 2010). In these mice Cre-recombinase is expressed in endothelial cells as well as in a subset of mesenchymal cells of the atrioventricular canal and the outflow tract from E.8.5 onwards, where it catalyses excision of the floxed alleles to abrogate Tbx2 expression.

PCR-based genotyping was used to confirm presence of the Tie2-Cre transgene, the *LoxP* sequences, as well as efficient excision of the second exon of Tbx2. Surprisingly, mice heterozygous for Tie2-Cre and homozygous for the floxed *Tbx2* allele were born in normal Mendelian ratios and were viable and fertile (Fig.

4.7). Further, defects in vascular remodelling were not apparent in the endothelial specific knockout of Tbx2.

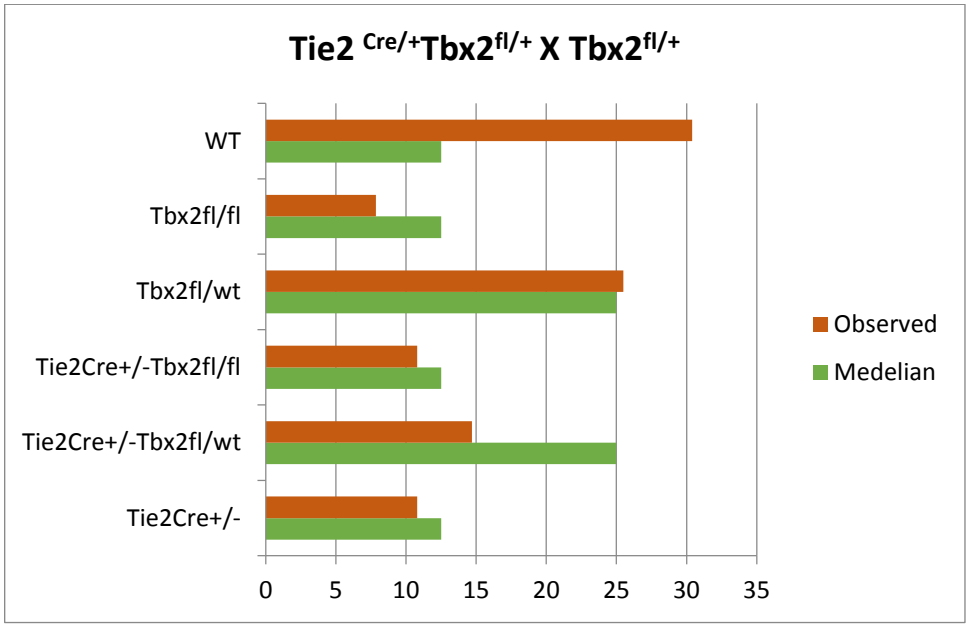


Figure 4.7: Tie2$^{Cre/+}$Tbx2$^{fl/fl}$ are born in normal Mendelian ratios

Breeding of Tie2$^{Cre/+}$Tbx2$^{fl/+}$ X Tbx2$^{fl/+}$ mice. Expected Mendelian ratios in orange, observed ratios in green. n=102

4.2.6 Analysis of the endothelial-specific deletion of Tbx2

The lack of a vascular phenotype after endothelial-specific knockout of Tbx2 in mice suggests two possibilities. First, Tbx2 may not be essential for vascular remodelling in the mouse, meaning that the observed vascular phenotype in the global Tbx2 null was entirely attributable to secondary effects caused by cardiac abnormalities. Alternatively, the lack of a phenotype in the endothelial-specific knockout may instead indicate that Tbx2 has a role in other cell types within the vasculature. To address this question, ISH for was performed on tissue sections of $Tie2^{Cre/+}Tbx2^{flox/flox}$ animals to inspect expression of Tbx2 *mRNA*. These experiments revealed robust expression of Tbx2 in the vasculature of $Tie2^{Cre/+}Tbx2^{flox/flox}$ mice at E13.5 in the same arterial pattern and comparable intensity as in littermate controls which did not express Cre-recombinase, despite the fact that any Tbx2 expression in endothelial cells would have been ablated (Fig. 4.8). This demonstrates that Tbx2 is expressed by another cell type in developing arteries, most likely by vascular mural cells, in which the Tie2 promoter used for the conditional knockout does not drive Cre expression. However, the low cellular resolution of the *in situ* staining used to visualize Tbx2 expression makes this technique unsuitable to clearly identify different vascular cell types.

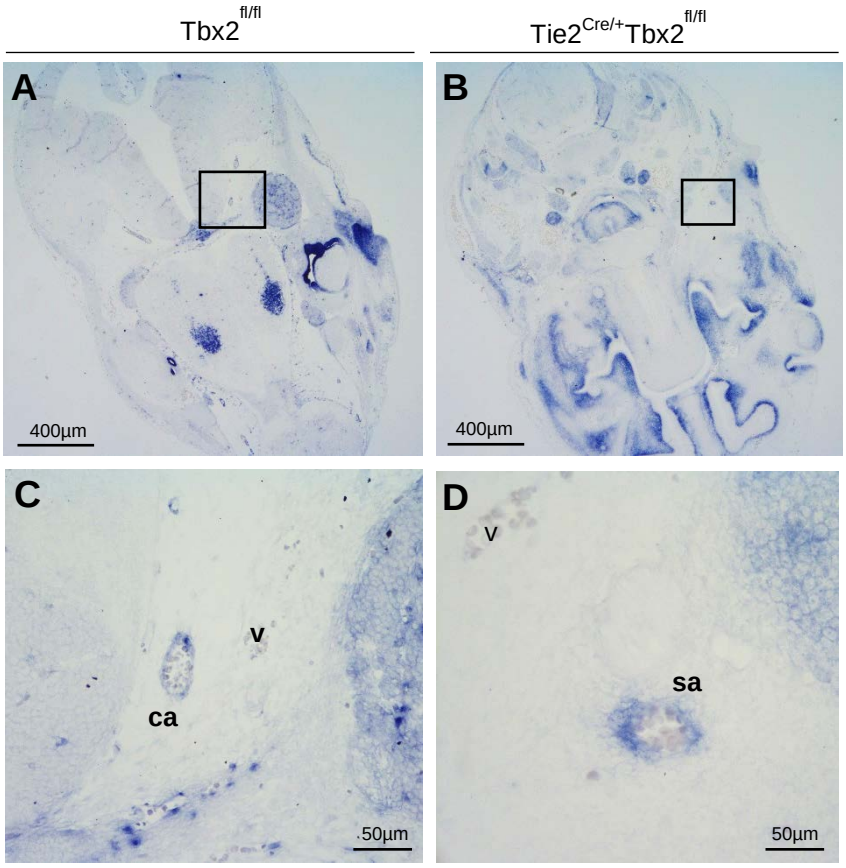


Figure 4.8: Tie2$^{Cre/+}$Tbx2$^{fl/fl}$ mice express Tbx2 in arteries

ISH for Tbx2 on 5µm paraffin sections of E13.5 embryos. Endothelial-specific Tbx2 nulls (B, D) and controls (A, C) which do not carry the Tie2Cre transgene both show positive staining for Tbx2 *mRNA* in arteries (a) but not veins (v). ca = carotid artery; sa = stapedial artery

4.2.7 Role of Tbx2 in Arterial Pericytes

Since an endothelial-specific deletion of Tbx2 in mice did not result in a loss of vascular Tbx2 expression, it is likely that Tbx2 is expressed in a different vascular cell type. In ISH even relatively small vessels in the developing brain showed expression of Tbx2. The only cell type, apart from endothelial cells, that is known to be associated with vessel at this stage are vascular pericytes and vascular smooth muscle cells, collectively referred to as mural cells. As mural and endothelial cells are closely associated – in the case of pericytes they even share the same basement membrane - the low cellular resolution of ISH makes it challenging to determine if the signal originates from one or the other. Fortunately, a newly generated antibody against Tbx2 became available mid-way through this project that could be used in immunohistochemistry to more closely examine Tbx2 expression at single-cell resolution.

In line with results from ISH for Tbx2 mRNA, immunofluorescence staining with this antibody on sections of E11.5 mouse embryos shows expression of Tbx2 in arterial vessels except for the dorsal aorta (Fig. 4.9 – 4.12). Immunostaining also revealed that Tbx2 was expressed in the nuclei of dispersed cells around the vascular lumen as visualized by co-staining using IsolectinB4 which specifically binds the endothelial glycocalyx (Ismail et al 2003). The nuclei of these cells had a cobblestone-like shape unlike endothelial cells (which have characteristic cigar-shaped nuclei). This suggested that Tbx2 was not expressed in endothelial but more likely in mural cells. To further exclude endothelial expression of Tbx2, sections were also treated with antibodies against the Ets-family transcription factor Erg which is constitutively expressed in endothelial but not mural cells (Birdsey et al 2008, McLaughlin et al 1999). In these experiments, Erg expression was detected in cigar-

shaped nuclei of cells which formed a continuous layer around the vascular lumen, but was not seen in cells which stained positive for Tbx2 (Fig. 4.10). This indicates that Tbx2 is not expressed in endothelial cells.

As previous experiments suggest that Tbx2 is expressed in arterial mural cells, immunofluorescence was performed with antibodies against the proteoglycan neural/glial antigen 2 (NG2), a marker of pericytes, and against α-smooth muscle actin (α-SMA), which is detected in more mature mural cells (Armulik et al 2011, Ozerdem et al 2001). As reported previously, NG2 marked mural cells of the microvasculature as well as of more mature arteries but not of veins (Murfee et al 2005). Tbx2-positive nuclei were observed in NG2-positive vessels except in the dorsal aorta where Tbx2 was not present (Fig. 4.11). In agreement with previous studies αSMA was detected in mural cells of arterioles, arteries and large veins but not in capillaries (Ozerdem et al 2001). Tbx2 was co-localized with α-smooth muscle actin only in arteries. Again, the dorsal aorta, which is heavily invested with α-SMA-expressing mural cells did not contain any Tbx2-positive nuclei (Fig 4.12). α-SMA was also expressed in other structures, such as the bronchi, but did not overlap with Tbx2 staining in these tissues. Conversely, Tbx2 expression was observed in tissues where α-SMA was not detected, such as the lung, indicating that expression of these proteins is not inherently linked and only overlaps in mural cells supporting the vasculature.

Together, these results suggests that Tbx2 is expressed in pericytes of the microvasculature and in other mural cells supporting more mature arteries, but not in the dorsal aorta.

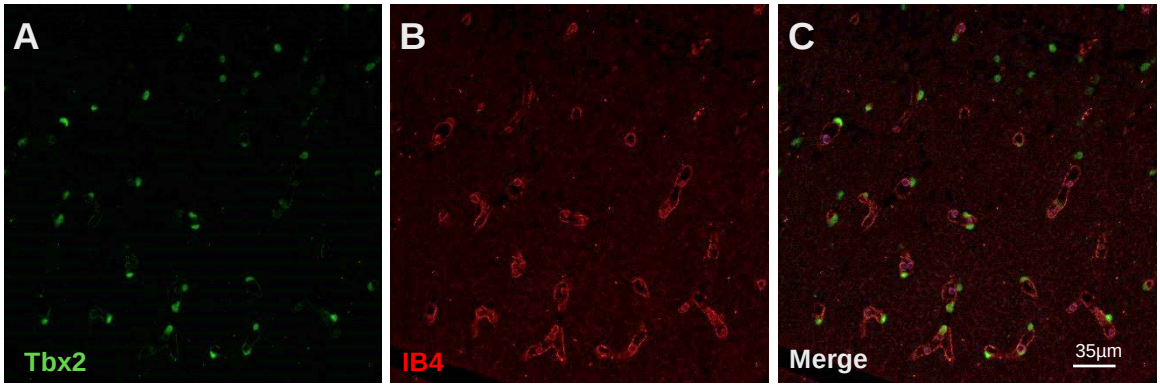


Figure 4.9: Tbx2 is expressed in disperse nuclei in the vasculature

5µm paraffin sections of E11.5 embryos were labelled with IsolectinB4 (IB4, B), a marker of endothelial cells and antibodies against Tbx2 (B). Tbx2 is detected in dispersed nuclei around the vascular lumen which is surrounded by a continuous layer of endothelial cells marked by IB4 (C).

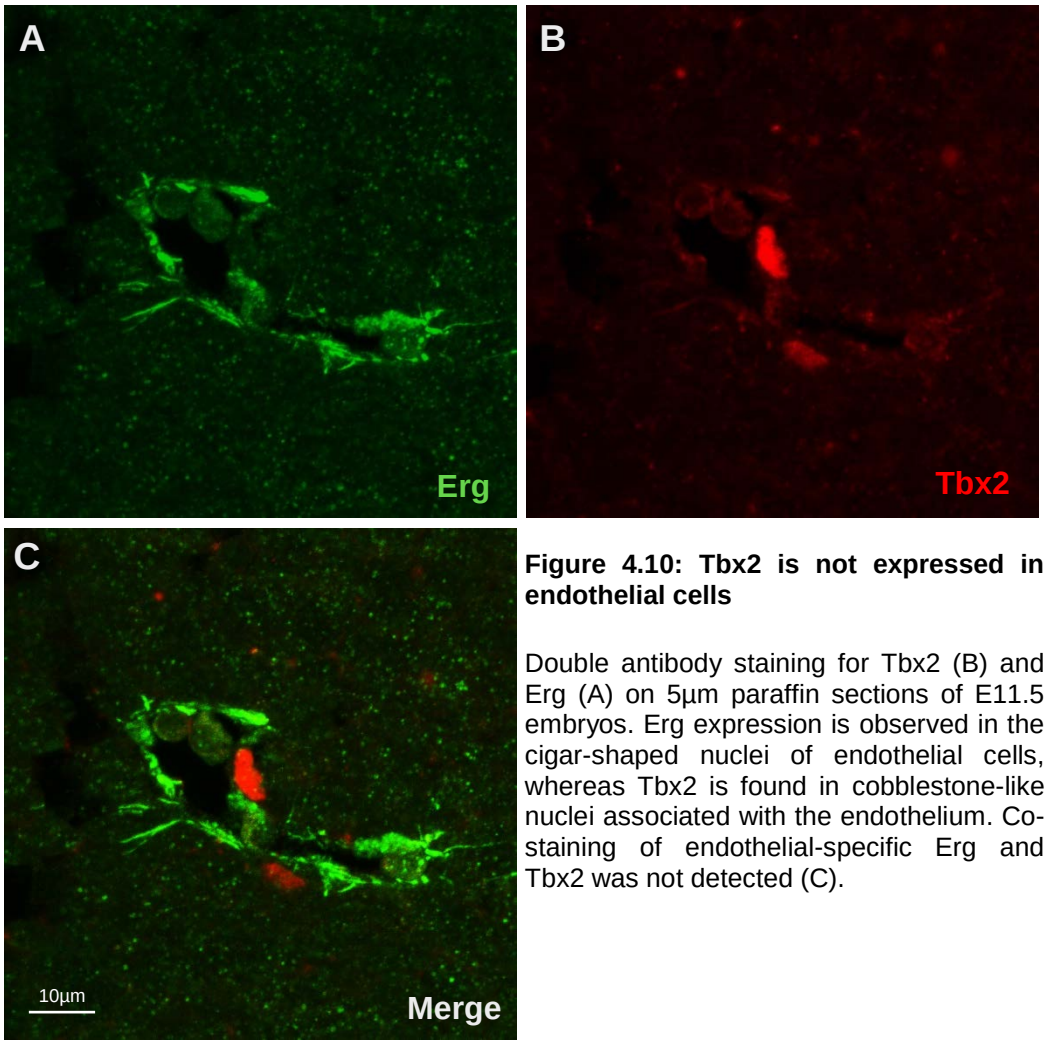


Figure 4.10: Tbx2 is not expressed in endothelial cells

Double antibody staining for Tbx2 (B) and Erg (A) on 5µm paraffin sections of E11.5 embryos. Erg expression is observed in the cigar-shaped nuclei of endothelial cells, whereas Tbx2 is found in cobblestone-like nuclei associated with the endothelium. Co-staining of endothelial-specific Erg and Tbx2 was not detected (C).

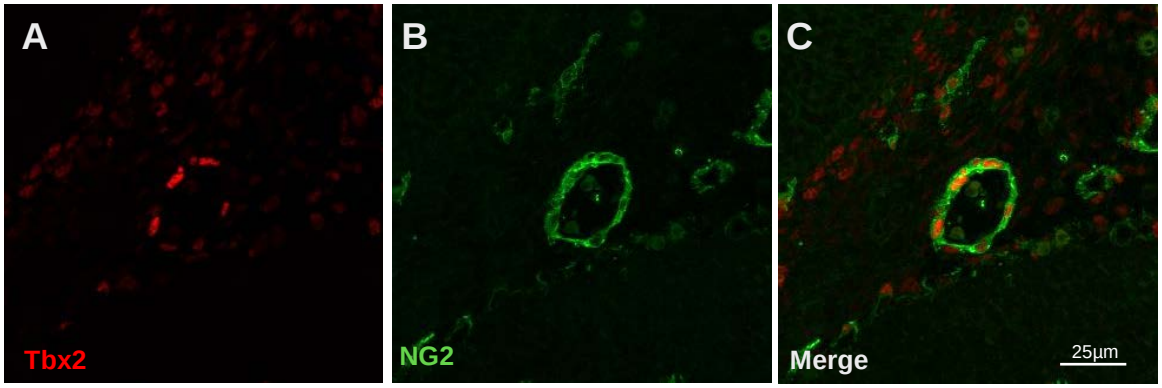


Figure 4.11: Tbx2 is expressed in vascular mural cells

Double antibody staining for Tbx2 (A) and NG2 (B) on 5µm paraffin sections of E11.5 embryos. Tbx2 staining is observed in cells expressing the pericyte and mural cell marker NG2 (C).

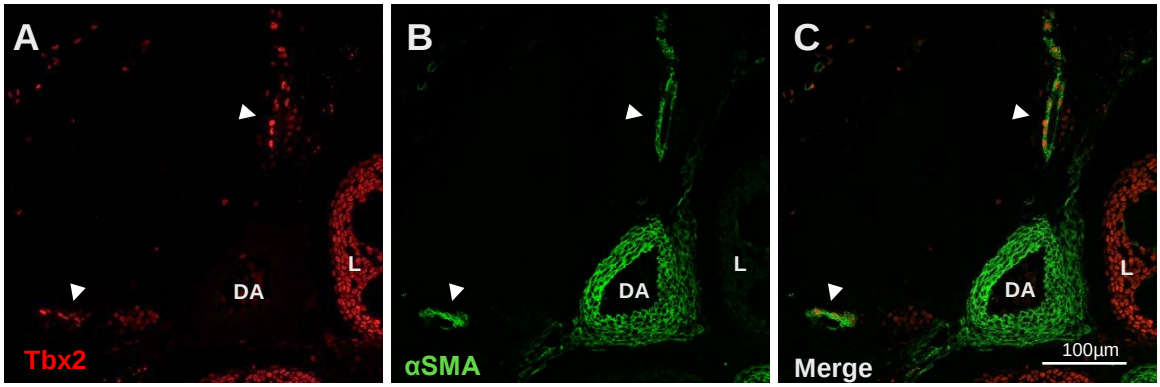


Figure 4.12: Tbx2 is expressed in the smooth muscle layer of peripheral arteries

Double antibody staining for Tbx2 (A) and αSMA (B) on 5µm paraffin sections of E12.5 embryos.Tbx2 is expressed in the smooth muscle layer of peripheral arteries (arrowheads) but but not in the wall of the dorsal aorta (DA). Tbx2 expression is also observed in lung tissue (L) where αSMA is not expressed.

4.2.8 Generation of a mouse model for inducible deletion of Tbx2 in embryos

Global deletion of Tbx2 results in embryonic mortality between E9.5 and E11.5 with severe defects in heart development. As cardiac function and hemodynamics are essential for the proper development of the vasculature it is difficult to delineate direct effects of a loss of Tbx2 on the vasculature from secondary effects caused by insufficient cardiac output. To study the effects of a loss of Tbx2 at later stages of development, a tamoxifen-inducible Tbx2 knockout was generated by crossing mice carrying a Cre recombinase-oestrogen receptor T2 (Cre-ERT2) cassette under the control of the constitutive, ubiquitous Rosa26 promoter with $Tbx2^{flox/flox}$ animals. The ERT2 moiety retains the Cre recombinase in the cytoplasm until tamoxifen administration releases this inhibition, thus permitting the recombination of genomic *loxP* sites. Efficient recombination in embryonic mice upon intra-peritoneal injection of tamoxifen into pregnant females was assessed by crossing $Rosa^{CreERT2}$/+ mice with a Cre reporter strain ($Rosa26^{LacZ/+}$), in which a *loxP*-flanked DNA STOP sequence prevents expression of the downstream *LacZ* reporter gene. Upon tamoxifen-induced Cre activation, the STOP sequence is removed and *LacZ* is expressed from the ubiquitous Rosa26 promoter.

Cre-reporter mice were injected with tamoxifen at E7.5/8.5 and, after harvesting of the embryos at E11.5, *LacZ* reporter gene expression was detected through X-Gal staining. Robust, ubiquitous staining was detected at this stage, which indicates efficient Cre activation after tamoxifen injection, whereas no staining was observed in animals which do not carry the Cre-ERT2 transgene (Fig. 4.13). This validates that

with this system, efficient inducible deletion of floxed genes can be achieved in developing embryos.

Figure 4.13: X-Gal staining of E11.5 Rosa$^{CreERT2/+}$ Rosa26$^{LacZ/LacZ}$

Tamoxifen was injected at E7.5/8.5. Ubiquitous expression of *LacZ* leads to developmental retardation in Rosa$^{CreERT2/+}$Rosa26$^{LacZ/LacZ}$ animals (B), while Rosa26$^{LacZ/LacZ}$ develop normally and show no X-Gal staining (A).

4.2.9 Analysis of inducible deletion of Tbx2

After induced deletion of Tbx2 through tamoxifen injections at E7.5 and E8.5, $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ embryos could be recovered at E11.5 at the expected Mendelian ratio of 1:1. However, $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ embryos were markedly smaller, reminiscent of the phenotype observed for the global deletion of Tbx2, when compared to littermate $Tbx2^{fl/fl}$ controls, which did not express Cre recombinase.

Antibody staining confirmed that Tbx2 was not expressed in tamoxifen injected $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ embryos, suggesting that Tbx2 was efficiently deleted upon induction with tamoxifen. Staining for the pericyte marker NG2 in E11.5 embryos after deletion of Tbx2 at E7.5/8.5 showed that pericytes were still detectable in the vasculature of the brain, suggesting that ablation of Tbx2 does not result in complete loss of pericytes (Fig. 4.14). However, in a closer examination of NG2 expression in several sectional planes of the embryo total pericyte coverage appeared markedly reduced in knockout animals (Fig. 4.15). Similar to the observations from the global Tbx2 deletion, co-staining with IsolectinB4 further revealed that the endothelium was equally developed in inducible knockouts and littermate controls, suggesting that angiogenesis is not affected by a loss of Tbx2. Immunostaining on the same embryos against α-SMA revealed that peripheral arteries were invested with mural cells after ablation of Tbx2 (Fig 4.16). However, in line with the observations from the global deletion of Tbx2, the arterial smooth muscle layer appeared discontinuous in induced Tbx2 knockouts. This may suggest that mural cell coverage is reduced after loss of Tbx2, either as a result of decreased proliferation, survival, or impaired mural cell recruitment.

As Cre induction at E7.5/8.5 lead to severe developmental defects in E11.5 embryos, secondary effects may affect vascular development as previously discussed for the global deletion of Tbx2. Therefore, induced deletion of Tbx2 through tamoxifen injections was also performed at the later time points of E9.5 and E10.5. $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ embryos were harvested at E13.5 with the expected Mendelian ratio of 1:1 and were of similar size as controls, although they displayed incomplete closure of the neural tube and generally appeared pale in comparison with littermates which did not carry the Cre-ERT2 transgene, suggesting that Tbx2 ablation still results in global developmental anomalies (Fig. 4.17). Antibody staining confirmed efficient knockout of Tbx2 in $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ (Fig. 4.18). Staining with α-SMA revealed that mural cells were present in knockout animals, however, in cross-sections of arterial vessels fewer mural cells appeared to be present in knockout embryos and in some cases mural cells displayed an irregular morphology compared to controls (Fig. 4.19). Again, this suggest that loss of Tbx2 may affect mural cell recruitment, proliferation, survival, and/or differentiation.

Overall, the results obtained from the inducible knockout further validate previous observation made in the constitutive Tbx2 knockout. Expression of both NG2 and α-smooth muscle actin further suggest that Tbx2 is not required for cell fate specification in mural cell progenitor cells. Instead, the results might point to a role for Tbx2 in mural cell proliferation, survival, differentiation, or recruitment. However, the inducible knockout did not offer a considerably improved model to examine the effects of Tbx2 deletion in the vasculature independent of confounding effects on other organ systems. While it did allow investigation of later stage embryos, it was only partially useful in circumventing the difficulties described for the analysis of the constitutive deletion of Tbx2. If Cre induction was performed prior to the onset of

vascular Tbx2 expression around E10.5, loss of Tbx2 still resulted in considerable cardiac abnormalities and lead to early embryonic death around E11.5. If Cre induction was performed later, developmental abnormalities were less pronounced, although the observed incomplete closure of the neural tube does suggests that Tbx2 deletion has considerable detrimental effects on multiple organ systems which complicates identification of a primary vascular phenotype. Moreover, Cre deletion after E9.5 may allow for initial vascular Tbx2 expression to occur, which would attenuate any detrimental effects of the loss of Tbx2 on mural cell development. In line with the observations of Begum and Papaioannou, vascular expression of Tbx2 was not detected in adult mice (not shown), which further shortens the window in which an inducible deletion of Tbx2 would produce an observable vascular phenotype.

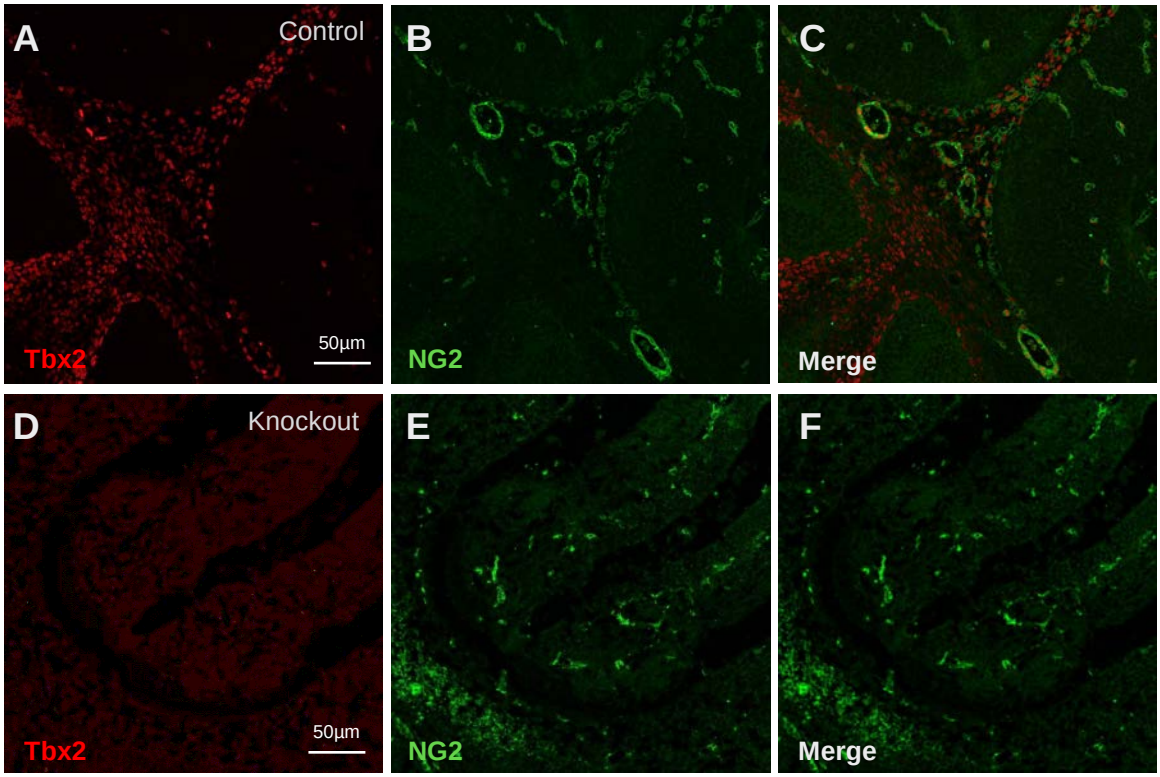


Figure 4.14: Tbx2 is efficiently deleted after tamoxifen-induced knockout

Double antibody staining for Tbx2 and NG2 on 5µm paraffin sections of E11.5 $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ (D, E, F) and $Tbx2^{fl/fl}$ controls (A, B, C)). Cre-mediated excision of floxed Tbx2 allele was induced by intraperitoneal injection of tamoxifen at E7.5 and E8.5. Controls do not carry the Cre-ERT2 transgene.
No Tbx2 staining is observed after Cre induction in $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ embryos whereas Tbx2 is expressed in controls. NG2 positive cells are still observed in the vasculature of the brain after loss of Tbx2

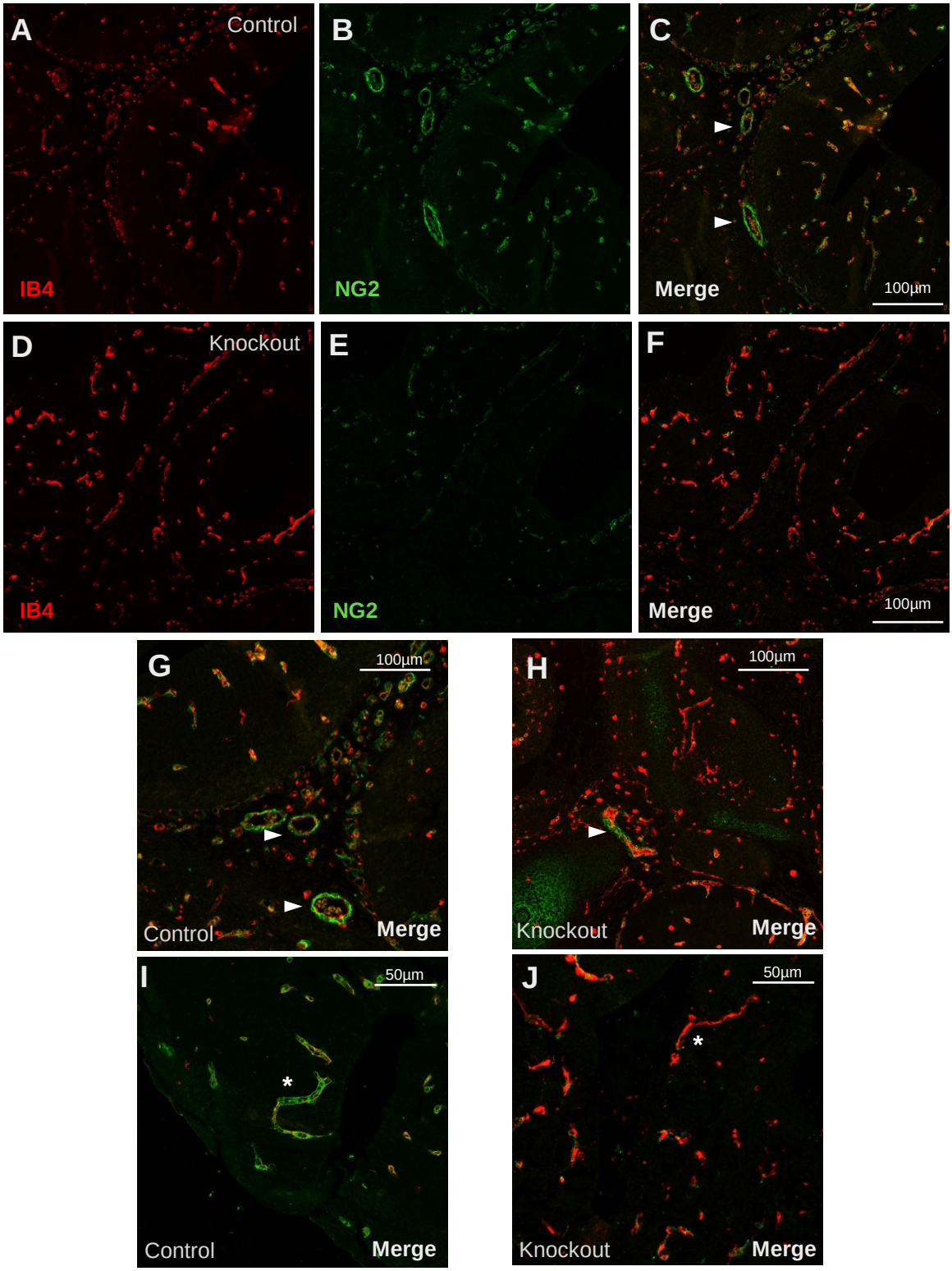


Figure 4.15: Pericyte coverage is reduced after Tbx2 knockout

Double fluorescence staining for IsolectinB4 (IB4) and NG2 on 5µm paraffin sections of E11.5 $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ (D, E, F, H, J) and $Tbx2^{fl/fl}$ controls (A, B, C, G, I). Cre-mediated excision of floxed Tbx2 allele was induced by intraperitoneal injection of tamoxifen at E7.5 and E8.5. Controls do not carry the Cre-ERT2 transgene.
Pericyte-specific NG2 staining is reduced in $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ embryos while the endothelial network is equally well developed.
White arrowheads = cerebral arteries; * = cerebral microvessels

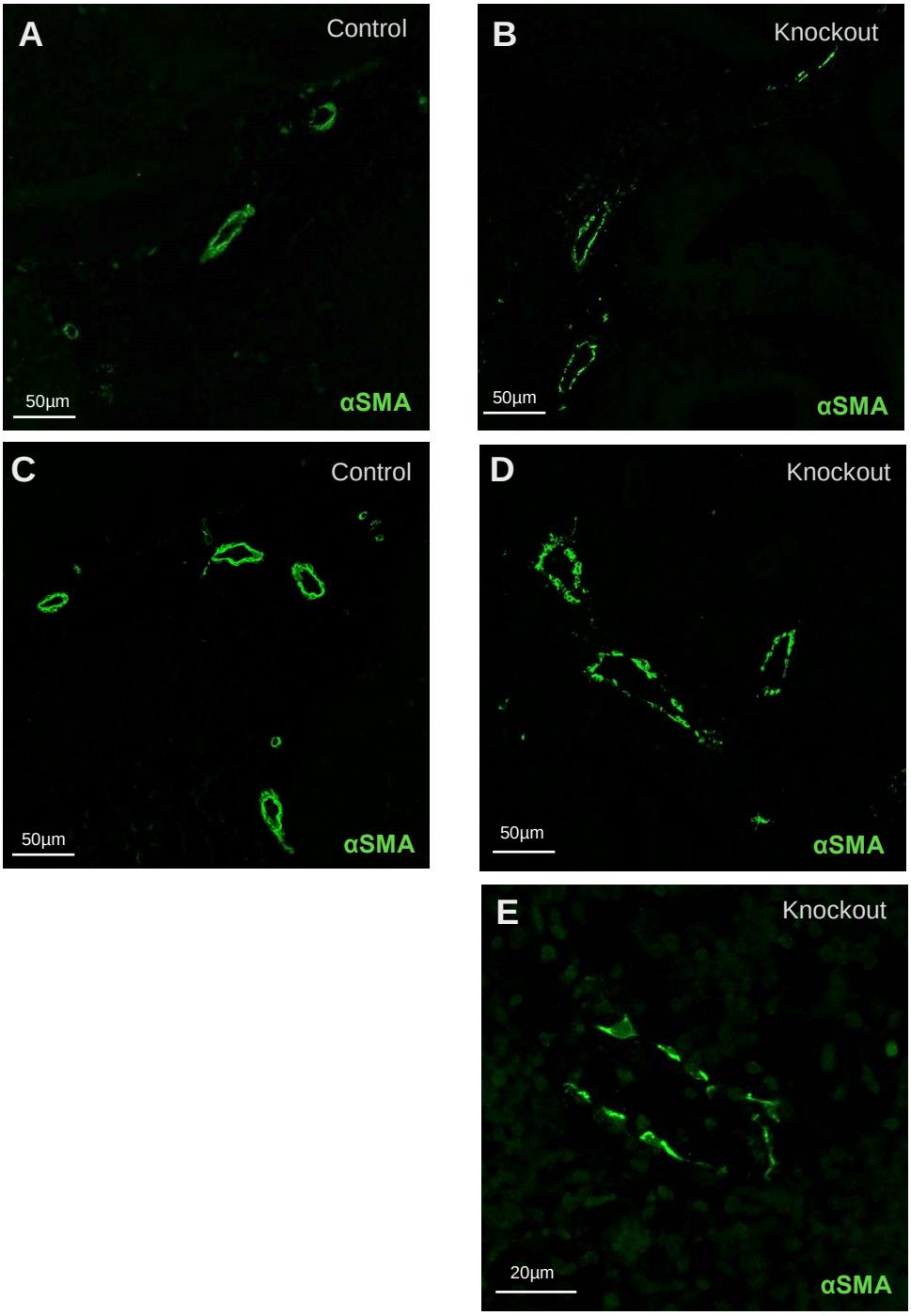


Figure 4.16: Vascular smooth muscle coverage is reduced after knockout of Tbx2

Antibody staining for αSMA on 5µm paraffin sections of E11.5 Rosa$^{CreERT2/+}$Tbx2$^{fl/fl}$ (left panel) and Tbx2$^{fl/fl}$ controls (right panel). Cre-mediated excision of floxed Tbx2 allele was induced by intraperitoneal injection of tamoxifen at E7.5 and E8.5. Controls do not carry the Cre-ERT2 transgene.
Expression of αSMA is seen in the cerebral arteries of both induced knockouts (B, D, E) and controls (A, C). However, the smooth muscle layer appears patchy in Tbx2 knockouts.

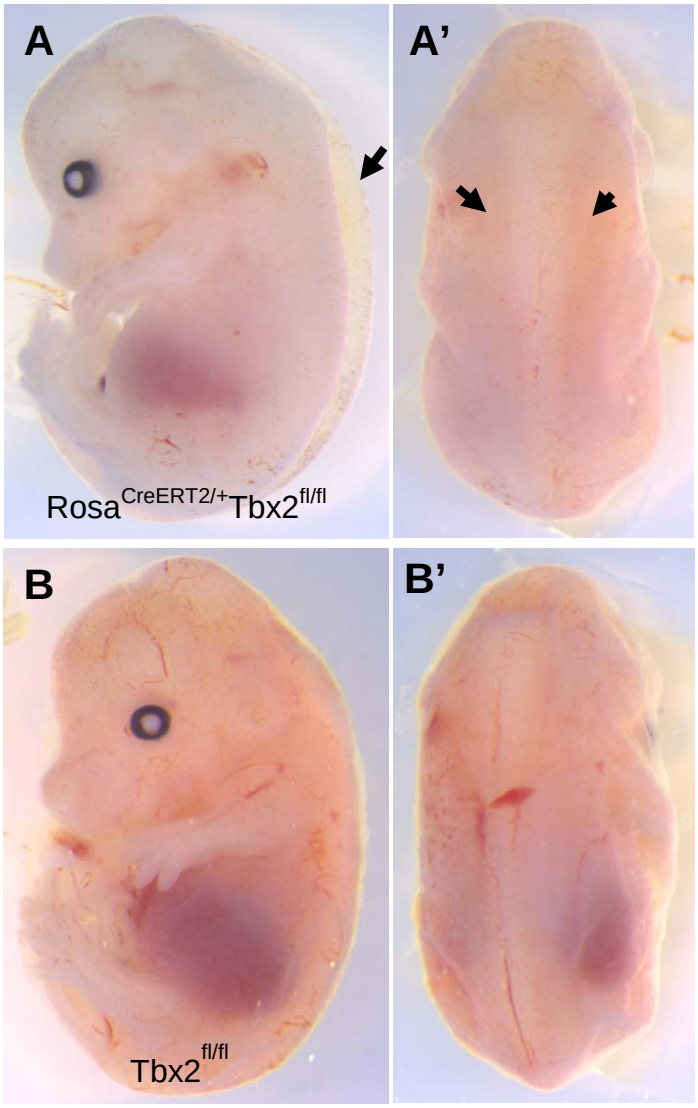


Figure 4.17: Induced deletion of Tbx2 leads to incomplete closure of the embryonic neural tube

E13.5 $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ (A) and Cre-$Tbx2^{fl/fl}$ control embryos (B). Cre-mediated excision of floxed Tbx2 allele was induced by intraperitoneal injection of tamoxifen at E9.5 and E10.5. Controls do not carry the Cre-ERT2 transgene. Deletion of Tbx2 leads to incomplete closure of the neural tube (black arrows). Tbx2 knockouts further appear pale compared to controls.

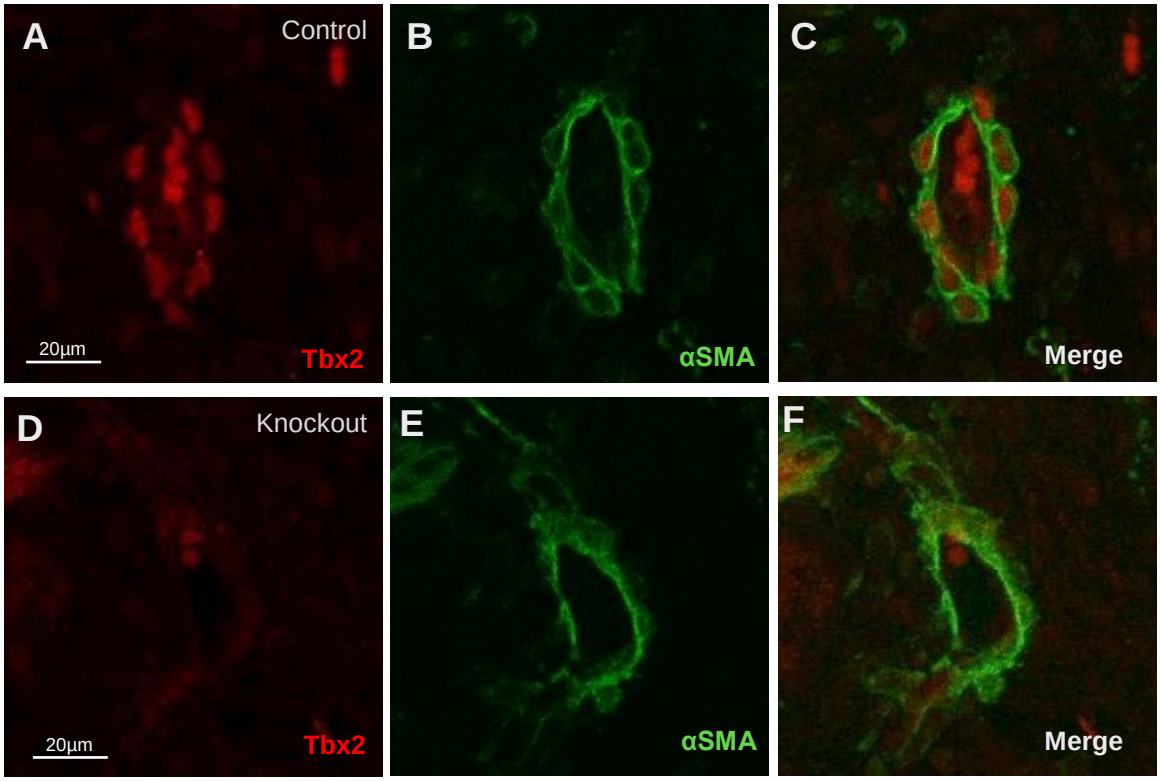


Figure 4.18: Tbx2 is absent after tamoxifen-induced Cre expression

Double antibody staining for Tbx2 and αSMA on 5µm paraffin sections of E13.5 $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ (D, E, F) and $Tbx2^{flox/flox}$ controls (A, B, C). Cre-mediated excision of floxed Tbx2 allele was induced by intraperitoneal injection of tamoxifen at E9.5 and E10.5. Controls do not carry the Cre-ERT2 transgene.
Tbx2 staining in mural cell nuclei of the vertebral artery is absent after Cre induction in $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ embryos but present in controls. Expression of αSMA is observed in both knockouts and controls.

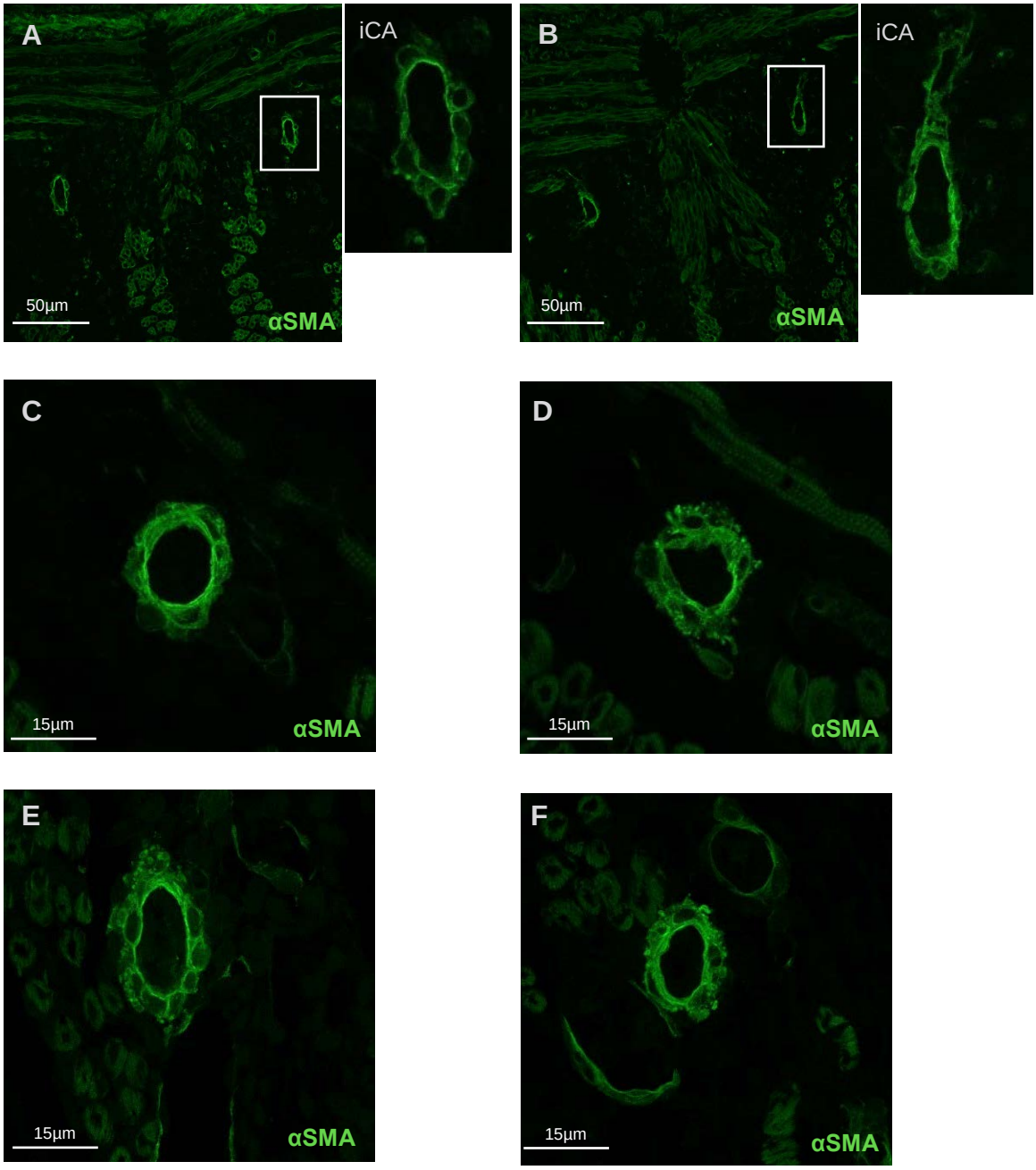


Figure 4.19: αSMA-expressing mural cells appear fewer and irregular in Tbx2 knockouts

Antibody staining for αSMA on 5µm paraffin sections of E13.5 $Rosa^{CreERT2/+}Tbx2^{fl/fl}$ (B, D, F) and $Tbx2^{fl/fl}$ controls (A, C, E). Cre-mediated excision of floxed Tbx2 allele was induced by intraperitoneal injection of tamoxifen at E9.5 and E10.5. Controls do not carry the Cre-ERT2 transgene.
Expression of αSMA is observed in both knockouts and controls, however mural cells in the knockout appear fewer (compare E and F and enlargements in A and B) and irregularly shaped in some cases (especially D).

4.2.10 Knock-down of Tbx2 expression in pericytes *in vitro*

Genetic deletion of Tbx2 in mice indicated that the loss of Tbx2 may affect mural cell behaviour. As a transcription factor, Tbx2 may therefore play a role in the regulation of gene expression in mural cells. To examine whether Tbx2 is important for the expression of a panel of genes with known roles in pericyte biology, we performed shRNA knockdown of Tbx2 in cultured human brain vascular pericytes (HBVP). These cells were recommended as a suitable model for in vitro studies of pericyte biology (C. Betsholtz, personal communication; Franco et al 2011). In a first step, antibody staining for Tbx2 was performed to ensure that these cells express detectable levels of Tbx2 (Fig. 4.20). Indeed, robust nuclear expression of Tbx2 was observed in cultured pericytes suggesting that they are an appropriate model to study the effects of Tbx2 knockdown *in vitro*. For shRNA knockdown, cells were infected with lentiviral particles (made by L. Nikitenko) to mediate insertion of a DNA cassette containing a shRNA template for targeting of Tbx2 mRNA along with a GFP reporter and a puromycin resistance gene into the host genome. Following puromycin selection, RNA was isolated and used to generate cDNA as a template for qRT-PCR, which was performed for Tbx2 to validate efficient knockdown in a total of six different shRNA (see Materials and Methods for details of shRNA clones). The two shRNA clones which produced the most robust knockdown of Tbx2 were used for further qRT-PCR analyses of the expression of the pericyte markers NG2 and αSMA, the TGF beta receptors Alk1 and Alk5, which have been implicated in pericyte differentiation, Pdgfrβ, which is important for pericyte recruitment, as well as Notch3, which has a role in pericyte differentiation and maturation.

shRNA knockdown reduced expression of Tbx2 mRNA to approximately 50% - 20% of the level observed in the non-silencing control (Fig. 4.21). Expression levels

of pericyte markers NG2 and αSMA were not convincingly altered after knockdown of Tbx2. This is consistent with observations in mice, where both markers were still expressed after ablation of Tbx2. Equally, an effect on the expression of the Tgfβ receptors Alk1 and Alk5 was not observed following knockdown of Tbx2 *in vitro,* suggesting that Tgfβ signalling is not affected by Tbx2. Levels of Pdgfrβ were consistently reduced to about 80% - 50% of the expression seen in the non-silencing control after knockdown of Tbx2. Likewise, expression levels of Notch3 were lowered to approximately 50% - 15% of expression observed in controls. Although the low number of only two biological replicates is not sufficient for meaningful statistical analysis, the results indicate that both Pdgfrβ and Notch3 are down-regulated after knockdown of Tbx2 *in vitro* which suggests that they may potentially be subject to regulation by Tbx2 in pericytes. As Pdgfrβ signalling is essential for mural cell recruitment and survival, and Notch3 has a role in mural cell differentiation and proliferation, regulation of either gene by Tbx2 may be important for the observed mural cell phenotype in Tbx2 knockout animals, which suggest an effect of Tbx2 deletion on mural cell recruitment, proliferation, survival, and/or differentiation.

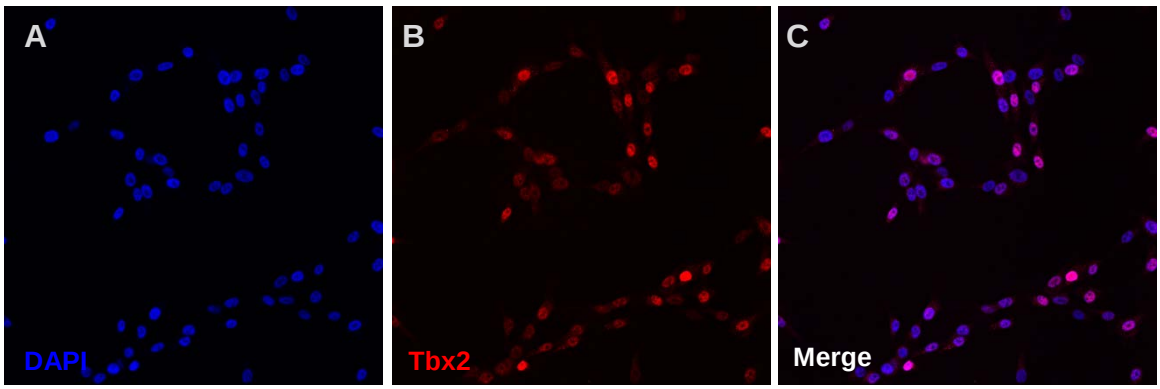


Figure 4.20: Tbx2 is expressed in human brain vascular pericytes

Antibody staining for Tbx2 (B) on cultured, PFA-fixed human brain vascular pericytes (HBVP). Staining is clearly visible in the nuclei of HBVPs, although with varying intensity (C). Nuclei are counter stained with DAPI (A).

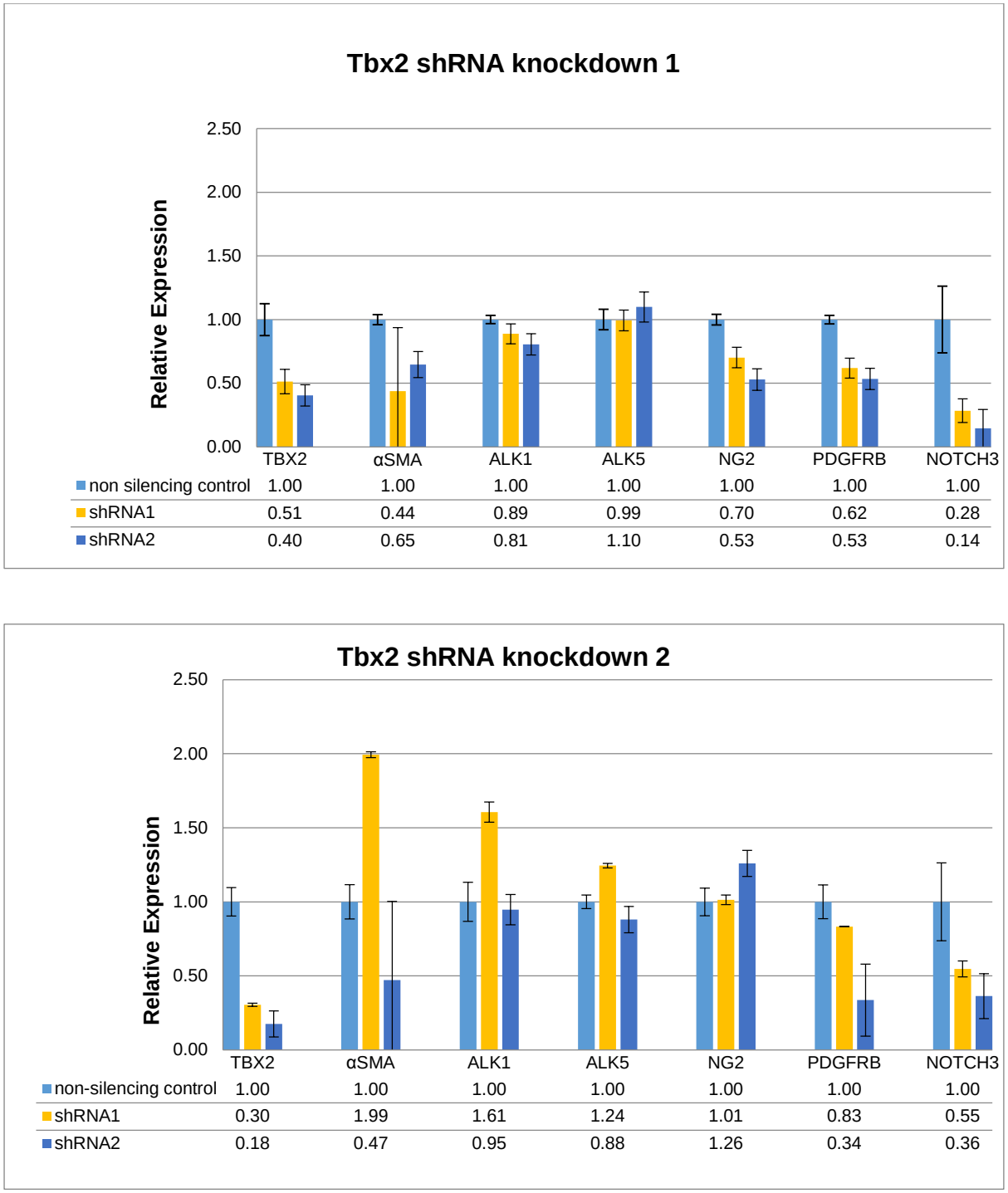


Figure 4.21: shRNA knockdown of Tbx2 in two independent experiments

2 different shRNAs were used to target Tbx2 *mRNA* in human brain vascular pericytes. Cells were selected with 4µg/ml puromycin over 3 days and grown to 80% confluency after which total RNA was isolated and used for q-RT-PCR against Tbx2, αSMA, Alk1, Alk5, NG2, Pdgfrβ, and Notch3. For each biological replicate, qPCR was performed in duplicates. Results were normalized against the Beta-actin housekeeping gene and expression relative to the non-silencing control was calculated using the comparative C_T Method ($\Delta\Delta C_T$ Method). Error bars = 1 standard deviation

4.2.11 Search for vascular-specific cis-regulatory enhancers in the Tbx2 locus

This study has identified Tbx2 as a gene expressed in mural cells of arteries during murine embryonic development. As very little is known about the transcriptional regulation of mural cell-specific genes, examination of the cis-regulatory elements driving Tbx2 expression could potentially identify upstream transcriptional regulators of mural cell fate. It has been reported previously that transgenic mice carrying the murine Tbx2 promoter together with a 6kb upstream fragment in front of a *LacZ* reporter drive expression of β-Gal analogously to the expression pattern of Tbx2 at E9.5 (Kokubo et al 2007). Since vascular Tbx2 expression is only detected after E9.5, and reporter gene expression at later stages of development was not assessed in this previous study, the role of this characterized enhancer in the regulation of Tbx2 expression in the vasculature was not clear. To investigate whether vascular Tbx2 expression is also regulated by this 5' enhancer region, transgenic mice were generated using the vector Tbx2-D3-*LacZ* (gift of H. Kokubo), which contained the 6kb 5' enhancer region cloned into a *LacZ* expression cassette (Kokubo et al 2007). Transgenic embryos were harvested at E11.5 and E13.5 and stained with X-Gal to visualize the activity of the enhancer through expression of the *LacZ* reporter gene. Vascular X-Gal staining was not detected at either E11.5 or E13.5 (Fig. 4.22). Expression of the *LacZ* reporter was, however, detected in the eyes, the pharyngeal mesoderm and parts of the brain as previously described (Kokubo et al 2007, Mesbah et al 2012, Pontecorvi et al 2008). This suggests that the extended promoter region alone is not sufficient to drive expression of Tbx2 in mural cells and that other, distal cis-regulatory regions outside the 6kb upstream region are involved in the regulation of Tbx2 transcription.

The FANTOM5 transcribed enhancer atlas was interrogated to identify cis-regulatory regions associated with Tbx2 expression. The enhancer atlas contains information about ~40,000 enhancers, detected by bidirectional cap analysis of gene expression (CAGE) using the FANTOM5 CAGE expression atlas which encompasses 135 primary cell and 432 tissue samples from human (Andersson et al 2014). The enhancer atlas contains three potential enhancers associated with Tbx2, all of which are located in regions of increased vertebrate sequence conservation suggesting evolutionary constraint (Fig. 4.23). One, located about 2.5 kb upstream of the promoter, falls within the extended promoter region that was already tested in transgenic mice. Interestingly, this enhancer shows significant overrepresentation in both cultured human pericytes and blood vessel tissue in the FANTOM5 transcribed enhancer atlas even though analysis of transgenic mice did not reveal enhancer activity in these tissues. However, transgenic mice were generated using the 6kb extended promoter region of the mouse sequence, whereas the FANTOM5 transcribed enhancer atlas uses human samples. Therefore, despite apparent conservation on the sequence level, the ability of this upstream enhancer to drive expression in mural cells may not be conserved in mice. Two further enhancers predicted by the FANTOM5 transcribed enhancer atlas are located over 70kb upstream in the 22nd intron of the neighbouring gene Bcas3. However, neither of these enhancers showed significant overrepresentation in human pericytes or blood vessel tissue, suggesting that they are not involved in the regulation of Tbx2 expression in these tissues.

The Tbx2 locus was further analysed with the help of ENCODE data providing information about DNase1 hypersensitivity (DNase1 HS) in cultured human brain vascular pericytes and human brain vascular smooth muscle cells (Consortium

2011). DNase1 HS is associated with regions of open chromatin often found at non-coding regulatory regions. However, this analysis did not reveal sites of increased DNase1 HS at any of the three predicted enhancers or in other non-coding regions of the Tbx2 locus which suggests that the 2.5kb upstream enhancer is not in a region of open chromatin in the tested cell types (Fig. 4.23). Therefore, *in silico* analysis of the Tbx2 locus did not reveal any regions with potential to regulate expression of Tbx2 in mural cells.

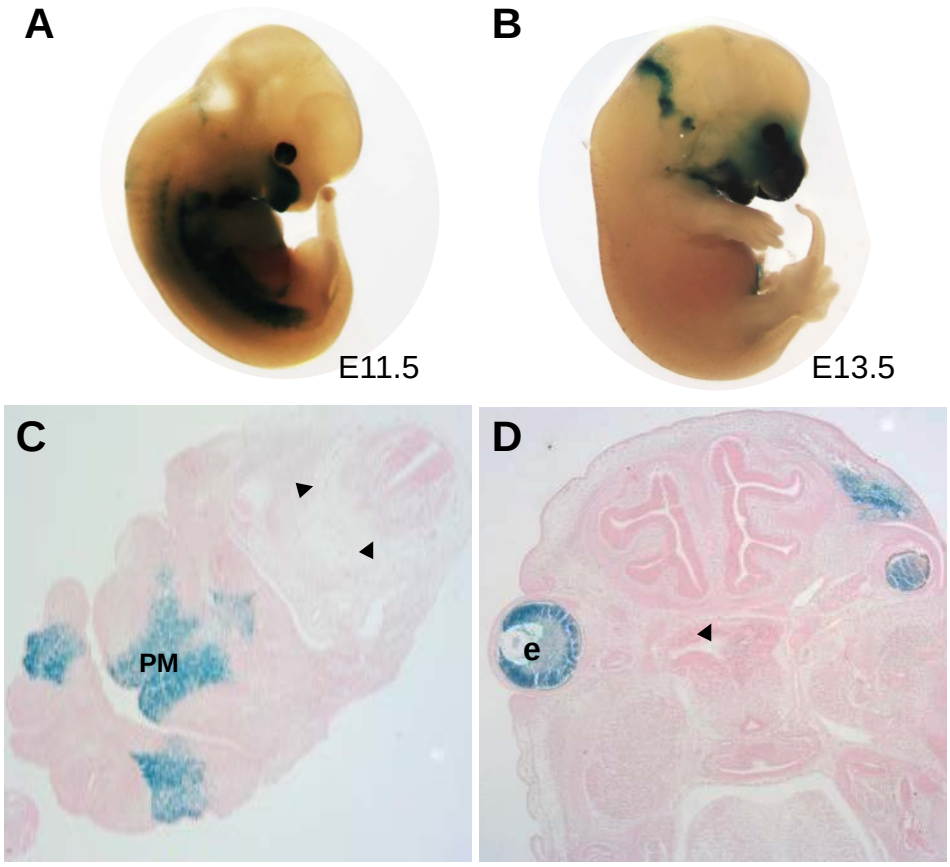


Figure 4.22: 6kb upstream region of Tbx2 does not drive vascular expression

A, b: X-Gal staining of E11.5 and E13.5 Tbx2-D3-*LacZ* transgenics. Tbx2-D3-*LacZ* contains a 6kb upstream region of the mouse sequence of the Tbx2 locus in front of a *LacZ* expression cassette.
C, D: 5µm section paraffin sections. Reporter gene expression is can be detected in eyes (e) and pharyngeal mesoderm (PM) but not in the vasculature. Black arrows indicate cerebral arteries where Tbx2 expression is reliably detectable with ISH and antibody staining.

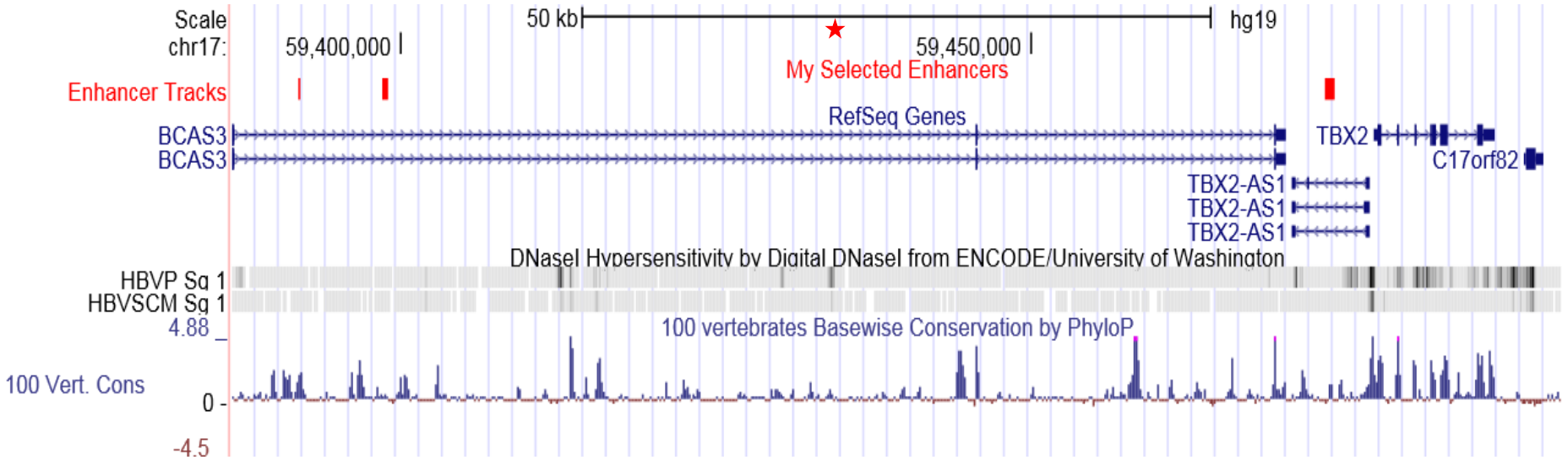


Figure 4.23: A potential enhancer regulating Tbx2 expression in mural cells is located ~ 2.5kb upstream of the Tbx2 TSS

The FANTOM enhancer atlas predicts three enhancers regulating Tbx2 expression (red track). The 2.5kb upstream enhancer (red star) is significantly overrepresented in human pericytes and blood vessel tissue.
DNase1 hypersensitivity analysis (greyscale track) in human brain vascular pericytes (HBVP) and human brain vascular smooth muscle cells (HBVSMC) did not detect regions of open chromatin for any of the three enhancers.
All three enhancers are located in regions of peak vertebrate conservation (lower track).

4.2.12 Identification of downstream targets of Tbx2

In vitro experiments indicate that Notch3 and Pdgfrβ *mRNA* levels are decreased upon shRNA knockdown of Tbx2. This raises the possibility that these genes are subject to direct transcriptional regulation by Tbx2. Therefore, the loci of *Notch3* and *Pdgfrβ* were analysed for regulatory regions containing Tbx2 consensus binding motifs (5'-G(G/T)GT(G/T)A-3').

4.2.12.1The Notch3 locus

The FANTOM5 transcribed enhancer atlas contains three enhancers associated with Notch3 expression, all of which are located in regions of open chromatin in human brain vascular pericytes and human brain vascular smooth muscle cells according to ENCODE DNase1 HS data (Fig. 4.21). One enhancer lies approximately 9kb upstream of the *Notch3* TSS and two further, adjacent enhancers are located approximately 20kb upstream of the TSS. The two far-upstream enhancers were both overrepresented in human pericytes and vascular smooth muscle cells according to the FANTOM5 enhancer atlas. However, inspection of the sequence of both enhancers did not detect high affinity binding sites for Tbx2 at either of the two elements. Therefore, sequence analysis of the two 20kb upstream enhancers does not suggest they are direct targets of Tbx2.

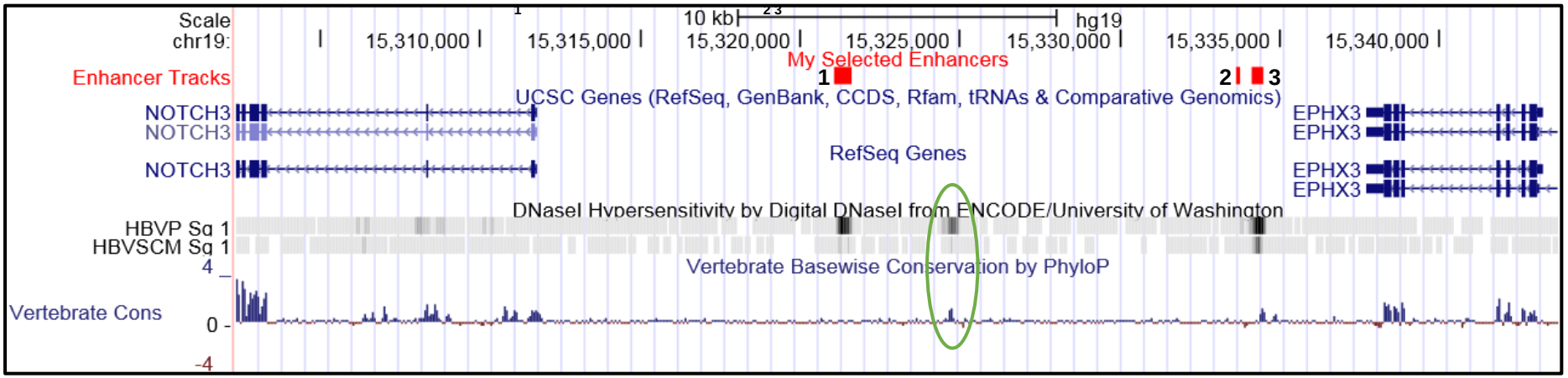
The 9kb upstream enhancer was not overrepresented in either mural cells or blood vessel tissue in the FANTOM5 enhancer atlas. However, the enhancer is located in a region of open chromatin in cultured mural cells, suggesting that it may be an active regulatory region in this cell type (Fig. 4.21). Additionally, analysis of the

sequence revealed the presence of two high affinity Tbx2 binding sites. Therefore, this enhancer has the potential to be directly regulated by Tbx2 in mural cells.

Another region of open chromatin about 11kb upstream of the Notch3 gene was not suggested as an enhancer by the FANTOM5 enhancer atlas. Interestingly, this region also showed a peak in vertebrate conservation suggesting that it may function as a regulatory element. Sequence analysis, however, did not reveal Tbx2 binding motifs in this region.

An inspection of the Notch3 promoter proximal regions with detectable vertebrate conservation did not identify any high affinity binding sites for Tbx2 suggesting that the Notch3 promoter is not a direct target of Tbx2.

A



B

CACTGCAGCCTGGCCAACAGAGTGACCTTATTAAAAAAAAAAAAAAGGAAGAAAAAGAAAAAAAATGAACCTGGGTCTCAGTG
ACTCCCGGCCACATGGAGTACAGGGGCGGGGTAGGGGTTGGGCAGAGGAATAACAGAAGTTGCCTAAGAAGGGGCACACACTG
ACTCACGGTGACTGTGCTGAGTCAAGAGGCCCATGGGGACAGGCACAGATCTAGTCCTGCGTCATGCCTACCACCCAGGAAGG
AAATCCAAGGCCCTCAGTCCTGCATTTGGGCCCCTTGGGCCGGGACTGACCCTCTCCTTCCTAACCCTCCCCACCAAACCCCC
AGCAAAAACAACAAACAAAAAACCATTGTCACACCATGTCAAACCCCAGATGCAACCAGCAGGCCTTGTGCTCCCATGGTCTT
TGCCTGGGTGCTTCCCTCTGCCTGAGACACCCAGCTGCACAGAAACACCCTTTTGCTTCCTCCCCAAGCAGGAACTCACAGCC
AGTGTGACCTCTTCAAAAATTCATTTTCTTCCAGCTAGAGGGA

Figure 4.24: A potential enhancer ~ 9kb upstream of the Notch3 gene contains binding sites for Tbx2

A: The FANTOM enhancer atlas predicts three enhancers (1,2,3) regulating Notch3 expression (red track). Two enhancers (2,3) ~ 20kb upstream of Notch3 are significantly overrepresented in human pericytes and blood vessel tissue but contain no TBX2 binding motifs.
A 9kb upstream enhancer (1) is in a region of open chromatin as predicted by DNase1 hypersensitivity analysis (greyscale track) in human brain vascular pericytes (HBVP) and contains binding sites for Tbx2.
Another region of open chromatin is evolutionary conserved (green oval) but does not contain binding sites for Tbx2.
B: 427bp sequence of the 9kb upstream enhancer containing two TBX2 binding motifs (5'-G-(G/T)-G-T-(G/T)-A-3') marked in red.

2.12.2The *Pdgfrβ* locus

For the *Pdgfrβ* locus, the FANTOM5 enhancer atlas lists five enhancers, two in the first intron and one in the 14th intron of *Pdgfrβ* as well as two further enhancers in the fourth and ninth intron of the neighbouring gene CSF1R (Fig. 4.22, A). One enhancer in the first intron of *Pdgfrβ* was overrepresented in human pericytes, vascular smooth muscle cells and blood vessel tissue, suggesting this region may be involved in the regulation of Pdgfrβ expression in these cell types. However, neither this, nor any of the other predicted enhancers contained any high affinity binding sites for Tbx2 in their sequence, suggesting that they are not direct Tbx2 targets. Further, sequence analysis of the proximal promoter also did not detect any Tbx2 binding sites.

Inspection of ENCODE data for DNase1 HS in cultured human brain vascular pericytes and human brain vascular smooth muscle cells revealed five separate regions of open chromatin within the fifth intron of the *Pdgfrβ* gene (Fig. 4.22, B). All of these regions also showed some degree of vertebrate conservation suggesting evolutionary constraint. One of these regions, located approximately 10kb downstream of the TSS contained three Tbx2 binding motifs and another region about 17kb downstream of the TSS contained a single Tbx2 binding site. In the latter regions, however, the DNase1 HS signal was weak compared to the other regions.

Collectively, analysis of the *Notch3* and *Pdgfrβ* loci using the FANTOM5 enhancer atlas, ENCODE DNase1 HS data, and evolutionary conservation revealed ten potential mural cell-specific enhancers, four for Notch3 and six for of Pdgfrβ. Out of these, one Notch3 enhancer and two of Pdgfrβ enhancers contained potential

Tbx2 binding sites, making them interesting candidates for further analysis in transgenic enhancer-reporter assays.

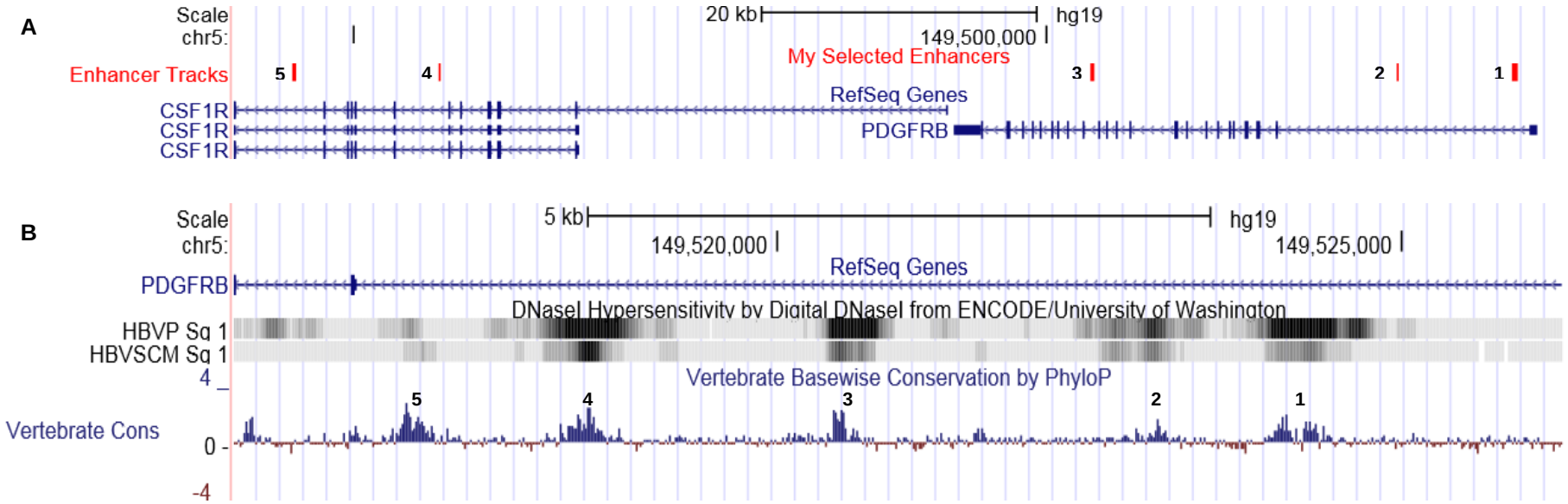


Figure 4.25: Two conserved regions of open chromatin in the first intron of *Pdgfrβ* contain biding sites for Tbx2

A: The FANTOM enhancer atlas predicts five enhancers (1,2,3,4,5) regulating Pdgfrβ expression (red track). One enhancer (1) in the first intron of *Pdgfrβ* is significantly overrepresented in human pericytes, vascular smooth muscle and blood vessel tissue but does not contain Tbx2 binding motifs. The remaining enhancers also contain no Tbx2 motifs.
B: Five regions (1,2,3,4,5) of open chromatin as predicted by DNase1 hypersensitivity analysis (greyscale track) in human brain vascular pericytes (HBVP) and human brain vascular smooth muscle cells (HBVSMC) in the first intron of *Pdgfrβ* show peaks of vertebrate conservation.
3 Tbx2 binding motifs are found in region 1 and 1 Tbx2 binding site is present in region 5.

4.3 Discussion

4.3.1 Tbx2 expression in mural cells is restricted to particular sub-populations

This study identifies the T-box protein Tbx2 as a transcription factor expressed in a sub-population of mural cells in peripheral arteries and microvessels during embryonic development in mice. This was shown by both *in situ* hybridisation for Tbx2 *mRNA* and immunohistochemistry for Tbx2 protein. Expression of Tbx2 was not observed in venous mural cells during any of the developmental stages examined. This is not the first report of a factor specifically expressed in arterial mural cells. Both NG2 and the regulator of G-protein signaling 5 (Rgs5), for example, have been reported to be specifically localized to arterial, but not venous, pericytes and vascular smooth muscle cells (Cho et al 2003, Li et al 2004, Murfee et al 2005). While this strongly suggests that, just like endothelial cells, mural cells are phenotypically different in the arterial and venous branches of the circulation, the question of how mural cell identity is established and maintained has not been rigorously addressed so far. Consequently, very little is known about the transcriptional regulation of mural cell fate. Deletion of Notch3 in mice has been shown to produce mainly arterial defects and result in vein-like arterial walls, suggesting that Notch3 is involved in the regulation of arterial mural cell fate (Domenga et al 2004). However, a different study reported expression of Notch3 also in venous mural cells, thus questioning the idea that Notch3 alone can establish arterial mural cell identity (Jin et al 2008). As a transcription factor known to be

expressed in arterial but not venous mural cells, Tbx2 is therefore an interesting candidate for a transcriptional regulator of arterial mural cell identity.

Although found in most arterial-associated mural cells, expression of Tbx2 was not detected in the wall of the aorta during any stage of development. While this work does not provide evidence to explain this observation, it is not the first report of differentially expressed genes among mural cells in different parts of the vasculature. Arteriolar mural cells, for example, do not always express smooth muscle myosin heavy chains and a careful study of the developing vasculature of the rat retina revealed differential expression of mural cell markers throughout vascular development in different vessel types (Hughes and Chan-Ling 2004, Nakamura et al 1999, Price et al 1994). Mural cells are also heterogeneous in their ontogeny with progenitors from as many as eight different origins described in the literature (Majesky et al 2011). The mural cells of the aorta alone have at least four independent origins in the secondary heart field, the neural crest, the somites, and the splanchnic mesoderm (Armulik et al 2011). Little is known about how progenitor cells from such different developmental origins become specified for a common cellular fate. However, discrete mural cell origins may present one obvious explanation for the absence of Tbx2 in the dorsal aorta. The mural cells associated with the aorta may belong to a distinct population with transcriptional networks that do not rely on Tbx2. For example, a different member of the T-Box family might be expressed in mural cells of the dorsal aorta, as shown recently for Tbx18 in mural cells of the kidney vasculature (see below) (Xu et al 2014). Alternatively, Tbx2 might fulfil a unique function in mural cells of peripheral arteries and microvessels and, therefore, its expression may be the result of a specific requirement in mural cells of peripheral arteries and mircrovessels that is not present in those of the dorsal aorta.

These two alternatives are also not mutually exclusive. While the dorsal aorta is classically considered part of the arterial circulation it differs considerably from peripheral vessels in terms of formation (vasculogenesis vs. angiogenesis), function (e.g. hemogenic endothelium), and environment (blood pressure and shear stress) (Sato 2012). Therefore, the aorta can be viewed as a separate organ within the vasculature which, perhaps, makes it less irreconcilable that arterial and aortic mural cells are molecularly distinct.

4.3.2 What is the vascular phenotype of Tbx2 deletion?

Histological observations from both the constitutive and inducible deletion of Tbx2 in mice reveal abnormalities in vascular remodelling and arterial wall formation. Mural cells in Tbx2 knockout mice still expressed the common pericyte and vascular smooth muscle cell markers NG2 and α-smooth muscle actin, demonstrating that cell fate specification of mural cells is not dependent on Tbx2. However, the smooth muscle layer in arteries of knockout mice appears discontinuous and the vasculature seems disorganized and, in parts, dilated. This might suggest that mural cell coverage is reduced after Tbx2 ablation in mice. Absence of mural cells during vascular development has been shown to result in abnormal vascular remodelling with endothelial hyperplasia, microaneurysms, vessel dilation, and oedema, all of which are reminiscent of the vascular phenotype observed in Tbx2 null embryos (Bergers and Song 2005, Hellström et al 1999, 2001, Lindahl et al 1997, Uemura et al 2002). Reduced mural cell coverage may be the result of impaired mural cell recruitment or reduced proliferation or survival. A recent study by Xu et al. has identified Tbx18 as a transcription factor expressed in vascular smooth muscle cells

of the developing kidney (Xu et al 2014). Similar to the observations in Tbx2 knockout mice, the investigators reported vascular abnormalities and reduced mural cell coverage in Tbx18$^{-/-}$ mouse embryos. Xu et al. demonstrated increased cell death during vascular smooth muscle cell differentiation and suggest a role for Tbx18 in the regulation of cell survival (Xu et al 2014). Therefore, Tbx2 may fulfil a similar function in mural cells of peripheral arteries. Indeed, Tbx2 has previously been identified as a survival factor in certain cancers (Ismail and Bateman 2009, Wansleben et al 2013). Further, roles have been described for Tbx2 in the regulation of cellular proliferation *in vitro* as well as in the regulation of cell migration during development of the neural plate in zebrafish (Fong et al 2005, Jarod Li et al 2014, Peres et al 2010). However, the exact effects of a loss of Tbx2 on mural cell development have to be studied in more detail, ideally using mural cell specific deletion of Tbx2 in mice.

4.3.4 Does Tbx2 interact with known regulators of mural cell behaviour?

The conditional, double homozygous deletion of the transcription factors Msx1 and Msx2, using Sm22α-Cre (driving Cre expression in mural cell precursors) causes decreased mural cell coverage in peripheral arteries and defects in vascular maturation in a manner similar to the Tbx2-ablated mice (Lopes et al 2011, Zhang et al 2006). Interestingly, Msx1/2 show a similar expression pattern in mural cells as Tbx2, with Msx2 preferentially expressed in mural cells of peripheral arteries, and Msx1 expressed in peripheral arteries, arterioles and capillaries (Goupille et al 2008). The mural cell-specific loss of Msx1/2 leads to reduced levels of Bmp and

subsequent downregulation of the metalloprotease genes Mmp2 and Mmp9, which are essential for cell migration and integration into the mural layer (Kenagy et al 1997, Newby 2006). Interestingly, Tbx2 has previously been shown to directly interact with both Msx1 and Msx2 *in vitro (Boogerd et al 2008)*. Moreover, Tbx2 has been shown to interact with Msx1 during tooth development, where the two transcription factors antagonistically regulate expression of Bmp4, which is repressed by Tbx2 and induced by Msx1 (Saadi et al 2013). This is in line with observations in vascular smooth muscle cells where the combined deletion of Msx1/2 leads to a dramatic reduction of Bmp4 expression (Lopes et al 2011). As Tbx2 is expressed in the vasculature in a similar pattern as Msx1/2, it seems possible that an interaction of these factors similar to the regulation of Bmp4 expression in the dental mesenchyme may also exists in mural cells. Since Tbx2 acts as a repressor of Bmp4 expression, knockout of Tbx2 may also result in deregulation of Mmp2 and Mmp9 expression (Mason et al 1999). While both Msx1/2 and Tbx2 have been shown to interact in the regulation of Bmp4 expression, it has also been reported that, conversely, epithelial Bmp4 induces mesenchymal Msx1/2 as well as Tbx2 expression (Bei 2009, Saadi et al 2013). This, therefore, may place Tbx2 downstream of Bmp signalling in mural cell development.

Tbx2 and Msx1/2 have further been shown to interact in the suppression of the expression of the gap-junction protein Connexin43 in myocardial cells (Boogerd et al 2008). Loss of Connexin43 gap-junctions lead to impaired endothelial-mural cell communication with inhibition of mural cell differentiation (Hirschi et al 2003). In Tbx2 null mice, ectopic expression of Connexin43 was observed in the myocardium (Aanhaanen et al 2011). Deregulation of Connexin43 expression as a consequence of a loss of Tbx2 may therefore result in dysfunctional endothelial-mural cell

communication. Endothelial-pericyte connections involving Connexin43 are also observed in the blood brain barrier (Winkler et al 2011). As Tbx2 is expressed in microvessels of the developing brain, it may have a role in the establishment of the blood brain barrier or in the regulation of its permeability. However, the effects of Connexin43 overexpression on endothelial-mural cell gap-junction formation have not been assessed.

It has been suggested that Tbx2, together with Tbx5 and the MYST family histone acetyltransferase TIP60, regulates the expression of serum response factor (SFR), a member of the MADS box superfamily of transcription factors, through binding of regulatory element in the SRF 3'-UTR (Barron et al 2005). Though not a focus of their research, the investigators demonstrate that this element drives vascular gene expression when used in a transgenic enhancer-reporter assay in transgenic mice (Barron et al 2005). SRF is widely expressed in the developing mouse embryo and is involved in the regulation of muscle-specific gene expression in cardiac, skeletal and smooth muscle cells (Miano 2003). SRF is important for the differentiation of vascular smooth muscle cells into contractile cells and smooth muscle-specific deletion of SRF in adult mice resulted in decreased vascular tone and vessel contractility (Galmiche et al 2013, Retailleau et al 2013). In cultured vascular smooth muscle cells, depletion of SRF resulted in increased migration and proliferation (Kaplan-Albuquerque et al 2005). Therefore, Tbx2 dependent activation of SRF expression may regulate the transition from a mobile progenitor state to a stationary, contractile state in vascular mural cells which would be in line with the previously mentioned potential regulatory role in the expression of metalloproteinases.

4.3.5 What is the relationship between Notch3, **Pdgfrβ**, and Tbx?

The results show that knockdown of Tbx2 in cultured pericytes causes a reduction in the expression of the membrane receptor proteins Notch3 and Pdgfrβ. Notch3 deletion in mice causes arterial defects reminiscent of those observed in Tbx2 mutant mice (Domenga et al 2004). While common mural cell markers are present, the vascular smooth muscle layer was thin and discontinuous and mural cells were irregularly shaped in *Notch3*$^{-/-}$ mice (Domenga et al 2004). However, these effects were only apparent after birth which may suggest that Notch3 has a more important role during later stages of development, and that the defects seen in Tbx2-ablated mice may not be entirely accounted for by downstream effects on Notch3.

Notch3 has been shown to directly activate Pdgfrβ expression in vascular smooth muscle cells *in vivo* and Pdgfrβ expression was reduced in arterial smooth muscle cells of *Notch3*$^{-/-}$ mice (Jin et al 2008). Therefore, the observed reduction of Pdgfrβ expression after knockdown of Tbx2 may be secondary to the low levels of Notch3. Interestingly, Notch3 and Pdgfrβ seem to have opposing roles during mural cell migration. While Pdgfrβ is essential for mural cell recruitment *in vivo* and increases migration of vascular smooth muscle cells *in vitro*, Notch3 activation had a negative effect on migration despite elevated levels of Pdgfrβ, suggesting that mural cell migration is subject to complex regulation (Jin et al 2008). This is further supported by the fact that, in cultured vascular smooth muscle cells, treatment with Pdgf lead to a 5-fold decrease in Notch3 *mRNA* which indicates that Notch3 expression is also regulated by Pdgfrβ signalling (Campos et al 2002).

Tbx2 has been shown to be robustly down-regulated in distal arteries of Notch3 null mice (Fouillade et al 2013). In contrast to our observation that Tbx2 is not

expressed in adult mice, downregulation of Tbx2 in Notch3$^{-/-}$ mice was shown for 1-month old animals. However, the sensitivity of the DNA-based methods used in this transcriptome analysis likely exceeds that of ISH. Consequently, Tbx2 expression may persist in the mural cells of older animals at lower levels. Notably, when the investigators repeated their analysis in mice with a smooth muscle-specific, SMMHC-CreERT2-driven deletion of the Notch downstream effector Rbpj, a significant downregulation of Tbx2 could no longer be observed, while most other genes were equally reduced in both models. This might suggest that Tbx2 is regulated by Rbpj-independent, non-canonical Notch signalling (Andersen et al 2012). Alternatively, this result could suggest that Tbx2 is mainly expressed in pericytes and smooth muscle cell precursors which do not express SMMHC. Nonetheless, our results may suggest a role for Tbx2 in the regulation of a Notch3/Pdgfrβ signalling loop as well as an interdependency of Tbx2 and Notch3 expression in arterial mural cells. However, a direct effect of Tbx2 on the expression of either factor has not been demonstrated. Perhaps the fact that Tbx2 has mostly been shown to act as a repressor of target genes suggests that Notch3 is unlikely to be a direct target as its expression is decreased as a result of Tbx2 knockdown. On the other hand, Tbx2 has been shown to also act as an activator of transcription in certain contexts (Paxton et al 2002).

4.3.6 Does Tbx2 directly regulate expression of mural cell genes?

A potential *cis*-regulatory element approximately 9kb upstream of the Notch3 promoter is predicted by the FANTOM5 enhancer atlas and contains two high-affinity Tbx2 biding sites (Andersson et al 2014, Carreira et al 1998). This element was not

conserved among vertebrates but in cultured human brain vascular pericytes and human brain vascular smooth muscle cells this element was located in a region of open chromatin as detected by DNaseI HS. This suggests that the element is accessible to Tbx2 in mural cells. However, no currently available method for enhancer identification predicts regulatory elements with absolute certainty. And even the presence of a high affinity motif does not guarantee that it can be bound by Tbx2. Moreover, not all transcription factor binding events are necessarily functional. Therefore, to validate an effect of Tbx2 on Notch3 expression through binding sites in the 9kb upstream enhancer, the element has to be tested in an enhancer-reporter assay, and the contribution of the Tbx2 motifs to its activity should be assessed through targeted mutagenesis.

Pdgfrβ is another potential direct target of Tbx2 in mural cells. There are two regions of open chromatin in the fifth intron of the Pdgfrβ gene, approximately 10kb and 17kb downstream of the TSS that contain Tbx2 binding motifs. Both elements also show peaks of vertebrate conservation. While regulatory elements are not subject to the same evolutionary constraints as protein-coding sequences, it is still seen for the majority of enhancers identified to date. Although there may be some bias towards selecting only conserved regions to be tested for regulatory function in the first place, conservation is, without a doubt, a useful predictor of functional sequences even if its absence does not disprove regulatory activity (Nelson and Wardle 2013). However, sequence conservation of regulatory elements does not necessarily imply functional conservation (Shen et al 2012). Just as with the putative Notch3 enhancer, the two regions in the fifth intron of Pdgfrβ have to be examined in an enhancer-reporter model and Tbx2 binding has to be verified in a biological assay.

Other potential regulatory elements were also identified in the Notch3 and Pdgfrβ loci that do not contain Tbx2 binding motifs. As Tbx2 has been shown to interact with other transcription factors such as Msx1/2, MyoD and Nkx2.5, it is possible that Tbx2 is recruited to these elements even in the absence of suitable Tbx2 binding motifs as long as binding sites for the binding partner are present (Boogerd et al 2008, Habets et al 2002, Bo Zhu et al 2014).

4.3.7 How is Tbx2 expression regulated in mural cells?

There is evidence from other studies suggesting that Notch and Bmp signalling may regulate Tbx2 expression (Fouillade et al 2013, Saadi et al 2013). The FANTOM5 enhancer atlas predicts a regulatory element 2.5 kb upstream of the Tbx2 TSS that is overrepresented in pericytes. The same element has previously been shown to direct expression to the atrioventricular canal in a Bmp/Smad-dependent manner (Singh et al 2009). Further, activity of this enhancer region was repressed by binding of the Notch target gene Hey2 in the chamber myocardium (Stefanovic et al 2014). However, vascular reporter gene expression was not detected in any part of the vasculature in an enhancer-reporter assay in transgenic mice. Yet, for reasons unknown, construct lengths can sometimes affect regulatory activity in reporter assays such that larger constructs mediate weaker activation than shorter ones (Yin et al 2005). As the fragment tested in this study was fairly large (>6kb) compared to enhancer-reporter constructs which contain only core enhancer sequences (<1kb) activity may have been reduced in this context. On the other hand, predictions in the FANTOM5 enhancer atlas are based on CAGE data from human cells and tissues whereas the tested construct contained the murine sequence of the extended

promoter. As mentioned earlier, function of regulatory elements is not always conserved even if conservation is apparent on the sequence level. Therefore, while the human sequence may have enhancer activity in pericytes as suggested by the FANTOM5 atlas, the mouse sequence may not. Further inspection of the Tbx2 locus based on a combination of both evolutionary conservation and biochemical activity did not reveal other regions with obvious potential to regulate Tbx2 in mural cells. However, data on histone modifications, which is extremely useful for the prediction of regulatory elements, is not currently available for pericytes or vascular smooth muscle cells which complicates the identification of regions with regulatory activity in these cells. Therefore, other elements with regulatory activity in mural cells may not have been detectable with currently available data.

3.8 Should we study Tbx2 in mural cells?

Overall, identification of Tbx2 as a transcription factor in arterial mural cells opens up several promising lines of investigation, as it has the potential to regulate the expression of key players in mural cell biology such as SRF, Notch3, and Pdgfrβ. The ability of Tbx2 to form heterodimers with other transcription factors, to interact with different epigenetic modifiers, and to mediate both activation and repression make it an ideal candidate for the regulation of complex cell-state transitions such as the differentiation from a mural cell progenitor to a contractile vascular smooth muscle cell. Therefore, the role of Tbx2 in mural cells should be closely examined in future studies. Moreover, because of its arterial-specific expression pattern Tbx2 may be involved in the transcriptional regulation of arterial mural cell identity. As our understanding of establishment and maintenance of mural cell fate is very limited

and few other transcription factors have been described in mural cells, the role of Tbx2 in the regulation of an arterial-specific mural cell phenotype will be an interesting subject for further research.

4.4 Future studies

4.4.1 Conditional knockout of Tbx2 in mural cells

Early deletion of Tbx2 leads to severe developmental retardation including cardiac malfunction. This makes it difficult to establish whether the observed vascular abnormalities in $Tbx^{-/-}$ embryos are a direct result of Tbx2 ablation in mural cells. Without a doubt the most informative experiment to elucidate the role of Tbx2 in mural cell development involves the deletion of Tbx2 specifically in mural cells. There are several different approaches, all using the Cre-lox system, that have been utilized for mural cell-specific gene deletions in mice.

A number of Cre mouse models have been developed for the study of mural cells that could potentially be useful in future research. However, each come with caveats. The SM22α-Cre, which uses a 2.8kb fragment of the extended SM22α promoter of the smooth muscle-specific cytoskeletal protein SM22α to drive Cre expression, can be detected from E9.5 in smooth muscle cells of the dorsal aorta as well as in the outflow tract but also myocardium (Lepore et al 2005). Therefore, detrimental effects on the heart caused by Tbx2 deletion may persist in this model.

SMMHC-Cre and αSMA-Cre transgenic mice have both also been used to generate vascular smooth muscle cell-specific knockout models, but the expression of the Cre protein only occurs during the later stages of mural cell development and therefore probably express Cre too late to be useful to study Tbx2 function in mural cells (Wirth et al 2008, Wu et al 2007).

Other mural cell specific Cre models have been developed using genome fragments from both Pdgfrβ and NG2 loci which are better suited for gene deletion at earlier time points. Two different Pdgfrβ-Cre have been developed, although only one has been has a documented expression pattern (Cuttler et al 2011, Foo et al 2006). This Pdgfrβ-Cre uses a −4.7/+0.1 kb extended promoter region of the Pdgfrβ gene as a driver of Cre expression (Cuttler et al 2011). Unfortunately, the investigators did not give a detailed report of the expression pattern of their Pdgfrβ-Cre in the vasculature during early embryonic development. However, Cre expression was detectable in the myocardium and lung at E10.5, indicating that this Cre transgene may also be unsuitable for our purposes, since it could lead to considerable non-vascular defects when used to generate Tbx2 knockout mice. Upon request, we were informed that the second Pdgfrβ-Cre mentioned in the literature has since been rendered unusable after re-derivation of the line resulted in the development of a mosaic expression pattern (R. Adams, personal communication).

Two different NG2-Cre models are also available, one of which was generated with the bacterial artificial chromosome modification technique, while the other is a knock-in of an inducible CreERT2 into the endogenous NG2 gene (Huang et al 2014, Xiaoqin Zhu et al 2011). It is these Cre models that appear best suited to the study of Tbx2 in mural cells. NG2-Cre has no reported expression in the heart, is expressed early in mural cell development, and Cre expression appears to correspond very well with the expression of Tbx2 in the vasculature. Consequently, the NG2-Cre would be ideal for the generation of NG2-Cre^{+}Tbx$^{flox/flow}$ mice to examine the loss of Tbx2 in mural cells without deleterious effects on the development of other organ systems.

However, it will be important to consider that mural cell-specific gene deletion models often have a relatively mild phenotype. Knockout of Notch3, example produced viable and fertile mice and defects in vascular development were only revealed after careful histological analysis. Therefore, the conditional knockout of Tbx2 in mural cells will have to be thoroughly documented. It will be interesting to investigate the expression the potential Tbx2 targets Notch3, Pdgfrβ, Bmp4, and SRF as well as expression of Mmp2, Mmp9, and Connexin43. A transcriptome analysis, as performed by Foillade et al. for the conditional Notch3 deletion, would also be instrumental to identify novel targets (Fouillade et al 2013). In some cases, mural cell-specific gene deletions have very obvious phenotypes when vascular function is challenged as, for example, in reperfusion injury models, which may also be useful to assess vascular integrity after loss of Tbx2 (Proweller et al 2007).

4.4.2 Tbx2 in vascular pathology

Smooth muscle cells in the adult vasculature are usually quiescent but can be stimulated by injury to again express certain genes characteristic of earlier developmental states and become migratory and proliferative (Rzucidlo et al 2007). As vascular expression of Tbx2 was only observed during embryonic development it will be interesting to examine its expression during this dedifferentiation of vascular smooth muscle cells in response to vascular injury. Furthermore, study of Tbx2 expression during neovascularization in the adult, as seen in solid tumours, may also prove insightful.

4.4.3 Mural cell research in zebrafish

Mural cell development has not been studied in zebrafish in detail until recently, but reports indicate that it is a suitable model for mural cell research (Santoro et al 2009, Wang et al 2014, Whitesell et al 2014). The Tol2 transposon system would be ideal for the investigation of mural cell-specific gene regulation, as it allows medium-throughput generation of transgenics, and rapid screening of regulatory sequences in enhancer-reporter assays (Kawakami 2005). Validation of mural cell-specific activity of the potential cis-regulatory elements identified in this study in Tol2 transgenic zebrafish would establish this model as a useful tool for the study of mural cell gene regulation. Further, genetic manipulation in zebrafish using Morpholino oligonucleotides or CRISPR/Cas9 mediated mutations would provide a powerful tool to investigate the role of individual or combinations of genes for mural cell development.

Conclusion

The aim of this study was to investigate the transcriptional regulation of arterial blood vessel identity both through the study of an enhancer regulating Flk1 (encoding Vegfr2) expression in arterial endothelial cells and by describing a novel role for the transcription factor Tbx2 in mural cells.

The Flk1i10 enhancer receives inputs from both GATA and Rbpj to drive arterial-specific expression

Analysis of the *Flk1* locus identified a novel enhancer in the tenth intron (Flk1i10 enhancer) driving expression specifically in arterial endothelial cells during mouse development. The regulatory activity of this element clearly differs from two previously described enhancers, which drive Vegfr2 expression throughout the endothelium and in mesodermal progenitors, in that it allows for differential regulation of Vegfr2 expression in veins and arteries. This establishes a regulatory landscape of the *Flk1* locus which governs Vegfr2 expression both generally across the endothelium and specifically in arterial endothelial cells. In agreement with this, reports found both a general requirement of Vegfr2 for endothelial survival, as well as specific requirements for differential expression of Vegfr2 in certain cases, such as tip/stalk cell selection during sprouting angiogenesis. Targeted deletion of both endothelial Flk1 enhancers will be instrumental to establish their individual roles for endothelial cell behaviour.

Arterial specificity of the enhancer is achieved by integration of positive inputs from GATA binding sites and repressive inputs from RBPJ binding sites. This was demonstrated by the disruption of Rbpj binding, resulting in the expansion of Flki10 activity into the venous endothelium and disruption of Gata binding which resulted in a loss of endothelial enhancer activity. This suggests that Gata factors are a general activators of transcription in endothelial cells, whereas Rbpj can repress transcription specifically in veins. This is supported by reports that the other described regulatory elements in the Flk1 locus also require inputs from Gata factors but do not contain RBPJ binding motifs. However, a role of Notch signalling for Flk1i10 enhancer activity could not be convincingly determined in this study. While repression of Flk1i10 in veins agrees with current models which assume that Rbpj acts as a repressor in the absence of Notch receptor activation and that Notch receptor expression itself is repressed in veins, inhibition for Notch signalling did not result in a loss of Flki10 enhancer activity in arteries. This may suggest a role for non-canonical Notch signalling or a Notch-independent role for Rbpj. However, further investigation is required to elucidate the role of Notch and Rbpj for Flki10 enhancer activation specifically and for regulation of endothelial identity in general.

Finally, we show that the Flk1i10 enhancer does not drive transcription in the absence of Vegfr2 signalling. As Vegfr2 receptor signalling is required for arterial specification this further supports the idea that Flk1i10 enhancer activation does not take place outside of a clearly established arterial identity in fish.

Tbx2 is a novel regulator of arterial mural cell behaviour

Examination of the vascular expression pattern of the T-box transcription factor Tbx2 in this study has demonstrated that Tbx2 is exclusively expressed in mural cells of peripheral arteries and microvessels during embryonic development in mice. To my knowledge, this is the first report describing Tbx2 expression in vascular mural cells.

Global deletion of Tbx2 resulted in discontinuous mural cell coverage of peripheral arteries as well as in irregular mural cell morphology, suggesting that Tbx2 is important for normal mural cell behaviour. A conditional deletion of Tbx2 in mural cells will be instrumental to further elucidate this role of Tbx2.

In an *in vitro* knockdown screen Notch3 and Pdgfrβ were identified as potential downstream targets of Tbx2 in mural cells and *in silico* analysis of the regulatory landscape of both genetic loci revealed the presence of potential *cis*-regulatory elements with binding sites for Tbx2. Whether Tbx2 can regulate the expression of Notch3 or Pdgfrβ by binding to these elements will have to be established in further studies.

A transgenic mouse model failed to demonstrate a role for the upstream promoter region of Tbx2 in mural cells. However, an element contained within the examined region shows high potential as a mural cell-specific element *in silico*. Therefore, this element should be re-inspected individually to rule out confounding factors in the original experimental design. Further *in silico* analysis of the *Tbx2* locus did not reveal other regions with the potential to drive expression of Tbx2 in the vasculature. Establishment of a zebrafish model for medium throughput analysis

would be a useful tool for the future identification of mural cell-specific enhancers both in the *Tbx2* locus as well as in other loci.

Overall, this research contributes to our understanding of arterial development by providing original insights into mechanisms of arterial cell fate determination in endothelial cells and identifying potential novel transcriptional regulators of arterial identity in mural cells. Importantly, it provides a solid basis and direction for further scientific investigation.

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