Exploring the role of transmembrane 4 L six family member 1 (Tm4sf1) in the control of tip cell behaviour during sprouting angiogenesis

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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List of Abbreviations

ADAM, a disintegrin and metalloproteinase
ACD, antibody drug conjugate
Alk-1, Activin receptor-like kinase 1
ANG, angiopoietin
AP, alkaline phosphatise
BMP, bone morphogenic protein
CIP, 5-Bromo 4-chloro 3-indolyl phosphate;
CDC42, cell division control protein 42
COUP-TFI, chicken ovalbumin upstream promoter transcription factor II
CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats
CV, cardinal vein
CXCR4,
DA, dorsal aorta
DIG, digoxigenin
DLAV, dorsal longitudinal anastomotic vessel
DLL, delta-like
DMSO, dimethyl sulfoxide
EC, endothelial cell
ECM, extracellular matrix
EDTA, ethylenediaminetetraacetic acid
EGFR, epidermal growth factor receptor
FAK, focal adhesion kinase
FEVR, familial exudative vitreoretinopathy
FISH, fluorescent in situ hybridization
Flt, fetal liver kinase
GFP, green fluorescent protein
HB-EGF, heparin binding epidermal growth factor
HD-EGF, heparin binding epidermal growth factor
HEY, Hairy/enhancer-of-split related with YRPW motif protein
HGF, hepatocyte growth factor
HHT, hereditary haemorrhagic telangiectasia
HIF-1, hypoxia inducible factor-1
HM, horizontal myoseptum
hpf, hours post fertilization
HR, homologous recombination
HRM, high resolution melt
HUVEC, human umbilical vein endothelial cell
Hyb, hybridisation solution
IGFR, insulin growth factor receptor
IPTG, isopropyl β-D-1-thiogalactopyranoside
ISH, in situ hybridisation
ISV, intersegmental vessel
Kdr, kinase insert domain receptor
Lef1, Lymphoid enhancer-binding factor 1
MAPK, mitogen activated protein kinase
MCeV, mid-cerebral vein
MCP-1, monocytic chemotactic protein-1
MeOH, methanol; MIB, mind bomb
MIB, mind-bomb
MMPs, matrix metalloproteinase
MO, morpholino oligonucleotide
NBT, Nitro blue tetrazolium
NECD, Notch extracellular domain
NHEJ, non-homologous end joining
NICD, Notch intracellular domain
NRP, neuropilin
PAM, protospacer adjacent motif
PAR, partitioning defective protein
PBS, phosphate buffer solution
PBT, phosphate buffer solution plus tween
PDGF, platelet derived growth factor
Pecam-1, platelet endothelial cell adhesion molecule-1
PFA, paraformaldehyde
PHBC, primordial hindbrain channel
PI3K, phosphoinositide 3-kinases
PIK3R1, phosphatidylinositol 3-kinase
PLCγ, phospholipase C-γ
PLGF, placental growth factor
pLR, last repeat plasmid
PODXL, Podocalyxin-like protein
PPrA, primitive prosencephalic artery
PTU, phenylthiourea
qPCR, quantitative polymerase chain reaction
RBPJ, Recombination signal binding protein for immunoglobulin kappa J region
RE, restriction endonuclease
RVD, repeat-variable diresidue
Shh, Sonic hedgehog
SSC, saline sodium citrate
TALEN, transcription activator-like effector nucleases
TC, tip cell
TEM, tetraspanin enriched micodomain
TGF, transforming growth factor
TIMP, tissue inhibitors of metalloproteinases
TM4SF, transmembrane 4 superfamily
SC, stalk cell
VEG vascular endothelial growth factor
VEGFR, vascular endothelial growth factor receptor
Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZFN, zinc finger nuclease
ZO-1, zonula occludens-1
ABSTRACT

Angiogenesis is the process of new blood vessel sprouting from pre-existing vessels and is responsible for generating the majority of nascent vessels during development, tissue regeneration and disease. During angiogenesis, sprouting endothelial cells (ECs) are organised into leading 'tip' cells (TCs) and trailing 'stalk' cells (SCs). This hierarchal organisation of TCs and SCs is essential for the coordinated collective migration of ECs during sprouting. However, the precise mechanisms that define TC versus SC behaviour and identity remains uncertain. Transcriptomic analysis of sprouting vessels in zebrafish embryos led to the identification of a novel TC-associated gene, transmembrane 4 L six family member 1 (tm4sf1). We find that tm4sf1 expression is tightly spatiotemporally restricted to migrating TCs during intersegmental vessel (ISV) sprouting in zebrafish. Furthermore, TC tm4sf1 expression is controlled by the vascular endothelial growth factor receptor (Vegfr) - Notch signalling axis. Morpholino oligonucleotide (MO)-mediated knockdown of tm4sf1 reveals a subtle delay in ISV sprouting upon loss of tm4sf1 expression. Moreover, using multiplexed, real-time imaging approaches and in-depth analysis of TC and SC behaviours at single cell resolution, we reveal that the delay in ISV sprouting is specifically due to reduced TC motility. Furthermore, we find that tm4sf1 functions to induce TC motility in the leading daughter cell following TC mitosis, to rapidly re-establish post-mitotic TC behaviour. Generation of tm4sf1 mutant zebrafish lines using both transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) confirms that Tm4sf1 modulates TC behaviour. Additionally, mechanistic studies in human ECs reveal that tm4sf1 regulates VEGFR-mediated signalling upon VEGF-stimulation, which subsequently controls cell migration and expression of the TC determinants, DLL4 and VEGFR2. Hence, our results suggest that tm4sf1 is a novel modulator of the TC-SC hierarchy and collective EC migration during ISV sprouting. Overall, these findings have potential therapeutic implications since tm4sf1 may be a promising target for the manipulation of pathological angiogenesis in disease.
DECLARATION

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1.1 Development of the vasculature

Endothelial cells (ECs) generate an extensive vascular network that consists of five different types of blood vessels; namely arteries, arterioles, capillaries, venules and veins, which are defined by their size and structure. This complex branching network functions to facilitate the transport of gases, nutrients, blood cells and essential macromolecules to almost all tissues of the body. ECs line the luminal surface of all blood vessels, forming an interface between the blood and vessel wall (Michiels 2003). During embryogenesis and in adults, ECs and mesoderm-derived EC precursors (or angioblasts) possess the ability to generate the entire vasculature via two distinct morphogenetic mechanisms; vasculogenesis and angiogenesis (Carmeliet & Jain 2011; Herbert & Stainier 2011).

Vasculogenesis represents the initial construction of the first primitive vascular tree by mesoderm-derived angioblasts during embryogenesis (Figure 1.1). In the vertebrate embryo, angioblasts receive local differentiation, maturation and proliferative cues in the lateral plate mesoderm (Gore et al. 2012; Coultas et al. 2005). These signals enable the specification, coalescence and assembly of angioblasts to form the first embryonic vessels; namely, the dorsal aorta (DA) and cardinal vein (CV) (Lawson et al. 2002; Coultas et al. 2005; Verma et al. 2010). Initially, Sonic hedgehog (Shh)-induced expression of Vascular endothelial growth factor a (Vegfa) in zebrafish and subsequent activation of Notch (Lawson et al. 2002), in addition to other factors (Pendeville et al. 2008), was proposed to be the major regulator of this process. However, more recent work in zebrafish found Vegfa to be dispensable for angioblast migration to the midline and intrinsic expression of Apelin receptors was reportedly essential for angioblast migration, triggered by the recently discovered ligand, Elabela (Helker et al. 2015). Very early in development, ECs are determined as acquiring either arterial or venous EC fate, which is controlled by a series of interacting haemodynamic stimuli and genetic factors. Examples include signalling via Ephrin-B2-EphB4 (Wang et al. 1998; Adams et al. 1999), Notch, and vascular endothelial growth factor receptors (Vegfrs) (Zhang et al. 2008; Lanner et al. 2007), as well as via the transcriptional regulators chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) (You et al. 2005) and Hairy/enhancer-of-split related with YRPW motif protein 1 (HEY1) and 2 (HEY2) (Fischer et al. 2004; Rocha & Adams 2009). Subsequent arterial/venous sorting and segregation, whereby one common
precursor vessel gives rise to the unconnected DA and CV in zebrafish, is mediated by the expression of Ephrin-B2 and its receptor EphB4, in arterial-fated and venous-fated progenitors, respectively (Herbert et al. 2009). Additionally, angioblasts assemble to form blood islands which subsequently establish the primary capillary plexus (Ferguson 2005; Adams & Alitalo 2007). Following establishment of the primitive vasculature by vasculogenesis, fabrication of an extensive and mature vascular network is established predominantly by angiogenesis. Angiogenesis is defined as the remodelling or sprouting of new vessels from pre-existing vessels (Figure 1.1) (Herbert & Stainier 2011; Ferguson 2005; Adams & Alitalo 2007). Similarly, the formation of the lymphatic vasculature system (Figure 1.1), which plays a critical role in normal fluid homeostasis, occurs via a process known as lymphangiogenesis and originates from embryonic veins (reviewed in detail by Tammela & Alitalo 2010).

1.2 Discovery and characterisation of angiogenesis

The process of angiogenesis was first recognised in 1794 by Scottish anatomist and surgeon, John Hunter. It was observed that the degree of tissue vascularisation was proportional to the metabolic requirements of a tissue. Specifically, if a tissue was little used, there were fewer blood vessels associated with that tissue. However, if a tissue was growing, the tissue became more vascular (Hunter 1963). This relationship is clear during development, with the cardiovascular system being the first organ system to form during embryogenesis. For the development of most organ systems to commence or mature, a blood supply transporting oxygen, hormones and nutrients are required (Michiels 2003). However, an in-depth understanding of the process of angiogenesis did not arise from studying the physiological process of blood vessel development, but originated from the realisation that new blood vessels penetrate tumours and wounds (Algire 1943; Algire et al. 1945). Shortly after these observations were made, it was realised that tumours possessed the property of promoting new blood vessel formation, described as the ‘angiogenic factor’. The significance of these findings, in addition to a wealth of knowledge on the implications of angiogenesis for tumour progression (Folkman et al. 1971; Wolf & Harrison 1973; Greenblatt & Philippe 1968), led to the discovery of human VEGFA, described then as vascular permeability factor, after recognition of its ability to increase vascular permeability (Senger et al. 1983). VEGFA was subsequently found to be the central regulator of angiogenesis (Leung et al. 1989; Keck et al. 1989) and VEGFRs,
Figure 1.1: Blood vessel morphogenesis; vasculogenesis and angiogenesis. (A) The process of vasculogenesis promotes the de novo formation of the initial embryonic blood vessels, the DA and CV, via the aggregation and differentiation of angioblasts into primitive vessels and arterial/venous segregation. Angioblasts in the yolk sac blood islands also differentiate and remodel to form a primitive vascular plexus. (B) Angiogenesis denotes the formation of new blood vessels by the sprouting new vessels from pre-existing vessels and is responsible for expansion of the vasculature during development to form almost all adult arteries, arterioles, capillaries, veins and venules. As new vessels form, they are stabilised by mural cells to form a mature vascular network. Additionally, venous ECs give rise to lymphatic vessels in a process known as lymphangiogenesis.
as well as their downstream signalling pathways, were subsequently characterised (Shibuya & Claesson 2006; Millauer 1993). An ensuing high profile publication evoked great interest in the therapeutic potential of VEGF, when it was reported that tumour growth in mice was inhibited by treatment with a monoclonal antibody targeting VEGFA (Kim et al. 1993).

1.3 Angiogenesis and disease

The deregulation of angiogenesis can have severe pathological consequences since normal organ development and function relies on an adequate blood supply. The most widely studied angiogenesis-associated disease is tumour angiogenesis, in which the ‘angiogenic switch’ is turned on by growing tumours upon alteration of the balance of angiogenic activators and inhibitors. This promotes the growth of new blood vessels to supply the tumour with oxygen and nutrients, and in turn facilitates the growth of primary tumours as well as their subsequent invasion and metastasis (Hanahan & Weinberg 1999; Kaipainen et al. 1995). Other angiogenesis-associated diseases include diabetic retinopathy, whereby impaired neovascularisation characterised by abnormal vessel architecture and enhanced permeability can lead to visual impairment and eventual blindness. With the increasing prevalence of diabetes mellitus in the western world, diabetes is the leading cause of blindness amongst adults in the UK (Diabetes UK, 2010; Cai & Boulton 2002). Insufficient or excessive angiogenesis has also been implicated in the pathogenesis of a number of other disease states, some of which are summarised below in table 1.1.

Some disease states associated with excessive angiogenesis respond well to anti-angiogenic treatments. For example, a number of anti-VEGFA antibodies are available for the treatment of macular degeneration (Avery et al. 2014). However, the development of anti-angiogenic therapies for other conditions, such as cancer, have encountered a number of unexpected set-backs or unwanted side effects; most notably, resistance (Bergers & Hanahan 2008), but also increased blood clotting and incidence of stroke (Garber 2002).

Studying angiogenesis during development can thus give novel insights into the mechanisms involved in angiogenesis-associated disease. A deeper understanding of this process could enable the identification of novel therapeutic targets for the manipulation of pathological angiogenesis in multiple disease states.
### Table 1.1: Examples of diseases associated with excessive or insufficient angiogenesis

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<th>Angiogenesis</th>
<th>Disease</th>
<th>Description</th>
<th>Reference</th>
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<td>Insufficient</td>
<td>Gastric ulcers</td>
<td>Production of angiogenic inhibitors by Helicobacter - reduced VEGFA levels linked to recurrent ulcerations</td>
<td>(Jenkinson et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Stroke</td>
<td>Lack of angiogenesis post injury is associated with poor survival</td>
<td>(Krupinski et al. 1994)</td>
</tr>
<tr>
<td></td>
<td>Osteoporosis</td>
<td>Impaired bone formation due to compromised angiogenesis as a consequence of declining levels of VEGFA with age</td>
<td>(Martinez et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>Tumour cells secrete large amounts of VEGFA to promote neovascularisation to enable tumour growth and invasion</td>
<td>(Hanahan &amp; Weinberg 1999)</td>
</tr>
<tr>
<td>Excessive</td>
<td>Obesity</td>
<td>Obesity can be controlled in mice by limiting the vascular supply to adipose tissue by using anti-angiogenic agents</td>
<td>(Rupnick et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Rheumatoid arthritis</td>
<td>Soluble VEGFR1 chimeric protein suppresses excessive angiogenesis in the rheumatoid synovium</td>
<td>(Sekimoto et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Endometriosis</td>
<td>Enhanced levels of VEGFA in the peritoneal fluid of patients with endometriosis potentially promotes the ectopic migration and settlement of endometrial cells</td>
<td>(McLaren et al. 1996)</td>
</tr>
</tbody>
</table>

#### 1.4 ECs: the building blocks of a new blood vessel

**1.4.1 Tip cell and stalk cell characteristics**

Sprouting angiogenesis occurs in response to pro-angiogenic environmental cues. In particular, new blood vessel sprouting is primarily driven by the pro-angiogenic factor, VEGFA (Gerhardt et al. 2003). VEGFA and other pro-angiogenic cues are sensed by ECs in quiescent vessels, which are arranged in a monolayer lining the luminal surface of the vessel (Figure 1.2A). Pro-angiogenic signals confer a highly motile and invasive phenotype on a small sub-population of cells, known as ‘tip cells’ (TCs; figure 1.2B). TCs lead a new sprouting vessel whilst extending filopodia; long cytoplasmic protrusions which sense the immediate environment for further guidance cues (Gerhardt et al. 2003; Herbert & Stainier 2011). The ECs that trail behind the motile TCs are known as stalk cells (SCs; figure 1.2C). SCs support vessel sprouting; they provide the building blocks of the new vessel trunk and maintain vessel integrity and connectivity to the vessel of origin. Importantly, evidence also suggests that SCs are responsible for lumen formation of the
new vessel (Iruela-Arispe & Davis 2009; Kamei et al. 2006) and thus maintain perfusion of the new vascular sprout.

### 1.4.2 Initiation and elongation of the vascular sprout

On activation of vascular sprouting, TCs are thought to release enzymes and proteases that degrade the basement membrane and extracellular matrix (ECM) (Carmeliet & Jain 2011). Specifically, matrix metalloproteinases (MMPs) are released by human ECs in response to the major activator of angiogenic sprouting, VEGF, which are known to degrade the surrounding ECM (Pufe et al. 2004; Carmeliet & Jain 2011). TC sprouting is also facilitated by VEGFA induced VE-cadherin tyrosine phosphorylation, which allows the loosening of EC junctions and increased EC permeability (Esser et al. 1998). Altogether, these TC features enable the directional sprouting of a new blood vessel. Although SCs ‘trail’ behind the leading TCs during vessel sprouting, it was previously thought TC division occurs rarely. Elongation of the vascular sprout was attributed to SCs proliferation (Gerhardt et al. 2003; Ausprunk & Folkman 1977). Hence, it has often been assumed that SCs ‘push’ the TCs towards their target vessel. However, initial sprouting of TCs has been observed in the absence of SC divisions. Moreover, evidence suggests that TCs exert a pulling force by local interaction with the surrounding ECM (Phng & Gerhardt 2009; Phng et al. 2009; Ausprunk & Folkman 1977). Additionally, we and others (Blum et al. 2008; Herbert et al. 2012) have shown that TCs undergo extensive proliferation during ISV sprouting. Thus, in various vascular beds it is clear that TC divisions are a major contributing factor to elongation of a new vessel.

### 1.4.3 Formation of a vascular lumen

As the new vascular sprout migrates towards its target vessel, SCs simultaneously form the vascular lumen (Figure 1.2D). ECs first establish apicobasal polarity, a process mediated by β1-integrin and its downstream target, partitioning defective protein 3 (Par3) (Wang et al. 2010; Zovein et al. 2010). Par3 is a well-established determinant of polarity and forms a functional complex with Par6, atypical protein kinase C (aPKC) and cell division control protein 42 homolog (CDC42) in epithelial cells (Suzuki 2006). In mouse retinal ECs, loss of β1-integrin results in decreased Par3 and cells are unable to form a lumen (Zovein et al. 2010), which was previously observed in studies utilising pharmaceutical inhibitors of β1-integrin (Drake et al. 1992). Additionally, disruption of the
Figure 1.2: The cellular mechanisms of angiogenesis. A. Existing vessels are maintained in a quiescent state in the absence of pro-angiogenic factors and presence of angiogenic inhibitors. B. An EC sensing high levels of pro-angiogenic factors such as VEGF begins to respond and is specified as a TC. The TC releases MMPs to degrade the surrounding ECM, cellular junctions are loosened and mural cells are lost to enable the sprouting of a new vessel. C. The TC leads the new vascular sprout and SCs trail behind. TC and SC divisions contribute to the elongation of the vessel. The sprouting vessel is guided by gradients of pro-angiogenic factors D. β1-integrin-mediated lumen formation is initiated. TCs reach their target vessel and upon contact, fuse by anastomosis potentially mediated by macrophages. E. Mural cells are recruited to support newly formed vessels, tight junctions are re-established, lumen formation completes and the vessels become perfused. ECM is deposited and the new blood vessel becomes quiescent.
CDC42-PAR3- PAR6-aPKC complex in human ECs hinders luminogenesis and the formation of vascular tubes (Koh et al. 2008). This is possibly due to the abnormal distribution of multiple junctional adhesion proteins including CLAUDIN-5, platelet endothelial cell adhesion molecule 1 (PECAM-1), VE-cadherin, and cluster of differentiation 99 (CD99) (Wang et al. 2010; Zovein et al. 2010). These data suggests that PAR3 may mediate the tight regulation of EC junctional proteins which are required for the polarised organisation of ECs prior to vascular lumen formation. Following PAR3-mediated polarisation of ECs, studies in mouse embryos have found that proteins necessary to initiate lumen dilation are recruited. VE-Cadherin is essential for the recruitment of CD34-sialomucins to the apical membranes. Podocalyxin-like protein (Podxl) is also recruited to the apical surface by both β1-integrin and VE-Cadherin (Strilic et al. 2009; Zovein et al. 2010). CD34 and Podxl are localised in vesicles and it has been postulated that VE-Cadherin may facilitate their deposition at the apical surface via exocytosis (Strilic et al. 2009). Following the relocalisation of CD34-sialomucins and Podxl, Moesin is also recruited to the apical surface and may facilitate the accumulation of F-actin, which is thought to be involved in luminal expansion. Lumen formation is subsequently initiated, possibly due to the repulsion of negative charges on the apical surface due to the deposition of CD34-sialomucins (Wang et al. 2010; Strilić et al. 2010; Strilic et al. 2009). The lumen subsequently expands via a series of poorly understood mechanisms. Interactions between non-muscle myosin II and F-actin in a Vegfa-dependant manner are thought to induce EC shape changes to further establish the vessel lumen (Zovein et al. 2010; Strilic et al. 2009). This is supported by recent evidence that shows the scaffold protein, AmotL2, associates with VE-cadherin where it facilitates the coupling of adheren junctions to F-actin fibres (Hultin et al. 2014). Hence, association of VE-Cadherin-AmotL2 with F-actin or Myosin II fibres may facilitate lumen formation via a similar contractile mechanism as observed in myocytes. Additionally, intracellular vacuoles arise and fuse with the apical surface to form an intracellular space (Kamei et al. 2006; Wang et al. 2010). This process is thought to arise via β1-integrin-ECM-mediated pinocytosis (Kamei et al. 2006), consistent with the accumulation of intracellular vacuoles in β1-integrin negative ECs (Zovein et al. 2010). Finally, the establishment, but not stabilisation of a vascular lumen was found the be independent of blood flow (Wang et al. 2010).

1.4.4 Anastamosis

Sprouting continues towards the Vegfa gradient with the TC directing the path (Figure 1.2C-D); however when a TC makes contact with another vessel, the sprout fuses
with the vessel during a process known as anastamosis (Figure 1.2D). During anastamosis, intersegmental vessels (ISVs) fuse with neighbouring ISVs to form the dorsal longitudinal anastamotic vessel (DLAV) (Childs et al. 2002). Initially, TCs make contact with each other via filopodial extensions (Phng et al. 2013; Almagro et al. 2010; Lenard et al. 2013). Recent evidence from investigations in zebrafish suggest macrophages may interact with TCs to guide their contact and support new connections (Fantin et al. 2010; Rymo et al. 2011). However, albeit slower in the absence of macrophages, normal anastamosis still occurs, indicating that EC-autonomous mechanisms are the major determinants of vessel fusion (Fantin et al. 2010; Rymo et al. 2011). On TC-TC contact, TC polarisation and connection stability is facilitated by the Myosin-X-mediated co-localisation of VE-cadherin and Zonula occludens (ZO-1), since their rapid relocalisation to the contact point has been observed (Almagro et al. 2010; Blum et al. 2008; Lenard et al. 2013). Furthermore, Podxl2 also becomes localised at the site of contact and apical membrane invagination and fusion occurs. Subsequently, TCs lose their filopodia and apical membrane invagination continues creating luminal pockets and cell rearrangements that eventually generate a single lumen. When the process of anastamosis is complete, a seamless vessel is generated with no evidence of a fusion site (Lenard et al. 2013).

1.4.5 Vessel maturation

When the fusion of two vessels is established, remodelling and maturation of the vessel occurs. Angiopoietin-1 (ANG-1) is an essential regulator of maturation and remodelling (Suri et al. 1996). ANG-1 is constitutively expressed by pericytes and acts via the TIE-2 receptor on ECs to induce the release of monocytic chemotactic protein-1 (MCP-1). MCP-1 is a chemokine essential for the recruitment of pericytes to the maturing vasculature (Kim et al. 2000; Aplin et al. 2010). Pericytes stabilise the immature vessel by releasing additional factors such as tissue inhibitors of metalloproteinases (TIMPs) to prevent matrix degradation. ANG-1 also recruits smooth muscle cells by inducing EC expression of heparin binding epidermal growth factor (HB-EGF) and hepatocyte growth factor (HGF) (Iivanainen et al. 2003; Kobayashi et al. 2006). In addition, endothelial-derived platelet derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) contribute to the recruitment of mural cells to support the maturing vasculature (Jain 2003).
1.5 VEGF signalling positively regulates new sprout formation

Two signalling pathways that are fundamental to TC and SC phenotypic specification are the VEGFR and Notch pathways. VEGFA is described as the ‘master regulator’ of angiogenesis and belongs to the VEGF family of secreted glycoproteins, in addition to placental growth factor, (PLGF), VEGFB, VEGFC, VEGFD, and VEGFE, which bind specific cognate protein tyrosine kinase receptors (VEGFRs; Figure 1.3). In humans and other mammals, three VEGFRs have been identified, namely VEGFR1 (also named FLT1), VEGFR2 (also named KDR and FLK1) and VEGFR3 (also named FLT4). In the zebrafish, four genes encoding VEGFRs have been identified: the orthologues of VEGFR1, VEGFR3, and two genes with high similarity to VEGFR2 (KDR/FLK1). The initial VEGFR2 to be cloned and characterized has an essential role in angiogenesis in zebrafish (Bussmann et al., 2008; Habeck et al., 2002) and was originally specified as the KDR zebrafish orthologue. However, the second VEGFR2 orthologue to be identified, which plays a lesser role in vascular development (Bahary et al., 2007; Bussmann et al., 2008), is more similar to the human VEGFR2 gene. Thus, the first KDR orthologue to be identified in zebrafish that is instrumental for blood vessel development in zebrafish is now referred to as KDR-like (kdrl), and is referred to as Vegfr2/kdrl throughout this thesis. The presence of this second gene is not attributed to genome duplication; rather, the absence of the forth VEGFR gene in humans is most likely due to loss in the eutherian lineage, after the divergence of marsupial and placental mammals (Bussmann et al., 2008). VEGF ligands exist as homodimeric polypeptides although VEGFA and PLGF heterodimers have also been described (Conn 1995). Of this family, VEGFA plays the most significant role in angiogenesis, since its activity promotes the sprouting of new blood vessels during both physiological and pathological angiogenesis (Carmeliet et al. 1996; Leung et al. 1989).

1.5.1 VEGFA positively regulates new sprout formation

The essential role of VEGFA in vascular development was confirmed by embryonic studies in heterozygous Vegfa<sup>+/−</sup> mice, which display severely impaired vascular development and embryonic lethality mid-gestation (Carmeliet et al. 1996; Ferrara et al. 1996). The human gene for VEGFA resides on chromosome 6 and can be alternatively spliced to give rise to eight distinct isoforms. The isoforms are distinct due to the presence or absence of heparin sulphate proteoglycan binding domains that are encoded by exon 6 and exon 7. This gives the isoforms unique properties, such as differential bioactivity and patterns of expression between tissues (Robinson & Stringer 2001). VEGFA<sub>121</sub> is soluble
Figure 1.3: The binding specificities of VEGF ligands to their cognate protein receptor tyrosine kinases. VEGFs bind to the three VEGFRs, leading to the formation of VEGFR homodimers and heterodimers. VEGFA, VEGFB and PLGF bind to VEGFR1, VEGFA and VEGFE bind to VEGFR2, and VEGFC and D bind to VEGFR3. Proteolytic processing of the human VEGFC and D allows for binding to VEGFR2; however, these factors bind to VEGF2 with lower affinity than to VEGFR3. Neuropilin (NRP)-1 and NRP-2 act as co-receptors for both VEGFR1 and VEGFR2 whereas VEGFR3 interacts only with NRP-2. All three VEGFRs are involved in the control of angiogenesis, with VEGFR2/VEGFR3 and VEGFR1/sVEGFR1 positively and negatively regulating blood vessel formation, respectively. VEGFA-VEGFR2 signalling is the primary regulator of angiogenesis. VEGFR2 and VEGFR3 have been implicated in the control of lymphangiogenesis but VEGFC-VEGFR3 signalling is the primary regulator of lymphatic vessel formation.
and freely diffusible due to the absence of a heparin sulphate binding domain (Ng et al. 2001). It establishes a shallow concentration gradient and promotes EC proliferation (Ruhrberg et al. 2002). In contrast, VEGFA_{189} contains two heparin sulphate binding domains and is thus tethered to the cell surface or ECM. On the other hand, VEGFA_{165} contains one out of two potential binding domains and its resultant properties enable both diffusion and matrix association (Stringer et al. 2006; Park et al. 1993). VEGFA_{165} establishes a steep concentration gradient that encourages filopodial extension and cell migration (Ruhrberg et al. 2002). Additionally, a number of unconventional VEGFA isoforms exist known as β-isoforms. Their altered carboxyl termini consequently fail to efficiently activate VEGFR2 receptors and are thus considered anti-angiogenic (Kawamura et al. 2008; Ladomery et al. 2007). The presence of heparin sulphate binding domains, and thus the ability of the ligand to be sequestered to the matrix, not only determines the distribution of the VEGF ligands but also affects their signalling output (Chen et al. 2010). Hence, it is likely that the relative expression ratios and spatial regulation of VEGFA splice variants are involved in the tight control of vascular sprouting.

VEGFA expression is induced in response to hypoxia (Forsythe et al., 1996). When a cell is not receiving sufficient oxygen for metabolism, activation of the transcription factor, hypoxia-inducible factor-1 (HIF-1), facilitates the expression and subsequent secretion of VEGFA from the cell (Forsythe et al., 1996). This establishes a VEGFA gradient to which ECs in a quiescent vessel respond (Gerhardt et al. 2003; Shweiki et al. 1992; Pugh & Ratcliffe 2003). In response to VEGFA, an emerging TC extends filopodia, which contain high concentrations of VEGFR2 (Gerhardt et al. 2003). The filopodia extend towards the VEGF gradient to promote the directional migration of the new vessel. Disruption of this gradient hinders EC filopodial extensions in mice, resulting in impaired vascular sprouting (Ruhrberg et al. 2002).

VEGF ligands elicit a biological response by binding to their specific cognate protein tyrosine kinase receptors, VEGFRs (Figure 1.3). There are three VEGFRs denoted VEGFR1 (Flt1), VEGFR2 (also known as kinase insert domain receptor, Kdr, or fetal liver kinase 1, Flk-1), and VEGFR3 (Flt4). The receptors show specific patterns of expression with VEGFR1 expression being primarily observed in monocytes and macrophages, VEGFR2 in vascular ECs, and VEGFR3 in lymphatic ECs; however all three VEGFRs are expressed in ECs and play significant roles during angiogenesis (Olsson et al. 2006; Claesson-Welsh 2012; Lohela et al. 2009).
1.5.2 **VEGFR2 is the primary receptor responsible for angiogenic sprouting**

VEGFR2 is abundantly expressed on ECs and plays an essential role in new blood vessel formation, both physiologically and in disease (Carmeliet 2003; Gerhardt et al. 2003). Disruption of VEGFR2 by targeted homologous recombination in mouse embryos results in early embryonic lethality at E8.5 due to impaired haematopoetic and EC development (Shalaby et al. 1995). The primary ligand for VEGFR2 is VEGFA, which tightly binds the extracellular Ig-like domains 2 or 3 of the receptor (Fuh et al. 1998). VEGFR2 can also bind VEGFC and VEGFD which are activated by proteolytic cleavage of the N and C terminal propeptides to enable dimerisation of the receptor and subsequent activation (Joukov et al. 1997; McColl et al. 2003). On binding of the primary VEGFR2 ligand, VEGFA, VEGFR2 dimerisation is initiated which brings extracellular proximal domains into direct contact. This conformational change reveals the intracellular kinase domain ATP binding site, resulting in kinase activation by autophosphorylation of specific VEGFR tyrosine residues and receptor activation (Claesson-Welsh 2012; Lohela et al. 2009; Yang et al. 2010). The most important VEGFR2 phosphorylation site resides within the C-terminal domain at tyrosine Y1175. VEGFA dependent phosphorylation of Y1175 is crucial for phospholipase C-γ (PLC-γ) binding via its SH2 domain and subsequent downstream activation of the mitogen activated protein kinase (MAPK) signalling pathway (Takahashi et al. 2001). Investigations in mice confirm these findings since substitution of Y1173 (which corresponds to human Y1175) with phenylalanine induces embryonic lethality at E8.5-E.95 due to diminished hematopoietic progenitors and a lack of blood vessel formation. In contrast, substitution of Y1212 (Y1214 in humans) with phenylalanine does not affect mouse viability or fertility (Sakurai et al. 2005). Further phosphorylation sites of interest include tyrosines Y1054 and Y1059, which reside within the kinase domain and, Y951, found in the kinase insert domain. Auto-phosphorylation at these sites increases VEGFR2 tyrosine kinase activity (Olsson et al. 2006; Kendall et al. 1999). Together, the phosphorylation sites of VEGFR2 activate an array of signal transduction pathways including the MAPKs, the phosphoinositide 3-kinases (PI3K) and PLCγ, that impact cell proliferation, migration, survival and ultimately, angiogenesis (Olsson et al., 2006) (Figure 1.4).

1.5.3 **The role of VEGFR1 in angiogenesis**

The physiological role of VEGFR1 in ECs is to oppose the activity of VEGFR2 and thus, VEGFR1 is a repressor of ectopic vessel growth. VEGFR1 binds both VEGFA and
Figure 1.4: Key signalling cascades activated by VEGFA-VEGFR2. VEGFA signalling via VEGFR2 leads to the auto-phosphorylation of specific tyrosines, which lead to the activation of a complex, interconnected intracellular signalling cascade. Signalling pathways, including the MAPK, PI3K and PLCγ1 pathways, subsequently influence the expression of genes that control EC migration, survival, proliferation and vascular permeability; ultimately controlling angiogenesis.
PLGF, with the latter exclusive binding VEGFR1. VEGFR1 is alternatively spliced to give rise to a full-length, membrane localised form (mVEGFR1), and a soluble, secreted form (sVEGFR1) that effectively ‘mops up’ any excess VEGFA ligand thereby controlling the amount of VEGFA ligand available for VEGFR2 binding (Olsson et al. 2006; Kendall & Thomas 1993; Roberts et al. 2004). Interestingly, VEGFA has a higher affinity for both the soluble and membranous forms of VEGFR1 compared to VEGFR2. However, VEGFR1 tyrosine kinase activity is weak (Sawano et al. 1996), thus VEGFA binding to VEGFR1 is thought to negatively regulate angiogenic sprouting and is often described as a decoy receptor. The decoy characteristics of VEGFR1 have been mapped to a single amino acid change in the tyrosine kinase activation loop, that lowers the efficiency of VEGFR1 autophosphorylation on ligand binding (Meyer et al. 2006). Loss of flt1/Vegfr1 in zebrafish mildly enhances angiogenesis (Krueger et al. 2011) and similarly, Flt1/Vegfr1 deletion in mice is lethal at E9 due to vessel dysmorphogenesis characterised by hypersprouting and abnormal patterning of blood vessels (Fong et al. 1995; Kearney et al. 2002). However, mice lacking the tyrosine kinase domain of Vegfr1 developed normally (Hiratsuka et al. 1998), suggesting tyrosine kinase signalling via Vegfr is not required for normal blood vessel development.

Consistent with the negative regulatory role for Vegfr1 in blood vessel sprouting, Chappell et al (2009) described a role for sVegfr1 in the further refinement of local Vegfa gradients in the microenvironment of new vascular sprouts. EC expression of sVegfr1 adjacent to sprouting vessels generates sVegfr1 counter-gradients that restrict the amount of Vegfa availability for Vegfr2, and effectively guide sprouts from the parent vessel (Chappell et al., 2009). Hence, both mVegfr1 and sVegfr1 play an important role in the negative regulation and guidance of new sprout formation.

1.5.4 The emerging role of VEGFR3 during angiogenesis

VEGFR3 binds both VEGFC and VEGFD, and as described for VEGFR2, ligand binding leads to receptor dimerisation and phosphorylation of tyrosines residues which activates receptor signalling. Multiple phosphorylation sites in VEGFR3 that are activated by dimerisation have been identified, including Y1230, Y1231, Y1265, Y1337, and Y1363 located at the C-terminus (Dixelius et al. 2003), and suspected phosphorylation sites in the kinase domain activation loop, Y1063 and Y1068 (Salameh et al. 2005). VEGFC-VEGFR3 signalling plays a well established role in the development of lymphatic vessels (Alitalo et al. 2005). Lymphatic ECs sprout from the CV during embryogenesis and form a separate blind ended vascular network by a process known as lymphangiogenesis (Alitalo et al. 2005).
VEGF3 (FLT4) is almost exclusively expressed on lymphatic ECs during adulthood. However during development and in diseases such as cancer, VEGFR3 is also expressed by sprouting blood vessels (Kaipainen et al. 1995) and positively regulates angiogenesis (Tammela et al. 2008). Loss of Vegfr3 in mice leads to lethality at E9.5 due to defects in blood vessel formation, before the emergence of lymphatic vessels at E10.5 (Covassin et al. 2006; Tammela et al. 2008; Dumont et al. 1998). Specifically, Vegfr3/flt4 expression is high in sprouting TCs of the mouse retina and zebrafish ISVs (Covassin et al. 2006; Tammela et al. 2008; Siekmann & Lawson 2007). Inhibition of VEGFR3 signalling in mice using antibodies that block ligand binding to VEGFR3 mildly attenuated retinal vascular density (Tammela et al. 2008). Similarly, studies in zebrafish found morpholino oligonucleotide (MO)-mediated knockdown of flt4 caused a mild block in ISV sprouting (Covassin et al. 2006). Additionally, signalling via Vegfr3 may partially compensate for lack of Vegfr2 signalling (Zarkada et al. 2015; Tammela et al. 2008). Interestingly, a number of studies both in vitro and in vivo have also indicated that VEGFR3/Vegfr3 may also play a ligand-independent role during angiogenesis (Tammela et al. 2011; Haiko et al. 2008; Benedito et al. 2012; Dumont et al. 1998). While homozygous loss of Vegfr3 in mouse embryos is lethal, Vegfc−/− and Vegfd−/− embryos develop normal blood vasculature after E9.5 (Haiko et al. 2008). In vitro, VEGFR3 was found to be phosphorylated in human ECs cultured on collagen I, even in the presence of VEGFR3 blocking antibodies or inhibitors of tyrosine kinase activity (Tammela et al. 2011), suggesting VEGFR3-ECM interactions may promote VEGFR3 phosphorylation in the absence of endogenous ligand. Ligand-independent VEGFR3 phosphorylation was Src-dependent (Tammela et al. 2011) in agreement with previous reports (Galvagni et al. 2010). Benedito et al (2012) also proposed a ligand-independent role for VEGFR3 in SCs, since Vegfr3 was found to be active in the presence of ligand-blocking antibodies in cells with low Notch signalling, suggesting a potential role for Vegfr3 in SCs. However, the findings from this study are controversial (Zarkada et al. 2015). Furthermore, Tammela et al (2011) found that targeted deletion of VEGFR3 in human ECs resulted in excessive angiogenesis and filopodial protrusions, due to increased VEGFR2 activity and decreased Notch signalling (Tammela et al. 2011) and the reasons for this are unclear. Hence, VEGFR3 is clearly important for angiogenesis but contrasting findings suggest VEGFR3 could potentially play both positive and negative regulatory role in different contexts.
1.6 The neuropilin co-receptors

Neuropilin (NRP)-1 and NRP-2 are transmembrane glycoproteins that interact with VEGFRs to modulate VEGFR signalling (Staton et al. 2007) (Figure 1.3). NRPs were initially recognised for their essential role in axonal growth and guidance, a process with many similarities to angiogenesis (Neufeld et al. 2002). NRP-1 acts as a co-receptor for VEGFA but only those isoforms that contain exon 7 (Soker et al. 1998), thus NRP-1 selectively modulates VEGFR signalling depending on the presence of specific VEGFA isoforms. Although the role of NRPs in angiogenesis is not fully delineated, Nrp-1 knockout mice exhibit abnormal vascular branching suggesting they play an important role in vascular development (Kawasaki et al. 1999). Similarly, angiogenesis is also disrupted when nrp-1 is knocked down in zebrafish (Lee et al. 2002). Using the less conventional mouse hindbrain model to study angiogenesis, Nrp-1 was found to be required for TC identity during sprouting. Nrp-1 expressing cells to preferentially attain the TC position over Nrp-1 negative cells. Nrp-1 also regulates filopodia orientation but is not required for TC identify, since TCs are still specified in the absence of Nrp-1, as confirmed by the levels of TC markers such as Dll4 and Ang-2 (Fantin et al. 2015; Fantin et al. 2013; Gerhardt et al. 2004). Nrp-1 promotes TC filopodia formation by enabling ECM-induced activation of CDC42, a key determinant of filopodial extension (Fantin et al. 2015). More recently, an additional or alternative role has been described for Nrp-1. Nrp-1 was shown to be a negative regulator of Tgfβ/bone morphogenic protein (Bmp)-mediated Smad activation in TCs (Aspalter et al. 2015), as discussed further in section 1.8.

1.7 NOTCH signalling specifies TC and SC fate

1.7.1 The NOTCH signalling pathway

In response to VEGFA-VEGFR2 signalling, only a select proportion of VEGF stimulated ECs become TCs, whereas others may become SCs. This balance of TC and SC identity is finely tuned by NOTCH signalling, a highly conserved pathway known to play a role in cell communication and cell fate decisions (Figure 1.5). NOTCH receptors are a family of single pass transmembrane proteins of which three have been identified in ECs (NOTCH1, NOTCH3 and NOTCH4). In mammals, NOTCH receptors bind four transmembrane NOTCH ligands; namely, DLL1, DLL4, JAGGED1 and JAGGED2 ligands (Roca & Adams 2007; Phng & Gerhardt 2009). NOTCH receptors are first processed by furin-like convertase in the Golgi by proteolytic cleavage (Logeat et al. 1998)
Figure 1.5: A diagrammatic representation of the Notch signalling pathway. In the golgi, Notch receptors are processed by furin-like convertase and then are targeted to the cell surface. They form heterodimers consisting of NECD and NICD. NECD binds Notch ligands on adjacent cells and subsequent processing and cleavage of NICD by ADAM10, ADAM17, γ-secretase and presenilin, liberates NICD from the membrane. Notch ligands are ubiquitylated by Mind-bomb-1 and endocytosed, which facilitates efficient Notch activation. NICD translocates to the nucleus and binds to the RBPJ transcription factor. NICD-RBPJ displaces co-repressors and recruits MAML to form an RBPJ-NICD-MAML complex, which is responsible for the transcriptional activation of Notch target genes such as Hey1 and Hes1.
and modified receptors are then targeted to the cell surface where they form heterodimers that comprise a large extracellular domain (NECD) non-covalently linked to the intracellular domain (NICD) (Rand et al. 2000; Sanchez-Irizarry et al. 2004). The NECD region binds membrane associated NOTCH ligands that are expressed and presented on adjacent cells, thus direct contact by cells is essential for NOTCH signalling to commence (Rebay et al. 1991). Mind-bomb-1, an E3 ubiquitin ligase, interacts with the intracellular domain of NOTCH ligands to facilitate their ubiquitylation and endocytosis, an event essential for efficient activation of NOTCH signalling in adjacent cells (Itoh et al. 2003). Subsequent ectodomain processing by a disintegrin and metalloproteinase (ADAM)-10 and ADAM17, followed by γ-secretase and presenilin-mediated intramembrane proteolysis cleaves and releases the NICD (LaVoie & Selkoe 2003). The NICD fragment translocates to the nucleus of the receiving cell where is binds directly to Recombination Signal Binding Protein For Immunoglobulin Kappa J Region (RBPJ) transcription factor (Jarriault et al. 1995). RBPJ in turn displaces co-repressors and enables recruitment of Mastermind-like (MAML), a transcriptional co-activator that associates with both RBPJ and NICD to form a complex. This complex is responsible for the transcriptional activation of a large spectrum of NOTCH target genes, including transcription factors of the HES and HEY families (Kopan & Ilagan 2009; Jarriault et al. 1995).

1.7.2 NOTCH signalling controls TC and SC specification

The hierarchal organisation of ECs into leading TCs and trailing SCs during collective cell migration is paramount for the development of new blood vessels (Herbert & Stainier 2011). As indicated above, the DLL4-NOTCH signalling pathway was discovered to play a fundamental role in the coordination of EC behaviour and most importantly, the specification of TC and SC fate, during angiogenic sprouting (Hellström et al. 2007; Suchting et al. 2007; Siekmann & Lawson 2007). Critically, the control of TC and SC specification by NOTCH signalling is highly conserved between vertebrate species (Hellström et al. 2007; Siekmann & Lawson 2007) and since this crucial discovery, our understanding of angiogenesis has grown considerably.

In the absence of Notch signalling orDll4/dll4expression in the mouse retina or zebrafish ISVs, all sprouting ECs ectopically display TC-like characteristics, at the expense of SC identity (Hellström et al. 2007; Siekmann & Lawson 2007). Siekmann & Lawson (2007) generated mosaic zebrafish by transplanting transgenic cells with activated Notch into early WT embryos. It was found that ECs with activated Notch signalling
contributed to SC positions, but did not contribute to TC positions. Moreover, global activation of Notch signalling was also shown to prevent the formation of new sprouting vessels in zebrafish (Leslie et al. 2007). Similar studies performed in mouse with activated Notch by systemic administration a soluble Jagged-1 peptide led to fewer TCs and vessel branches and similarly, ECs deficient for Notch1 preferentially assumed TC characteristics (Hellström et al. 2007). Hence, ECs that exhibit high Notch signalling are destined for SC fate whereas those with low Notch signalling preferentially become TCs and lead new vascular sprouts (Figure 1.6). By use of in situ hybridisation (ISH) in mice, Gerhardt et al (2003) demonstrated Vegfr2 expression at the leading edge of the mouse retina. Similarly, it has also been shown that flt4, the ortholog for Vegfr3 in zebrafish, is expressed at higher levels in the tips of sprouting vessels in zebrafish (Siekmann & Lawson 2007) and is also up-regulated by Vegfr2 activity (Figure 1.6) (Zarkada et al. 2015; Benedito et al. 2012). Hence, in addition to low levels of NOTCH signalling, TCs also exhibit high levels of VEGFR signalling. Since enhanced NOTCH signalling as a consequence of DLL4 overexpression in human ECs hinders the expression of VEGFR2 and NRP-1 (Williams et al. 2006) and Dll4 haploinsufficiency in mice enhances the expression of Vegfr2 (Suchting et al. 2007), it can be assumed with confidence that high levels of Notch leads to a decrease in TC behaviour due to the downregulation of Vegfrs. Hence, DLL4-NOTCH lateral inhibition of VEGFRs in SCs prevents SCs from becoming TCs. In further support of this, VEGFR3/FLT4 is also regulated in a similar fashion to VEGFR2 by NOTCH signalling (Figure 1.6). In zebrafish embryos that lack Notch signalling due to loss of Rbpj by MO, flt4 is ectopically expressed in the DA and SCs (Siekmann & Lawson 2007). In contrast, flt4 expression is also negatively regulated by Notch signalling, since inducible ectopic activation of Notch in zebrafish represses the expression of flt4 mRNA (Siekmann & Lawson 2007; Lawson et al. 2001). Accordingly, these cells are less responsive to Vegfa ligand signals. Therefore, a reduction in Vegfr expression in SCs reinforces the SC phenotype by preventing TC specification. In contrast, Dll4-Notch signalling positively regulates FLT1/Flt1 expression (Figure 1.6), since inhibition of Notch signalling in both mouse and human cells was shown to reduce FLT1/Flt1 expression (Funahashi et al. 2010). Thus, the decoy characteristics of Vegfr1 in SCs as previously discussed (section 1.5.3), likely contribute to the prevention of Vegfa-mediated TC specification. In agreement with this, a hypersprouting phenotype is observed following MO-mediated knockdown of flt1/vegfr1 in zebrafish (Krueger et al. 2011). Interestingly, dynamic shuffling of TCs and SCs during blood vessel sprouting has been reported by multiple
Figure 1.6: Notch-mediated control of TC/SC specification. In the TC, VEGFR2 and VEGFR3 signalling is initiated by VEGFA and VEGFC ligand binding, respectively. Although VEGFR3 may also be active independently of endogenous ligand, VEGFR2 signalling is thought to enhance DLL4 expression in the TC, which activates Notch signalling in the adjacent SCs. VEGFR2 signalling also enhances the expression of VEGFR3. High Notch signalling in the SCs inhibits the expression of both VEGFR2 and VEGFR3; preventing VEGF ligand binding and thus reinforcing the SC phenotype. Furthermore, Notch signalling in the SC also promotes the expression of the decoy receptor, VEGFR1. This feedback loop ensures that low Notch signalling is maintained in TCs and high Notch signalling is maintained in SCs, reinforcing the respective phenotypes by lateral inhibition.
studies (Jakobsson et al. 2010; Arima et al. 2011). Time-lapse suggest TC-SC rapid positional changes occur before commitment to the TC or SC position and the dynamic shuffling is somewhat dependent on relative levels of Flt1Vegfr1 and Vegfr2 (Jakobsson et al. 2010). Since TCs appear to be regularly challenged by SCs during sprout elongation, this suggests TCs and SCs are versatile in their abilities to respond to signalling cues from the environment or adjacent cells (Jakobsson et al. 2010). These findings suggest that TC/SC fate may not be an audible decision; instead, the constant re-evaluation of the Vegfr-Dll4-Notch axis occurs. Altogether, these data indicate that DLL4-NOTCH signalling is the major determinant of TC/SC identities during angiogenic sprouting and this is achieved by NOTCH lateral inhibition of VEGFR expression and thus signalling.

1.7.3 JAGGED-1-NOTCH promotes TC formation

While DLL4-NOTCH signalling negatively regulates TC fate and sprouting, the NOTCH ligand JAGGED-1 is a positive regulator of sprouting angiogenesis. Jagged-1 overexpression in mice promotes sprouting and TC formation, characterised by increased vessel branching, EC density, proliferation, and filopodial extensions (Benedito et al. 2009). Furthermore, inactivation of the Jagged-1 gene in mice resulted in enhanced Notch signalling as determined by strong up-regulation of Hey1, a transcriptional repressor and downstream target of Notch (Benedito et al. 2009). These phenotypes were observed since Jagged-1 is a poor activator of Notch signalling and thus antagonises Dll4-Notch signalling. Hence, the presence of Jagged-1 expression dampens Notch signalling and promotes TC specification. The weak signalling induced by Jagged-1-Notch interactions in TCs is a consequence of post-translational modification of the Notch extracellular domain by Fringe glycosyltransferase (Panin et al. 1997; Benedito et al. 2009). In its modified state, Notch favours the binding of Dll4 as opposed to Jagged-1, thus Notch becomes activated in adjacent cells. The expression of Jagged-1 in SCs thus dampens Notch signalling in TCs, again reinforcing the TC phenotype (Benedito et al. 2009; Benedito et al. 2012; Eilken & Adams 2010). Thus the balance between Dll4 and Jagged-1 ligands also acts as an additional control mechanism for the selection of TCs and SCs and the spatio-temporal regulation of Notch signalling is a consequence of ligand post-translational modification by Fringe.

1.8 Modulators of the NOTCH Signalling Pathway

Recent studies have revealed additional mechanisms that support the NOTCH signalling pathway during TC-SC specification (Larrivee et al. 2012; Aspalter et al. 2015).
One such example is Activin receptor-like kinase 1 (Alk1), an EC-specific member of the Tgf-β/Bmp receptor family. Alk1 is mutated in Osler–Weber–Rendu disease (also known as hereditary haemorrhagic telangiectasia, HHT), which is characterised by abnormal blood vessel formation (McDonald et al. 2011). Recent findings suggest that Alk1 is a negative regulator of angiogenesis via interactions with the Notch pathway. Studies in the mouse retinal vasculature have shown hypersprouting in the retina and arterio-venous malformations when Alk1 is inhibited during postnatal development; these are features commonly encountered with HHT (Larrivee et al. 2012). Similar features are observed with EC-specific deletion of Smad1 or Smad5 (Moya et al. 2012) downstream targets of Alk1. Additionally, Bmp-9 and Bmp-10 signalling via Alk1 inhibits blood vessel sprouting by promoting the activation of Notch signalling and upregulation of Notch downstream targets, thereby promoting the SC phenotype (van Meeteren et al. 2012; Larrivee et al. 2012; Moya et al. 2012). Recently it was found that Nrp-1 is also a negative regulator of Tgfβ/Bmp-mediated Smad activation in TCs (Aspalter et al. 2015), whereas downregulation of Nrp-1 in SCs enables repression of TC fate via Alk1-Smad-Hey1/Hey2 signalling, thereby dampening Vegfr signalling in SCs (Aspalter et al. 2015; Larrivee et al. 2012). Hence, Notch and Alk1 signalling act in synergy to promote heterogeneity between TCs and SCs. The Wnt-β–catenin pathway has also been implicated in the regulation of EC Notch signalling. Wnt-β-catenin gain of function was found to positively regulate Dll4 expression in ECs. As a consequence, EC branching in mice was defective due to enhanced Wnt-β–catenin-mediated Notch signalling (Corada et al. 2010). Furthermore, Wnt-β-catenin also plays a role in vascular stability via interactions with the Notch pathway. Notch-regulated ankyrin repeat-containing protein (Nrar) is up-regulated in response to Notch-Dll4 signalling. Nrarp is an inhibitor of Notch signalling, but also a promoter of Wnt-β-catenin signalling in SCs via Lymphoid enhancer-binding factor 1 (Lef1). Furthermore, Nrarp+/ mice display defective retinal angiogenesis and MO-mediated knockdown of nnarpa and/or nnarpb in zebrafish causes defective ISV patterning. Abnormal angiogenesis in both mouse and zebrafish was due to the ectopic regression of blood vessels in the absence of Nrarp, as a consequence of reduced Wnt-β–catenin-mediated vascular stability (Corada et al. 2010; Phng et al. 2009). Hence, the Notch-mediated activation of Nrarp in SCs upregulates promotes the Lef1-dependent activation of Wnt signalling to control vessel stability during sprouting (Phng et al., 2009). Altogether, these findings emphasise an important role for Notch-Wnt-β-catenin communication in developing vessels.
1.9 Post-transcriptional control of angiogenesis by micro RNAs (miRNAs)

Micro RNAs (miRNAs) are small, non-coding RNAs that post-transcriptionally regulate the expression of many genes. By binding complementary sequences within mRNA targets, miRNAs can promote RNA degradation of block translation of specific transcripts (Small & Olson 2011). The first evidence implicating miRNAs in the regulation of angiogenesis arose when hypomorphic mice for the allele of DICER1 died between embryonic days 12.5 and 14.5. DICER is an endoribonuclease essential for activation of the RNA-induced silencing complex, RISC. The mutant mouse embryos had severely impaired blood vessel formation and altered expression of Vegfr1, Vegfr2 and Tie1 (Yang et al. 2004). Since this report, numerous studies have identified specific miRNAs that play a pivotal role in the regulation of angiogenesis (Urbich et al. 2011; Anand et al. 2010; Bonauer et al. 2009).

Functional screening in zebrafish identified miR-221 as an essential post-transcriptional modulator of TC behaviour (Nicoli et al. 2012). MO-mediated knockdown of miR-221 in zebrafish revealed a phenotype similar to the loss of flt4 (Covassin et al. 2006), with mildly delayed ISV sprouting and failure to form the primordial hindbrain channel (PHBC) vessels (Nicoli et al. 2012; Hogan et al. 2009). miR-221 was required for TC proliferation and migration and mosaic studies revealed a requirement for miR-122 for TC potential. Moreover, in the absence of Notch signalling, loss of miR-221 abrogated the typical Notch hypersprouting phenotype. Mechanistically, miR-221 maintains TC potential by repressing cyclin dependent kinase inhibitor 1b (cdkn1b) and phosphoinositide-3-kinase regulatory subunit 1 (pik3r1), the p85-alpha regulatory subunit of the phosphoinosotide-3-kinase (Pi3k) complex (Nicoli et al. 2012). Another miRNA, miR-27b, plays an essential role in controlling endothelial TC fate, in addition to venous specification (Biyashev et al. 2011). Silencing of miR-27b in zebrafish and mouse hinders angiogenic sprouting and filopodia formation due to the post-transcriptional upregulation of the miR-27b targets, dll4 and sprouty homologue 2 (sry2), which attenuates Map kinase activation and thus sprouting. Consequently, the phenotypes observed due to miR-27b knockdown could be rescued by blocking dll4 or sry2 expression (Biyashev et al. 2011). Additionally, dll4 was also identified as a target for another miRNA implicated in the control of TC specification, miR-30a (Jiang et al. 2013). Moreover, miR-126 is essential for angiogenesis in both zebrafish and mice (Kuhnert et al. 2008; Fish et al. 2008; S. Wang et al. 2008). miR-126 represses expression of sprouty-related evh1 domain contain 1 (Spred1) and pik3r2.
Spred1 and Pik3r2 negatively regulate Mapk and Pi3k signalling, respectively, thus miR-126 knockdown hinders VEGF-mediated signalling via these pathways (Kuhnert et al. 2008; Fish et al. 2008; Wang et al. 2008). Hence, post-transcriptional regulation of angiogenesis associated genes by miRNAs is essential for the fine tuning of angiogenesis and TC/SC specification.

1.10 Post-translational control of VEGFR signalling by endocytosis

Several studies in human cells and mice have highlighted the importance of VEGFR2/Vegfr2 endocytosis for efficient downstream signalling (reviewed in detail by Simons (2012)). Receptor endocytosis is a means of prolonging the duration and magnitude of VEGFR2 signalling, by removing VEGFRs from surface phosphatases and trafficking to endosome signalling platforms (Lampugnani et al. 2006). The significant contribution of Vegfr signalling from endosomes in TCs was highlighted by investigations from Nakayama et al (2013) in mice that revealed lower levels of Vegfr at the angiogenic front when compared to quiescent vessels. This was due to high turnover of Vegfr by endocytosis to enhance signalling (Nakayama et al. 2013). Thus, Vegfr2 is promptly internalised following Vegfa binding, facilitated by clathrin and dynamin-mediated endocytosis (Nakayama et al. 2013). Vegfr2 and Vegfr3 internalisation is mediated by Ephrin-B2, a ligand for the EphB receptor tyrosine kinases. In the absence of Ephrin-B2 in mice and humans cells, Vegfr2/3 internalisation is defective and downstream signalling via Rac1, Akt and Erk1/2 is impaired (Wang et al. 2010; Sawamiphak et al. 2010). Consequently, angiogenic sprouting in both the mouse retinal model and zebrafish ISV model are hindered (Wang et al. 2010). Furthermore, Vegfr2 and Vegfr3 were shown to form a complex with Dab2 and Par-3, and Dab2 also interacts with Ephrin-B2. Loss of Par-3 or Dab2 hindered the internalisation of Vegfr2 or Vegfr3 at the angiogenic tip and phenocopies the Ephrin-B2 mutants (Wang et al. 2010; Sawamiphak et al. 2010). Hence, Ephrin-B2-Dab2-Par-3 complex formation is essential for Vegfr2/3 internalisation and if any of these components are disrupted, Vegfr signalling is subsequently hindered. A number of other intracellular trafficking proteins are important for Vegfr endocytic trafficking, including Synectin, and Retrograde motor mysoin VI (Lanahan et al. 2014). In the absence of Synectin and Retrograde motor mysoin VI in mice, Vegfr2 still undergoes internalisation, but entry of the receptor into early endosomes is deferred. Consequently, retention of VEGFR2 at cell surface in human cells promotes de-phosphorylation of VEGFR2 at the key residue, Y1175, by the phosphatase, PTP1B. Hence, PTP1B reduces
the ability of the VEGFR2 to bind PLCγ1 and reduces ERK downstream signalling (Lanahan et al. 2014), which is critical for the formation of new blood vessels (Gourlaouen et al. 2013). In agreement with this, mice lacking Synectin exhibit reduced angiogenesis as a result of impaired Vegfa signalling (Lanahan et al. 2014; Chittenden et al. 2006). Hence, Synectin, and Retrograde motor mysoin VI are important for Vegfr intracellular trafficking to remove the receptor from surface phosphatases. In doing so, VEGFR enters endosomal signalling platforms that enhance Vegfr signalling duration and amplitude in the TC.

1.11 Additional determinants of TC and SC fate

While the VEGFR-NOTCH signalling axis predominantly controls TC-SC specification during blood vessel sprouting, new determinants of TC and SC identity are beginning to emerge from the transcriptomic analysis of TCs verses SCs. The emergence of these new players highlights the molecular complexity of TC/SC fate decisions and new, important questions are being raised (see section 1.12).

Laser dissection of mouse retinal TCs and subsequent transcriptomic analysis of retinal TCs and SCs led to the identification of CXC chemokine receptor 4 (Cxcr4), amongst a number of other TC enriched genes (Strasser et al. 2010). Cxcr4 is a receptor for the chemokine stromal-cell derived factor-1. Inhibition of Cxcr4 using a blocking antibody in neonatal mice decreased vascular density and hindered filopodia formation. Furthermore, lateral TC connections at the vascular front were attenuated, suggesting a role for Cxcr4 signalling in TC behaviour (Strasser et al. 2010). Interestingly, Strasser et al (2010) also identified Esm1 as a TC enriched gene. Subsequent studies by others have revealed Esm1 expression to be Vegfa-dependent and localised in TCs of the mouse retinal vasculature (Rocha et al. 2014). Esm1 knockout mice show a delay in retinal vessel sprouting, deceased filopodial protrusions and also reduced pErk1/2 in sprouting vessels. Esm1 also facilitates Vegfa-induced vascular permeability upon Vegfa165 stimulation. Interestingly, Esm1 functions to increase bioavailability of Vegfa165 and thus Vegfa-Vegfr signalling by binding to fibronectin and displacing Vegfa165 (Rocha et al. 2014). Hence, Esm1 is important for maintaining TC function. Conversely, h2.0-like homeobox-1 (Hlx-1) may be important for the maintenance of SC potential (Herbert et al. 2012). Hlx-1 was identified using a pharmacological strategy that manipulated Vegfr and Notch signalling in zebrafish embryos during ISV sprouting (discussed in section 1.13). Hlx-1 was expressed in sprouting ECs and essential for angiogenesis. Loss of hlx-1 reduced the numbers of ECs contributing to the ISVs and decreased EC proliferation. Moreover, mosaic transplant
experiments revealed that ECs lacking \textit{hlx-1} preferentially adopt the TC position and are less likely to acquire SC fate. Hence, Hlx1 may function cell-autonomously to maintain SC potential (Herbert et al. 2012). Similarly, the APJ endogenous ligand, Apelin, is also important for SC behaviour (del Toro et al. 2010). Apelin was identified by transcriptome analysis of retinal ECs isolated from Dll4 heterozygous mice, whereby all retinal ECs display TC features (Siekmann & Lawson 2007). Apelin is a secreted ligand and its loss in both mouse and zebrafish resulted in delayed sprouting and reduced proliferation of SCs. SCs express the Apelin receptor, APJ, thus TC release of Apelin is thought to regulate the proliferative behaviour of SCs via APJ (del Toro et al. 2010). Hence, the tight spatiotemporal control of the expression of certain TC/SC-specific genes plays a key role in determining resulting TC/SC behaviours.

\subsection{1.12 Remaining questions}

The cellular and molecular mechanisms that define and control the divergent behaviours of TCs and SCs during vascular morphogenesis are largely controlled by the VEGFR-NOTCH signalling axis. Despite our current insights, many open questions remain. Notch lateral inhibition plays an essential role in the control of angiogenic sprouting and TC-SC specification. However, the hierarchal organisation of cells in sprouting ISVs cannot be completely attributed to Notch lateral inhibition. Notch lateral inhibition alone would be predicted to specify more than one TC, such that a ‘salt and pepper’ distribution of TCs and SCs would be observed. Hence, the full extent of angiogenic control is most likely more complex and this suggests other control mechanisms must be working in parallel, some of which are already beginning to emerge. It is widely reported that NOTCH signalling is an essential regulator of VEGFR2 expression (Williams et al. 2006; Suchting et al. 2007; Siekmann & Lawson 2007; Suchting et al. 2007). Furthermore, while use of a Vegfa trap has been shown to reduce \textit{Dll4} RNA expression levels (Lobov et al. 2007), EC specific knock-out of Vegfr2 in mice maintain high levels of Dll4 protein expression at the angiogenic front of the retinal vasculature, suggesting \textit{Dll4} expression is only weakly modulated by Vegfr2 signalling (Benedito et al. 2012). Hence, these findings suggest \textit{Dll4} ligand expression may be under the control of unidentified mechanisms in the TC (Benedito et al. 2012). Furthermore, \textit{Dll4}-Notch signalling appears not to directly repress \textit{Vegfr} mRNA levels \textit{in vivo}, but may indirectly influence Vegfr protein expression. The mechanisms by which this occurs are not clear but is proposed to be post-translational (Zarkada et al. 2015; Benedito, et al. 2012). Hence, the specific mechanisms controlling NOTCH-mediated repression of
VEGFRs are yet to be determined. Altogether, it is clear that there are significant gaps in our knowledge of TC-SC specification and identification of novel mechanisms linking the VEGFR-NOTCH signalling pathways will be of paramount interest.

1.13 A strategy for the identification of TC-enriched genes

Previous work within our group developed a novel strategy for the identification of potential TC-enriched genes. As previously mentioned, Vegfr signalling promotes TC specification (Benedito, et al. 2012; Lobov et al. 2007), whereas Notch signalling represses TC fate to promote SC specification (Hellström et al. 2007; Siekmann & Lawson 2007). Hence, specific chemical inhibitors of Vegfr and Notch signalling were used to manipulate EC sprouting behaviour and gene expression to enrich for functional TC genes in zebrafish (Herbert et al. 2012). Specifically, inhibition of Notch signalling using the gamma-secretase inhibitor, DAPT, enhanced the numbers of sprouting TCs and thus increased expression of TC associated genes, as previously described (Herbert et al. 2012; Siekmann & Lawson 2007). Conversely, by incubation of embryos with the Vegfr tyrosine kinase inhibitor, SU5416, sprouting ECs were completely blocked and TC specific genes would be downregulated. Moreover, low levels of SU5416 were able to achieve intermediate EC numbers and partial sprouting (Herbert et al. 2012). These findings were exploited to identify novel genes whose expression correlated with increased TC numbers. Prior to blood vessel sprouting, Tg(kdrl:GFP)s843 x Tg(gata1:DsRed)sd2 were treated with DMSO, 2.5 μm SU5416, 0.625 μm SU5416, DAPT and a combination of SU5416 and DAPT (Figure 1.7A). At 30 hpf, fluorescence-activated cell sorting (FACS) analysis was used to isolate ECs (Figure 1.7A). ECs were identified as GFP positive; however, since the kdrl promoter has weak activity in primitive erythroid cells, any cells positive for gata1 were excluded (Figure 1.7A). Isolated, chemically-manipulated ECs were then subjected to transcriptomic analysis compared to DMSO controls, and genes associated with TC behaviour were identified (Figure 1.7B). Amongst 109 genes that were identified (Herbert et al. 2012), including genes already known to be enriched in the TC such as flt4 (Siekmann & Lawson 2007), was transmembrane 4 L six family member 1 (tm4sf1), a previously unknown TC-associated transcript which encodes for a tetraspanin-like protein.
Figure 1.7: Pharmacological strategy for the identification of potential TC-enriched genes. (A) At 22 hpf, \( Tg(kdrl:GFP)_{s843} \times Tg(gata1:DsRed)_{sd2} \) were treated with DMSO, 2.5 \( \mu \text{M} \) SU5416, 0.625 \( \mu \text{M} \) SU5416, DAPT and a combination of SU5416 and DAPT. At 30 hpf, \( kdrl:GFP \) positive/gata1:dsRed ECs were isolated by FACs analysis and subjected to transcriptomic analysis compared to DMSO controls. (B) The chemical treatments manipulated TC and SC numbers and thus the transcriptomic profile of sprouting ECs. TC-associated gene expression was down-regulated following VEGFR inhibition with SU5416, upregulated when Notch signalling was inhibited by DAPT treatment and downregulated with combined SU5416/DAPT treatment. 34 TC-associated genes were identified.
1.14 The emerging role of tetraspanins in ECs

Tetraspanins are transmembrane proteins of containing 200-350 amino acids (approximately 25-50 kDa) that are highly evolutionarily conserved (Hemler 2005). They are characterised by four transmembrane domains, two extracellular loops (EC1 and EC2), a cytoplasmic region which lies between transmembrane domain 2-3, and two termini which reside within the cytoplasm (Figure 1.8). The N- and C- termini are also post-translationally palmitoylated at membrane-proximal cysteine residues, which enables interaction with other tetraspanins for the formation of tetraspanin enriched microdomains (TEMs). TEMs, also known as tetraspanin webs, are complex networks of tetraspanins that associate with various transmembrane and intracellular molecules including integrins, receptors tyrosine kinases, protein and lipid kinases (Bailey et al. 2011; Hemler 2005). They have been implicated in regulating cell motility, cell-cell fusion and signalling events; largely attributed to their association with integrins (Sawada et al. 2003; Takeda et al. 2007). The large EC2 extracellular domain contains a highly conserved region with a CCG (Cys-Cys-Gly) motif and at least two additional cysteine residues, all of which contribute to two essential disulphide bonds that maintain the EC2 structure. The large EC2 loop consists of three conserved α-helices and a variable region, whereby the majority of known tetraspanin interaction sites have been mapped (Bailey et al. 2011; Hemler 2005). Since tetraspanins often don’t possess classic receptor-ligand activities, their importance has been largely discounted. However, increasing evidence is highlighting an essential role for tetraspanins in many major cellular processes, including cell morphology, motility, invasion, fusion and signalling (Bailey et al. 2011; Hemler 2005).

It is anticipated that tetraspanins play a major role in EC function since 23 of the 32 tetraspanins identified are expressed in ECs at the mRNA level (Bailey et al. 2011). For example, Tspan12 is exclusively expressed in the retinal vasculature in mouse autosomal dominant mutations in Tspan12 have been identified as causative mutations in familial exudative vitreoretinopathy (FEVR) (Poulter et al. 2010; Yang et al. 2011), a genetic condition caused by incomplete development and haemorrhage of the retinal vasculature. Tspan12 null mice showed striking similarities to vascular defects observed with loss of for Norrin, Fzd4 or Lrp5 and a defective vascular sprouting is observed in Tspan12/Norrin and Tspan12/Lrp5 heterozygotes. Furthermore, physical interactions between Tspan12 and Fzd4/Lrp5 were identified. Hence Tspan12 may modulate β-catenin signalling in the retinal vasculature (Junge et al. 2009). Additionally, tetraspanin CD63 has also been
Figure 1.8: The basic structure of tetraspanins. Tetraspanins consists of four transmembrane domains flanked by relatively short N and C terminal tails, which protrude into the cytoplasm. There is also another cytoplasmic loop which resides between transmembrane domains 2 and 3. Extracellularly, tetraspanins exhibit two extracellular loops; a small extracellular loop denoted EC1 and a large extracellular loop, denoted EC2. The N- and C- termini are post-translationally palmitoylated at membrane-proximal cysteine residues and the large EC2 extracellular domain contains a highly conserved CCG motif and between two and six additional cysteine residues. The large EC2 loop consists of three conserved α-helices and a variable region, important for known tetraspanin interactions.
implicated in controlling angiogenic sprouting. Loss of CD63 in HUVECs results in defective angiogenic sprouting and cell adhesion, and CD63 is predicted to interact with β1-integrin and VEGFR2. CD63 knockdown hindered the association of β1-integrin with VEGFR2. Loss of VEGFR2-β1 integrin interaction subsequently affected the VEGF-mediated activation of PLCγ, ERK1/2, and AKT. In vivo analysis of VEGFR2 phosphorylation in CD63 null mice showed a decrease in VEGFR2 phosphorylation at Tyr-949 and Tyr-1173. Thus, CD63 is important for VEGFR2-β1 integrin complex formation for the activation of VEGFR signalling during angiogenesis (Tugues et al. 2013). In summary, tetraspanin family member proteins are already known to play key roles in regulating VEGFR activity and new blood vessel formation. Hence, there is a precedent for the involvement of TM4SF1 in angiogenesis.

1.15 Transmembrane 4 L six family member 1 (TM4SF1)

Human TM4SF1 is a 21 kDa, four transmembrane domain protein that belong to the L6 family, which contains three other related proteins, TM4SF4 (intestinal and liver transmembrane protein, il-TMP), TM4SF5 and TM4SF18 (L6D) (Wice & Gordon 1995; Wright, et al. 2000; Muller-Pillasch et al. 1998; Wright & Tomlinson 1994). The L6 family members have comparable topology to proteins of the tetraspanin superfamily and were originally regarded as tetraspanins. However, in depth characterisation of L6 family members in comparison to tetraspanins revealed that they lack the CCG motif in the EC2 loop that is highly conserved between all tetraspanins (Wright et al. 2000) (Figure 1.9). TM4SF1 possesses a C-terminal consensus PSD-95/DlgA/ZO-1 (PDZ) binding motif (X-Tyr-X-Cys; figure 1.9) (Borrell et al. 2000). PDZ-binding motifs bind to PDZ domains by the formation of peptide-binding clefts. PDZ domains are typically present at the C-termini of membrane-associated proteins and facilitate the formation and localisation of multiprotein complexes for the control of cell signalling pathways (Fanning & Anderson 1999). Hence, PDZ-motif interactions may be important for the function of TM4SF1.

TM4SF1 was first identified in 1986 following a monoclonal antibody screen of antibodies raised against human lung carcinoma. The L6 antibody was found to react with most carcinomas of the breast and colon and was localised to the cell surface of tumour cells (Hellstrom et al. 1986). Since its initial identification, TM4SF1 has been reported to be expressed by many cancers, including gastric, lung (Kao et al. 2003), prostate (Allioli et al. 2011), and pancreatic cancer (Zheng et al. 2015). Its expression is associated with
Figure 1.9: The structure of TM4SF1. TM4SF1 is a tetraspanin-like protein. Like tetraspanins, TM4SF1 has four transmembrane domains (TM1-4) and the N-terminal, intracellular loop and C-terminal resides within the cytoplasm. TM4SF1 also contains two extracellular loops, EC1 (small) and EC2 (large). However, the large loop lacks the CCG motif common to all known tetraspanins, but does contain N-glycosylation site (indicated). TM4SF1 also possesses a C-terminal PDZ domain (indicated), at the far extremity of the C-terminal tail, most likely important for protein-protein interactions. Underlined numbers indicate the number of amino acids predicted to contribute to each region of TM4SF1.
enhanced tumour cell motility, invasive properties, metastatic properties and poor prognosis (Gordon et al. 2011; Allioli et al. 2011; Miao et al. 2013). Hence, much of the research into the functional role of TM4SF1 in cancer cells suggests a role in cell migration. In prostate cancer cells, siRNA-mediated knockdown of TM4SF1 rendered cells less motile in in vitro wound closure experiments (Allioli et al. 2011). In agreement with this, mRNA-141-mediated down-regulation of TM4SF1 in pancreatic cancer cells disrupts cell invasion and migration (Miao et al. 2013). Mechanistically, the role of TM4SF1 in cancer cells is not clear. However its presence in TEMs and association with the tetraspanins CD81, CD151 and CD63, suggests its cooperation with multiple membrane proteins in the modulation of cell motility (Lekishvili et al. 2008). Furthermore, siRNA-mediated knockdown of TM4SF1 causes an increase in CD81 and CD63 expression and this might be due to the compensatory upregulation of other genes (Lekishvili et al. 2008), a phenomenon that has been reported in the absence of other tetraspanins (Fradkin et al. 2002). The localisation of TM4SF1 within TEMs requires the three cytoplasmic portions of the protein. When these are mutated, TM4SF1 is not recruited to TEMs and cells lose their migratory phenotype (Lekishvili et al. 2008). Altogether, the upregulation of TM4SF1 in cancer cells appears to render the cells more motile and invasive.

1.16 The physiological role of TM4SF1 in ECs

As TM4SF1 is highly expressed on the surface of a numerous tumours from many origins, much research has been focused on the association of TM4SF1 with cancer. However, the physiological role of TM4SF1 is largely unknown. Under physiological conditions, the expression pattern of TM4SF1 in human tissue is weak but primarily restricted to ECs of the vasculature (Denardo et al. 1991; Marken et al. 1992) but has also been reported in keratinocytes (Storim et al. 2001). Similarly in mouse, Tm4sf1 transcripts were identified in numerous normal tissues at low levels but were most predominantly enriched in the endothelium, lung and skin (Edwards et al. 1995). ISH and immunofluorescence staining found TM4SF1 to be expressed at high levels in vascular ECs supplying several human cancers that originated from the kidney, ovary, breast and colon (Shih et al. 2009). Moreover, expression was also investigated in cultured ECs, including HUVECs, which revealed that TM4SF1 was expressed at around 70-90 mRNA copies per cell (Shih et al. 2009). Importantly, expression of TM4SF1 was enhanced upon stimulation of HUVECs with VEGFA or thrombin, whereas other stimulants, including
TNFα, TGFβ, FGFβ and HGF had no effect on \textit{TM4SF1} expression. This would be consistent with a VEGF-dependent induction of expression in TCs. TM4SF1 expression was localised at the plasma membrane, perinuclear vesicles and in particular, filopodial extensions, where TM4SF1 was expressed in regular banded clusters (Shih et al. 2009). By use of a TM4SF1 specific monoclonal antibody, 8G4, and nanogold transmission electron microscopy, a recent report from Sciuto et al (2015) confirmed the presence of TM4SF1 in cytoplasmic vesicles, but also proposed TM4SF1 was present in nuclear pores and nucleoplasm. Entry of TM4SF1 into intracellular vesicles by internalisation was dynamin-dependent and fluorescent images suggest TM4SF1 may be transported to the nucleus via microtubules (Sciuto et al. 2015). Hence, the subcellular localisation of TM4SF1 is tightly controlled and hints at roles in membrane trafficking and/or guidance of cells.

In addition to the localisation of TM4SF1 in ECs, \textit{in vitro} studies have suggested roles for TM4SF1 in EC function. Following siRNA knockdown of \textit{TM4SF1}, significant changes in ECs structure and function indicative or stress and senescence were observed (Zukauskas et al. 2011). Interestingly, loss of \textit{TM4SF1} has a detrimental effect on EC migration and filopodia formation \textit{in vitro} during a wound healing and tube formation assays (Zukauskas et al. 2011). Conversely, overexpression of TM4SF1 in HUVECs induces ectopic filopodial projections of longer length compared to control cells. An interesting observation is that introduction of GFP to N- or C- termini disrupts TM4SF1 localisation in filopodia (Zukauskas et al. 2011), suggesting that these regions may be important for its transport or function, in agreement with previous observations in cancer cells (Lekishvili et al. 2008). Taken together, the TM4SF1 loss and gain of function phenotypes observed in HUVECs suggest a potential role for TM4SF1 in EC migration and filopodia formation.

\subsection*{1.17 Tm4sf1 interaction partners}

Consistent with the potential localisation of TM4SF1 in TEMs, TM4SF1 has been found to interact with a number of proteins, typical of TEMs. Integrins αVβ3 and αVβ5 are known to play important roles angiogenesis by cooperating with VEGFR2 for the modulation of signalling and VEGFR2 recycling (Reynolds et al. 2009; Lakshmikanthan et al. 2011; Somanath et al. 2009). Co-immunoprecipitation experiments found integrin subunits α5 and β1 constitutively interact with TM4SF1 in a VEGFA-independent manner. In contrast, TM4SF1 only associated with αV and its two partners, β3 and β5, following stimulation of HUVECs with VEGF (Shih et al. 2009). This suggests TM4SF1-integrin
interactions may be important for EC function during angiogenesis. In addition to integrins, TM4SF1 also co-localises with CD63, CD81 and CD151 in TEMs (Lekishvili et al. 2008) and its presence in TEMs is important for filopodia formation and cell migration (Zukauskas et al. 2011; Shih et al. 2009). Hence, while the function of TM4SF1 is currently uncharacterised, its interactions with specific integrins and tetraspanins within TEMs may prove important.
1.18 Experimental Aims

During angiogenesis, ECs are hierarchically organised into leading TCs and trailing SCs that exhibit differential behaviours, essential for their collective migration and formation of a new vascular sprout. The differences in TC and SC behaviour are often attributed to DLL4-NOTCH lateral inhibition; however, the classical ‘salt and pepper’ pattern of TCs and SCs does not emerge. Hence, there must be factors contributing to the specification of ECs during sprouting. We have identified \textit{tm4sf1} as a potential TC-associated gene. The limited data available regarding the physiological role of human TM4SF1 in ECs is derived from \textit{in vitro} investigations and hints that it may be involvement in cell motility and filopodial dynamics. However, the function of TM4SF1 is completely unknown. The aim of this thesis characterise the physiological role of TM4SF1 \textit{in vivo}. To do this, we used the zebrafish embryo model for angiogenesis to: (i) determine the localisation of \textit{tm4sf1} mRNA and the mechanisms regulating \textit{tm4sf1} expression in zebrafish embryos, (ii) explore the consequences of \textit{tm4sf1} knockdown in zebrafish embryos using splice site-targeting MOs, (iii) generate a \textit{tm4sf1} loss of function mutant fish line for the confirmation of MO phenotypes and for in depth characterisation of \textit{tm4sf1} knock out, and (iv) begin to identify the mechanistic function of Tm4sf1 \textit{in vitro} using primary EC culture.
CHAPTER 2: Materials and methods

2.1 Embryo Manipulations

2.1.1 Animal subjects

The investigations described were performed using the following fish lines which were already established: Wild type (WT), Tg(kdrl:GFP)\textsuperscript{s43} (Jin et al. 2005), Tg(kdrl:nlsEGFP)\textsuperscript{zf109} (Blum et al. 2008), and cloche (clo\textsuperscript{e5}) mutants (Stainier et al. 1995). Animals were housed in a designated environment in the animal housing unit of the University of Manchester. Animal procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the University of Manchester Ethics Committee in accordance with the Home Office Regulations. All scientific procedures were carried out under project licence number 70/8132 in specified places using personal licence 40/10402.

2.1.2 Collection of zebrafish embryos

Pairs of male and female fish were set up in breeding tanks and kept separated overnight with a divider. The following morning, the dividers were removed to allow the fish to breed. Embryos were collected and raised at 28° C in petridishes containing embryo water (0.0645g tropic marin salts/ 1L double distilled water (ddH\textsubscript{2}O)). If required for live imaging studies, at 20 hpf embryos were incubated with embryo water containing 0.0045% Phenylthiourea (PTU), an inhibitor of tyrosinase, to prevent the formation of melanin pigments.

2.1.3 Morpholino oligonucleotide (MO) injection

1 mM MO stocks (Gene Tools) were diluted to 0.2 mM in distilled water (dH\textsubscript{2}O) (plus 1 µl phenol red) and injected at the indicated concentrations into the yolk of embryos at the one-cell stage. MO sequences were 5’-GCATCTGGAGATTTCAATAGAGCGC-3’ (tm4sf1 MO), 5’-GTTCGAGCTTACCGGCCACCCAAAG-3’ (dll4 MO) (Siekmann & Lawson 2007) and 5’-CCTCTTACCTCAGTTACAATTATA-3’ (control MO) (Herbert et al., 2012).

2.1.4 Tail clipping for genotyping

Individual fish were transferred into a petridish containing 3.75% tricaine diluted in embryo water (pH 7.4). When the fish became unconscious, they were transferred to a
clean pertridish and a small amount of tissue (approximately 1 mm²) was removed from the tail using a scalpel. The tissue was transferred to a microcentrifuge tube and immediately placed on ice. The fish were transferred into a temporary tank containing fish water at 28° C to recover. When the fish had fully recovered, they were sorted into individual tanks until their genotypes had been verified. The collected tissue was processed for genomic DNA extraction (section 2.5.1.3.).

2.1.5 Pharmacological treatments

Embryos were dechorionated manually and incubated with 2.5 µM SU5416 or 0.05% dimethyl sulfoxide (DMSO; vehicle) diluted in embryo water, for 1 to 3 h. After which, embryos were processed for RNA extraction as described in section 2.2.1.

2.2 RNA and DNA Manipulation

2.2.1 RNA extraction

20-50 whole embryos were transferred to microcentrifuge tubes and 250 µl Trizol (Invitrogen) was added. Embryos were lysed and homogenised using a mini pestle (Eppendorf) until embryos were no longer visible, before an additional 750 µl trizol was added and incubated for 5 min at room temperature. Samples were centrifuged for 15 min at 12,000 rpm to pellet cell debris. The supernatant was transferred to a fresh tube and 200 µl chloroform was added, vortexed and incubated at room temperature for 2 min before another 15 minute centrifugation at 12,000 rpm. The aqueous phase was transferred to another tube. 500 µl isopropanol was then added and incubated at room temperature for 10 min. Samples were centrifuged at 12,000 rpm for 10 min to pellet the RNA. The supernatant was removed completely and the pellet washed in 1 ml 75% ethanol. Samples were centrifuged for 5 min at 7500 rpm before the ethanol was removed and the pellet allowed to air-dry for approximately 10 min (time dependent on yield). The pellet was then re-suspended in 50 µl RNAse-free dH₂O and incubated at 55° C for 10 min, shaking frequently. Samples were then incubated at 37° C for 15 min with DNase1 to break down any remaining DNA within the samples. The RNA was purified using the RNeasy Mini Kit (Qiagen) as per manufacturer’s guidelines.

2.2.2 cDNA synthesis

1 µg mRNA was converted into complimentary DNA (cDNA) using either the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) or the SuperScript®
III First Strand Synthesis System (Invitrogen), as per manufacturers’ guidelines. cDNA concentration was determined using a NanoDrop spectrophotometer.

2.2.3 Polymerase Chain Reaction (PCR)

Both Taq and Pfx polymerases were used for PCR amplification. Taq polymerase reactions consisted of 0.2 mM dNTPs (Invitrogen), 0.5 μM each forward and reverse primer (Eurogentec, table 2.2), 1x amplification buffer (Roche), 1.25 U Taq polymerase (Roche) and a variable amount of cDNA or genomic DNA template (0.1 – 250 ng). Pfx polymerase reactions consisted of 1-2x Pfx amplification buffer, 0.3 μM each forward and reverse primer, 0.3 mM dNTPs, 1 mM MgSO₄, 1U Platinum Pfx DNA Polymerase (Invitrogen) and a variable amount of cDNA or genomic DNA template (0.1 – 250 ng). DNA was amplified on a T100 thermal cycler (Bio-Rad) using cycle parameters specific for Taq and Pfx reactions (table 2.1). Annealing temperatures were optimised for each set of primers and the length of the extension step was adjusted according to the length of the amplicon. Reaction products were loaded and run on a 1 or 2% agarose gel containing 0.01 μl/ml ethidium bromide and imaged using a transilluminator.

<table>
<thead>
<tr>
<th>Step</th>
<th>Taq</th>
<th>Pfx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temp (°C)</td>
<td>Time (min)</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94</td>
<td>2</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94</td>
<td>0.5</td>
</tr>
<tr>
<td>Annealing</td>
<td>*</td>
<td>0.5</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>**</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>7</td>
</tr>
</tbody>
</table>

*annealing temperature optimised for each set of primers; **extension time optimised for length of amplicon
Table 2.2: Primers used for PCR amplification and sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin F1</td>
<td>5’- TGTTTTCCCCCTCCATGGTTGG - 3’</td>
</tr>
<tr>
<td>β-actin R1</td>
<td>5’- TTCTCTGTGATGTTTCGAGGA - 3’</td>
</tr>
<tr>
<td>Tm4sf4 F</td>
<td>5’- CAACCTCCCCGGCACTTGTTAT - 3’</td>
</tr>
<tr>
<td>Tm4sf4 R</td>
<td>5’- AAATCGACTCACTATAGGGAATAAGGATCGCCCATCCAGCAGG - 3’</td>
</tr>
<tr>
<td>Egfp F</td>
<td>5’- AAATCGACTCACTATAGGGTTTACTGTTACAGCTCGTCCATGG - 3’</td>
</tr>
<tr>
<td>Tm4sf4 F</td>
<td>5’- TCCTTTCCAAGGCTGGGATAG - 3’</td>
</tr>
<tr>
<td>Tm4sf4 R</td>
<td>5’- AGAATACGACTCACTATAGGGTTTACTGTTACAGCTCGTCCATGG - 3’</td>
</tr>
<tr>
<td>Tm4sf1 F1</td>
<td>5’- ATGTGCTCTACAGGATTTGCC - 3’</td>
</tr>
<tr>
<td>Tm4sf1 R1</td>
<td>5’- TGTTGCTGAGGCTGGAGTATG - 3’</td>
</tr>
<tr>
<td>Tm4sf1 R2</td>
<td>5’- TGCTACACCTAAACACCAACC - 3’</td>
</tr>
<tr>
<td>pCR8_F1</td>
<td>5’- TTGATGCCCTGAGCTTCCT - 3’</td>
</tr>
<tr>
<td>pCR8_R1</td>
<td>5’- CGAACCAGAAGCCTTTATGCC - 3’</td>
</tr>
<tr>
<td>TAL F</td>
<td>5’- TGGCGTCGCGGCAACACGTTG - 3’</td>
</tr>
<tr>
<td>TAL R</td>
<td>5’- GGGGCGAGGTGGTGGTCGTTG - 3’</td>
</tr>
<tr>
<td>Tm4sf1 TALEN ID F</td>
<td>5’- CTGTTTTTCTCCACACAC - 3’</td>
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<tr>
<td>Tm4sf1 TALEN ID R</td>
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<tr>
<td>Tm4sf1 E2.CR.ID_F</td>
<td>5’- CACTAGCTTGGCAGGAGA - 3’</td>
</tr>
<tr>
<td>Tm4sf1 E2.CR.ID_F</td>
<td>5’- CTGTTTTCTCCCCACACAC - 3’</td>
</tr>
<tr>
<td>M13 F</td>
<td>5’- GGGCCGCCGTCGCTGTGTTG - 3’</td>
</tr>
<tr>
<td>M13 R</td>
<td>5’- CAGGAAAGACTGCTGATG - 3’</td>
</tr>
<tr>
<td>SP6 F</td>
<td>5’- ATTTAGGTGACACTATATAG - 3’</td>
</tr>
</tbody>
</table>

F, forward; R, reverse. **Bold** refers to T7 promoter sequence.

2.2.4 Quantitative real-time PCR (qPCR)

cDNA samples were diluted to a concentration of 50 ng/μl with dH2O. Each qPCR reaction was prepared in triplicate in a 48 or 96-well plate with each well consisting of 0.2 μM each forward and reverse primer (table 2.4), 50ng cDNA and SYBR Green Mastermix (Applied Biosystems). Reactions were run on an Eco Real-Time PCR System (Illumina) or Step One Plus Real-time PCR System (Applied Biosystems) alongside negative controls. The cycle parameters are shown in table 2.3. Real-time-PCR data was analysed by the ΔΔCt method (Livak & Schmittgen, 2001). Expression was normalised to β-actin and EF1α (zebrafish) and GAPDH (human). A relative quantification of gene expression was then determined using the formula 2ΔΔCt.

Table 2.3: Cycle parameters for the RT-PCR reactions

<table>
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<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Cycles</th>
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</thead>
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<td>Holding Stage</td>
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<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>0.25</td>
<td>40</td>
</tr>
<tr>
<td>Anneal/extend</td>
<td>60</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>Melting Curve</td>
<td>+3 each from 60, each cycle</td>
<td>0.25</td>
<td>10</td>
</tr>
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</table>

54
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>zβ actin F</td>
<td>5' - CGAGCTGTCTTTCCCATCCA - 3'</td>
<td>Tang et al (2007)</td>
</tr>
<tr>
<td>zβ actin R</td>
<td>5' - TCACAAACGTAGCTTCTTTCTG - 3'</td>
<td></td>
</tr>
<tr>
<td>zDll4 F</td>
<td>5' - TGGCCATTTATCCGTTCACC - 3'</td>
<td>Roukens et al (2010)</td>
</tr>
<tr>
<td>zDll4 R</td>
<td>5' - ACTACCTGCATCCCTCCAGAC - 3'</td>
<td></td>
</tr>
<tr>
<td>zEf1α F</td>
<td>5' - CTGGAGGCCAGCTCAAAACAT - 3'</td>
<td>Tang et al (2007)</td>
</tr>
<tr>
<td>zEf1α R</td>
<td>5' - ATCAAGAAGAGTAGTACCGCTACATTAC - 3'</td>
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</tr>
<tr>
<td>zFlt4 F</td>
<td>5' - CTGTCGAGTTTGAGTGGA - 3'</td>
<td>Covassin et al (2006)</td>
</tr>
<tr>
<td>zFlt4 R</td>
<td>5' - GGTGGAATCATGAATACCCCATTC - 3'</td>
<td></td>
</tr>
<tr>
<td>zKdrl F</td>
<td>5' - ACTTTGAGTGAGGTTTCATAAGGA - 3'</td>
<td>Covassin et al (2006)</td>
</tr>
<tr>
<td>zKdrl R</td>
<td>5' - TGGAGACCGGTGTTGTGCTA - 3'</td>
<td></td>
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<tr>
<td>zTm4sf1 F</td>
<td>5' - CTGGATACTGCTCCCTGATCTC - 3'</td>
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<tr>
<td>zTm4sf1 R</td>
<td>5' - AAAACAGATACCCGCCCTCAT - 3'</td>
<td></td>
</tr>
<tr>
<td>zTm4sf4 F</td>
<td>5' - AGCTAATAACGGCCACTTACC - 3'</td>
<td></td>
</tr>
<tr>
<td>zTm4sf4 R</td>
<td>5' - CAGGTGACAGCCGAGGA - 3'</td>
<td></td>
</tr>
<tr>
<td>zTm4sf5 F</td>
<td>5' - TGGAAATATCCATTTGAGGACA - 3'</td>
<td></td>
</tr>
<tr>
<td>zTm4sf5 R</td>
<td>5' - TGGAGACCGGTGTTGTGCTA - 3'</td>
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<tr>
<td>zCD63 F</td>
<td>5' - TGCAATCATTCTCTCCTCCTCA - 3'</td>
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<tr>
<td>zCD63 R</td>
<td>5' - GAGGGTTGTGCGGTATTCCT - 3'</td>
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<tr>
<td>zCD81 F</td>
<td>5' - ACAAGAATCTCTGCCACTGCT - 3'</td>
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<tr>
<td>zCD81 R</td>
<td>5' - GGTGTCATGAGCCACCTATC - 3'</td>
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<tr>
<td>zCD151 F</td>
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<tr>
<td>zCD151 R</td>
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<tr>
<td>zZgc:172079 F</td>
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<tr>
<td>zSi:ch211-137i24.10 F</td>
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<td>zLOC100002960 F</td>
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<td>hDll4 F</td>
<td>5' - CTGGAGAACGAGGTTTTGCT - 3'</td>
<td></td>
</tr>
<tr>
<td>hDll4 R</td>
<td>5' - CAGTTGAGAACGAGGTTTTGCT - 3'</td>
<td></td>
</tr>
<tr>
<td>hHES1 F</td>
<td>5' - CAGGCTACAGGTGTCAGA - 3'</td>
<td></td>
</tr>
<tr>
<td>hHES1 R</td>
<td>5' - TGGAAATATCCATTTGAGGACA - 3'</td>
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<tr>
<td>hGAPDH F</td>
<td>5' - TGGCGATGACGCTTCGT - 3'</td>
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<tr>
<td>hGAPDH R</td>
<td>5' - GGCATTGGAATGCTTGTA - 3'</td>
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</tr>
<tr>
<td>hVE-Cad F</td>
<td>5' - GAGGGCCAGCCCAAACGTT - 3'</td>
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<td>hVE-Cad R</td>
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<td>hJag1 F</td>
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<tr>
<td>hJag1 R</td>
<td>5' - ATCGCGGTGGGTTCGCAG - 3'</td>
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<tr>
<td>vVEGFR3 F</td>
<td>5' - GGGGTAGCTGAGCTTGTA - 3'</td>
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<tr>
<td>vVEGFR3 R</td>
<td>5' - AAAAGGAGGTTTTCACGCTA - 3'</td>
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<tr>
<td>hVEGFR2 F</td>
<td>5' - CGGGTCGTGTTGAGCTTGTA - 3'</td>
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<tr>
<td>hVEGFR2 R</td>
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<td>hVEGFR1 R</td>
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<td>hTM4SF1 F</td>
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<tr>
<td>hTM4SF1 R</td>
<td>5' - ATCGGGTCAGGTTTGTGCTGCT - 3'</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse; z, zebrafish; h, human
2.3 *In situ* hybridisation (ISH)

2.3.1 Embryo Fixation

Embryos were manually dechorionated with fine tweezers and fixed at 22, 24, 26, 28 and 30 hpf. For fixation, around 20 embryos were transferred to a microcentrifuge tube containing 1.5 ml 4% paraformaldehyde (PFA) diluted in 1x PBS. The embryos were incubated at 4° C overnight and gently rocked. If not being used immediately for experiments, PFA was removed and replaced with either 100% MeOH (for ISH) or 1 x PBS (for confocal imaging) and stored at -20° C or 4° C, respectively.

2.3.2 Generation of ISH RNA probes

For the *tm4sf1* whole mount chromogenic ISH probe, pCR-Blunt II-TOPO plasmid (Invitrogen) containing the *tm4sf1* open reading frame was linearised with EcoRV at 37° C for 3 h and linearisation was confirmed upon loading and running a sample on an agarose gel. The linearised plasmid was isolated using the QIAquick PCR purification kit (Qiagen) as per manufacturer’s guidelines. The concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). For the *tm4sf4* probe, primers were designed to amplify the full length of *tm4sf4* and included the T7 RNA polymerase promoter (5′-TAATACGACTCACTATAGGG-3′), located at 5′ extremity of the reverse primer (table 2.2). PCR was used to amplify the probe template from cDNA from zebrafish embryos. The PCR product was purified using the QIAquick PCR purification kit as per manufacturers’ guidelines.

Chromogenic ISH probes were synthesised, using DIG to label, for 2 h at 37° C using SP6 (NEB) or T7 (Promega) RNA polymerase, as per manufacturer’s guidelines (see table 2.5 for reaction components). 2 μl RNase-free DNase I (Roche) was then added to each reaction for 30 min at 37° C to degrade the DNA template. To terminate the reaction, 1 μl 0.5M ethylenediaminetetraacetic acid (EDTA) and 9 μl distilled water was subsequently added.

For the *tm4sf4* and *egfp* FISH probes, primers were designed to include the T7 RNA polymerase promoter (5′-TAATACGACTCACTATAGGG-3′; table 2.2), located at 5′ extremity of the reverse primer. PCR was used to amplify the probe template from *Tg(kdrl:nlsEGFP)zpf109* zebrafish cDNA. The *tm4sf4* RNA probe was synthesised as described above for ISH probes using T7 RNA polymerase. The *egfp* probe was
synthesised by T7 RNA polymerase with dinitrophenol (DNP) (PerkinElmer) in place of DIG at a final concentration of 100 μM.

**Table 2.5: Reaction components for the synthesis of RNA probes for ISH**

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>T7 RNA Synthesis Kit</th>
<th>SP6 RNA Synthesis Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
<td>1 μg</td>
<td>1 μg</td>
</tr>
<tr>
<td>10x NTP-DIG-RNA</td>
<td>2 μl</td>
<td>2 μl</td>
</tr>
<tr>
<td>Transcription buffer</td>
<td>2 μl (5x buffer)</td>
<td>2 μl (10x buffer)</td>
</tr>
<tr>
<td>0.1 M DDT</td>
<td>2 μl</td>
<td>-</td>
</tr>
<tr>
<td>RNAsein</td>
<td>1 μl T7</td>
<td>2 μl SP6</td>
</tr>
<tr>
<td>Polymerase</td>
<td>1 μl</td>
<td>2 μl SP6</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Up to 20 μl</td>
<td>Up to 20 μl</td>
</tr>
</tbody>
</table>

Probes were purified using a SigmaSpin column (Sigma Aldrich) as per manufacturer’s guidelines. 1 μl 0.5M EDTA and 9 μl RNAlater was added to the eluted probe and the probe concentration was determined using a NanoDrop spectrophotometer. The probe was aliquotted and stored at -80°C until use.

2.3.3 Whole mount ISH

Embryos were fixed in 4% PFA and incubated at 4°C overnight as described in section 2.1.4. Following fixation, embryos were dehydrated for 20 min by the addition of 1 ml 100% MeOH. The MeOH was replaced with fresh MeOH before storage of the embryos at -20°C for at least 24 h and up to 2 months. Embryos were rehydrated in 1 ml of the following vol/vol dilutions of MeOH in 1 x phosphate buffer solution (PBS): 5 min in 75% MeOH, 5 min in 50% MeOH and 5 min in 25% MeOH. These incubations were followed by four 5 minute washes in 1% PBT (1 x PBS + 0.1% Tween 20). PBT was replaced with 10 μg/ml proteinase K (diluted in 1 x PBT) to permeabilise the embryos. Proteinase K incubation time was dependant on the age of the embryos (table 2.6). Embryos were then fixed in 4% PFA for 20 min before four 5 minute PBT washes. PBT was then replaced with 700 μl hybridisation solution 1 (Hyb+; 50% deionised formamide, 5x saline sodium citrate (SSC), 0.1% Tween 20, 50 μg ml⁻¹ of heparin, 500 μg μl⁻¹ of RNase-free tRNA adjusted to pH 6.0 by adding citric acid) and then brought to 65°C in an incubator with 50 rpm rotation. Embryos were incubated for 2-5 h before Hyb+ was discarded and replaced with 200 μl pre-warmed Hyb+ solution containing 100 μg of tm4sf1
or *tm4sf4* pre-warmed probe. Embryos were then incubated overnight at 65°C with rotation.

<table>
<thead>
<tr>
<th>Embryo Age</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 hpf</td>
<td>10 min</td>
</tr>
<tr>
<td>22 hpf</td>
<td>10 min</td>
</tr>
<tr>
<td>24 hpf</td>
<td>15 min</td>
</tr>
<tr>
<td>26 hpf</td>
<td>20 min</td>
</tr>
<tr>
<td>28 hpf</td>
<td>20 min</td>
</tr>
<tr>
<td>30 hpf</td>
<td>25 min</td>
</tr>
</tbody>
</table>

Table 2.6: Proteinase K incubation time for embryo permeabilisation

The probe solution was discarded and embryos were washed briefly with pre-warmed hybridisation solution 2 (Hyb-; 50% deionised formamide, 5× SSC, 0.1% Tween 20, adjusted to pH 6.0 by adding citric acid). Embryos were subsequently washed in the following dilutions of Hyb- in 2x SSC for 10 min at each vol/vol dilution: 75% Hyb-, 50% Hyb-, and 25% Hyb-. SSC controls the stringency of wash buffer for the washing steps after hybridization. This was followed by a 10 minute wash with 2x SSC and two 30 minute washes with 0.2x SSC. Embryos were then removed allowed to cool to room temperature. This was followed by further washes in the following dilutions of 0.2x SSC in 1% PBT for 10 min at each vol/vol dilution: 75% 0.2x SSC, 50% 0.2x SSC, and 25% 0.2x SSC. After a 10 minute wash with PBT, embryos were incubated in 1 ml blocking solution (PBT, 2% sheep serum, 2 mg/ml bovine serum albumin (BSA)) at room temperature for 3-4 h. Blocking solution was discarded and replaced with 1.5 ml blocking solution containing a 1:10,000 dilution anti-DIG Ab. Embryos were incubated overnight at 4°C with agitation.

Embryos were washed briefly with PBT, before six 15 minute washes in PBT at room temperature with agitation. This was followed by three washes with alkaline phosphatase (AP) buffer (100 mM Tris HCl, pH 9.5, 50 mM MgCl₂, 100 mM NaCl and 0.1% Tween 20) for 5 min per wash. Embryos were transferred from microcentrifuge tubes to a 24 well plate and 1 ml staining solution (stock solution of 10 ml AP buffer + 200 μl Nitro blue tetrazolium (NBT)/5-Bromo 4-chloro 3-indolyl phosphate (BCIP)) was added to each well. Embryos were incubated in the dark, at room temperature without agitation.
until staining was observed. For \textit{tm4sf1}, this would take overnight whereas \textit{tm4sf4} took up to three days. When sufficient staining intensity was observed down the microscope, embryos were transferred back to fresh microcentrifuge tubes and washed three times in PBT for 5 min per wash. This was followed by fixation in 4% PFA for 1 h and a further six PBT washes of 5 min each. Embryos were transferred to a 24 well plate and cleared in 1 ml glycerol. Plates were agitated overnight in the dark prior to imaging the embryos.

2.3.4 Fluorescent ISH

Embryos were fixed at 26 hpf in 4% PFA and dehydrated in 100% MeOH as described in section 2.3.1. Prior to permeabilisation of embryos, they were rehydrated as described in section 2.2.1. Embryos were permeabilised in proteinase K for 20 min. Embryos were then fixed in 4% PFA for 20 min with rocking before four 5 minute PBT washes. PBT was then replaced with 700 μl hybridisation solution 1 (Hyb+) and then brought to 65° C in an incubator with 50 rpm rotation. Embryos were incubated for 1-5 h before Hyb+ was discarded and replaced with 200 μl pre-warmed Hyb+ solution containing 150 ng of each pre-warmed probe. Embryos were then incubated overnight at 65° C with rocking. The probe solution was discarded and embryos were washed briefly with pre-warmed hybridisation solution 2 (Hyb-; 50% deionised formamide, 5× SSC, 0.1% Tween 20, adjusted to pH 6.0 by adding citric acid). Embryos were subsequently washed in the following dilutions of Hyb- in 2x SSC for 10 min at each vol/vol dilution: 75% Hyb-, 50% Hyb-, and 25% Hyb-. SSC controls the stringency of wash buffer for the washing steps after hybridization. This was followed by a 10 minute wash with 2x SSC and two 30 minute washes with 0.2x SSC. Embryos were then removed allowed to cool to room temperature. This was followed by further washes in the following dilutions of 0.2x SSC in 1% PBT for 10 min at each vol/vol dilution: 75% 0.2x SSC, 50% 0.2x SSC, and 25% 0.2x SSC. After a 10 minute wash with PBT, an additional two washes were performed in maleic acid buffer (150 mM Maleic acid, 100 mM NaCl, 0.1% Tween-20, 7.9 g/L NaOH pH 7.5; MAB) for 5 min each prior to blocking. Embryos were incubated for 1 hour at room temperature in 0.5mL blocking buffer (MAB/2% Blocking Reagent (Roche, BM)/20% Lamb Serum) followed by incubation with 0.5 mL anti-DIG-POD antibody (1:1000; Roche) diluted in blocking buffer at 4° C, rocking overnight. The antibody solution was removed and the embryos were washed in MAB-0.1% Tween three times for 5 min. This was followed by 5 further washes in 1xMAB, each for 20 min. Embryos were then pre-washed in amplification buffer (Perkin Elmer Kit NEL744001KT) at room temperature for 10 min. For detection of the DIG-labelled probe, embryos were incubated
for 30 min in Tyramide Signal Amplification (TSA) - Cy3 solution (1 in 100 dilution in amplification buffer), rocking at room temperature. TSA-Cy3 uses horseradish peroxidase (HRP) to catalyze covalent attachment of Cy3 labels directly adjacent to the target-of-interest to enable the sensitive detection of RNA probes. Embryos were then washed three times for 10 min at room temperature in PBT with agitation. This was followed by four 5 minute washes with PBT, rocking gently. Embryos were incubated with 0.5 ml blocking buffer for 1 hour at room with gentle rocking. Blocking buffer was replaced with 0.5 ml blocking buffer containing anti-DNP-POD antibody (1:500; Perkin-Elmer) at 4° C overnight, rocking gently. Anti-DNP-POD antibody binds the egfp probe. After the overnight antibody incubation, embryos were washed five times for 20 min each in MAB. A subsequent wash for 10 min at room temperature in amplification buffer. For detection of the DNP-labelled probe, we incubated embryos for 30 min in TSA-Cy5 solution (1:100 dilution in amplification buffer), with rocking. Embryos were then washed three times for 10 min in PBT at room temperature and stored in PBT overnight at 4° C, rocking gently prior to imaging.

2.4 Microscopy

For confocal live imaging, zebrafish embryos were manually dechorionated and anesthetised in embryo water containing 3.75% tricaine and 0.0045% PTU. Embryos were then embedded in 1.0% low melt agarose (also containing tricaine) in a glass bottom dish at approximately 22 hpf. Embryos were imaged on a Zeiss LSM700 confocal microscope using a 20x dipping objective lens. Movies were analysed using ImageJ.

2.5 Genome Editing
2.5.1 Transcription activator-like effector nucleases (TALENs)
2.5.1.1 Design and assembly of TALENs

TALENs were designed to target exon 1 of zebrafish tm4sf1 using a predictive online tool (https://tale-nt.cac.cornell.edu/node/add/talen). The TALEN was selected and constructed using protocol described by Cermak et al (2011). Here we describe the construction of the TALEN in detail. This is also represented diagrammatically in figure 4.3. The target sequences chosen for the forward and reverse TALENs were 5’–TGTGCTCTACAGGATTTGCC-3’ and 5’-GCCCTGGTCCCTCTCGCCA-3’, respectively. Generation of the TALEN involved two steps.

Step 1: Assembly of RVDs into intermediate plasmids, pFUS_A and pFUS_B
Plasmids containing modules that encode RVDs 1–10 in the array were selected using plasmids numbered in that order. Thus, for the forward TALEN, the first 10 module plasmids selected for the forward TALEN were: pNN1 pNG2 pNN3 pHD4 pNG5 pHD6 pNG7 pNI8 pHD9 pNI10, and for the reverse TALEN: pNN1 pNN2 pHD3 pNN4 pNI5 pNN6 pNI7 pNN8 pNN9 pNN10. Modules from these plasmids were cloned into array plasmid pFUS_A using Golden Gate cloning (Cermak et al. 2011). Next, the remaining module plasmids for RVDs 11–n were selected, again starting with plasmids numbered from 1. Thus, for the forward TALEN, the eight module plasmids selected were: pNN1 pNN2 pNI3 pNG4 pNG5 pNG6 pNN7 pHD8, and for the reverse TALEN, pNI1 pHD2 pHD3 pNI4 pNN5 pNN6 pNN7. The modules encoding RVDs 11-20 were cloned into a pFUS_B array plasmid, which are numbered pFUS_B1-10, selected according to the number of modules going in. Thus, pFUS_B8 and pFUS_B7 were selected for the forward and reverse TALENS, respectively. The last RVD is encoded by a different plasmid, denoted the last repeat plasmid, and was added in the second step. 150 ng of each module and array plasmid were subjected to digestion and ligation in a single 20 µl reaction with 1 µl BsaI (NEB) and 1 µl T4 DNA Ligase (Roche) in T4 DNA ligase buffer (Roche). The reaction was incubated in a thermocycler for 10 cycles of 5 min at 37° C and 10 min at 16° C, and then heated to 50° C for 5 min and then 80° C for 5 min. Subsequent addition of 1 µl 25 mM ATP and 1 µl Plasmid Safe DNase (Sigma) were added to the reaction. This prevents linear DNA fragments, such as partial arrays, from recombining into array plasmids. The reaction mixture is incubated at 37° C for 1 hour, and then used to transform E.coli cells. Cells were plated on LB agar containing 50 µg/ml spectinomycin, with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl β-D-1-thiogalactopyranoside (IPTG) for blue/white screening of recombinants. Three white colonies from each transformation were selected and used for colony PCR using primers pCR8_F1 and pCR8_R1 and standard PCR reaction parameters. The primers flank the region whereby the TALEN RVDs should be inserted. PCR products were run on an agarose gel and correct clones were identified by a band of around ~1.2KB for pFUS_A arrays and slightly less for pFUS_B arrays, in addition to a smearing ‘ladder’ of bands starting at ~200 bp and every 100 bp up to ~500 bp. The smearing ladder is a consequence of the RVD repeats in the clones. Following isolation of the DNA plasmids by Miniprep (Qiagen) as per manufacturer’s guidelines, we also confirmed the clones by sequencing the region corresponding to the RVDs.
Step 2: Joining of the pFUS_A and pFUS_B containing RVDs into a destination vector to make the final construct

The second step involved the joining of intermediary arrays, pFUS_A and pFUS_B, containing the RVD modules in addition to the last repeat RVD, into the RCIscript-GoldyTALEN destination vector (D F Carlson et al. 2012). A 20 µl digestion and ligation reaction was prepared consisting of 150 ng pFUS_A, 150 ng pFUS_B, 150 ng RCIscript-GoldyTALEN destination vector, 150 ng of the last repeat plasmid, 1µl Esp3I, 1 µl T4 DNA Ligase (Roche) and 2 µl T4 DNA ligase buffer (Roche). The reaction was subjected to 10x(37° C/5min + 16° C/10min) followed by 37° C/15min and 80° C/5min. Plasmid Safe DNase treatment, as used following the first Golden Gate reaction described above, is not required for this step since the backbone plasmid termini has no homology with the array. The reaction was used to transform E. coli, and cells were plated on LB agar containing 100 µg/ml ampicillin with X-gal and IPTG. Similarly, three white colonies from each transformation were selected for colony PCR using primers TAL F and TAL R. The primers flank the region whereby the TALEN RVDs should be inserted. As previously described, a smeared/laddering effect was observed and a faint band of 2-3 KB indicated a correct clone. Plasmid DNA was isolated and inclusion of the final, full length repeat array in the RCIscript-GoldyTALEN destination vector was then confirmed by DNA sequencing.

2.5.1.2 TALEN RNA preparation

The RCIscript-GoldyTALEN plasmid containing the TALEN RVDs was digested with Sac1 restriction enzyme overnight and the linearised plasmid was purified using the PCR purification kit (Qiagen) as per manufacturers’ guidelines. TALEN mRNA was synthesised using mMessage Machine T3 kit (Ambion) as per manufacturer’s guidelines, using 1 µg linearised plasmid template. The reaction was incubated at 37° C for 2 h, and for a further 15 min following addition of 1 µl DNAse Turbo. mRNA was purified using the RNeasy Mini Kit (Qiagen) as per manufacturer’s guidelines.

2.5.1.3 Injection of embryos and high resolution melt (HRM) analysis

100 pg of both forward and reverse TALEN mRNA was injected into the single cell of WT zebrafish embryos alongside un-injected control embryos. At around 24-72 hpf, genomic DNA was extracted from individual embryos. Individual embryos were transferred into 0.2 ml tubes and excess embryos water was removed. Embryos were
incubated in 50 μl DNA extraction buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM KCl, 0.3% Tween-20, 0.3% NP-40 and 0.5 mg/ml proteinase K) for 4 h at 55° C, followed by 15 min at 95° C to inactivate the enzyme. 1-2 μl genomic DNA was used as template for PCR amplification. Following genomic DNA extraction (as described in section 2.5.1.3), a High Resolution Melt (HRM) reaction was performed on the Eco Real-Time PCR System (Illumina) consisting of 10 μl Meltdoctor HRM Mastermix (Applied Biosystems), 8 μl dH2O, 0.5 μl primers specific to tm4sf1 (10 mM TALEN ID F and R; table 2.2) and 1 μl genomic DNA. The cycle parameters are shown in table 2.7.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDG Incubation</td>
<td>50</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Polymerase activation</td>
<td>95</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>PCR Cycling</td>
<td>95</td>
<td>0.17</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>HRM Curve</td>
<td>55</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

Potential mutants were identified by assessing the melt curves of PCR products derived from TALEN injected embryos compared to un-injected embryos. Genomic DNA extracts from embryos with HRM curves that differed from WT were selected for Pfx PCR amplification of the TALEN targeted genomic region (as described in section 2.2.3). The PCR product was run on a 1% agarose gel and the DNA bands were extracted using the Gel Extraction Kit (Qiagen), as per manufacturer’s guidelines. The purified blunt PCR products were cloned into pCR-Blunt II-TOPO plasmids, as per manufacturer’s guidelines, and sequenced to identify mutations in tm4sf1.

2.5.2 The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

2.5.2.1 Design and assembly of CRISPR

CRISPR targets to exon 2 of tm4sf1 were identified using the Optimized CRISPR Design Tool’ by the Feng Zhang group (Ran et al. 2013). The selected target sequences are shown in table 2.8.
To generate the CRISPR constructs, we used protocol described by (Jao and W. Chen 2013). Briefly, two complementary oligonucleotides corresponding to the identified target sequences (table 2.8) were designed using specific criteria; forward TAGGN20 and reverse AAACN20 (table 2.9). Hence ‘TAGG’ was added to the 5’ end of the forward primer and ‘AAAC’ was added to the 5’ end of the reverse primer (table 2.9). These nucleotide additions to the guide sequence serve as overhang for subsequent cloning steps.

<table>
<thead>
<tr>
<th>Guide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>CCTGTGTGTTCCTGGGAATG</td>
</tr>
<tr>
<td>#2</td>
<td>GATTCCAGCCTGTGTGTTCC</td>
</tr>
<tr>
<td>#3</td>
<td>CCCCATTCAGGAACACACAC</td>
</tr>
<tr>
<td>#4</td>
<td>GCCTGTGTGTTCCTGGGAAT</td>
</tr>
</tbody>
</table>

Table 2.9: Primers designed for the generation of *tm4sf1* CRISPR guides

<table>
<thead>
<tr>
<th>Guide</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>F 5’ – TAGGCCTGTGTGTTCCCTGGGAAT – 3’&lt;br&gt;R 5’ – AAACATTCAGGAACACACACAGG – 3’</td>
</tr>
<tr>
<td>#2</td>
<td>F 5’ – TAGGGATTCAGCCTGTGTGTTCC – 3’&lt;br&gt;R 5’ – AAACGGAACACACAGCCTGGGAATGC – 3’</td>
</tr>
<tr>
<td>#3</td>
<td>F 5’ – TAGGCCATTCCCAGGAACACAC – 3’&lt;br&gt;R 5’ – AAACGTTGTGTTCCCTGGGAATGGG – 3’</td>
</tr>
<tr>
<td>#4</td>
<td>F 5’ – TAGGGCCTGTGTGTTCCCTGGGAAT – 3’&lt;br&gt;R 5’ – AAACATTCAGGAACACACACAGGC – 3’</td>
</tr>
</tbody>
</table>

BOLD indicates added nucleotides to generate cloning overhang

The pairs of complimentary oligonucleotides were diluted to 10 mM each in 1 x NEBuffer 1 (2 μl 100 μM primer stock each in a 20 μl 1 x NEBuffer 1) and annealed using the following cycle parameters: denaturation at 95° C for 5 min followed by ramping down to 50° C at 0.1° C/sec, holding at 50° C for 10 min, and cooling to 4° C at normal ramp speed (1° C/sec). 1 μl annealed oligonucleotides were mixed with 400 ng gRNA cloning vector (T7cas9sgRNA2), 1 μl 10x NEBuffer 3, 1 μl 10x T4 ligase Buffer, 0.5 μl BsmBI, 0.3 μl BglII, 0.3 μl SalI, 0.5 μl of T4 DNA ligase, and water to a total of 10μl. Digestion and ligation reactions were performed in a single step using the following cycle parameters: 3 cycles of 20 min at 37° C/15 min at 16° C, followed by 10 min at 37° C, 15 min at 55° C, and 15 min at 80° C. 2 μl of the ligation product was used for transformation.

Table 2.8: CRISPR Guide RNA Sequences
into competent *E.coli* cells. Correct clones, i.e. the T7cas9sgRNA2 vector containing the annealed oligonucleotide guide sequences were identified by sequencing, using the M13F or M13R primers.

### 2.5.2.2 CRISPR RNA synthesis and injection

Cas9 is the RNA-guided DNA endonuclease enzyme that associates with the CRISPR *in vivo*. For the synthesis of *nls-zCas9-nls* RNA, the template DNA plasmid pT3TS-nls-zCas9-nls was linearised using *XbaI* digestion and purified using a QIAprep column (Qiagen). Capped *nls-zCas9-nls* RNA was synthesised using the mMESSAGE mMACHINE T3 kit (Invitrogen) and purified using the RNeasy Mini kit (Qiagen) as per manufacturer’s instructions. For making *tm4sf1* guide RNA (gRNA), the template DNA was linearised by *BamHI* digestion and purified using a QIAprep column. gRNA was generated by *in vitro* transcription using the MEGAscript T7 kit (Invitrogen). After *in vitro* transcription, the gRNA was purified using MEGAclean Transcription Clean-Up Kit (Ambion). Injection mixes were made up at 100-150 ng/μl of *nls-zCas9-nls* RNA and 30-100 ng/μl of gRNA. 1 nl was injected into the single cell of zebrafish embryos mixed with a phenol red tracer. Genomic DNA was extracted from 24-48 hpf injected and uninjected embryos as previously described.

### 2.5.2.3 Identification of CRISPR-induced *tm4sf1* mutations

Taq DNA polymerase and primers which flanked the CRISPR target region were used for PCR amplification of genomic DNA as described in section 2.3.4. PCR product was purified using a PCR purification kit (Qiagen) and digested using *BsiI* (recognition site CCNNNNN^NNGG) at 55° C. Samples were separated on a 2% agarose gel to identify the presence of mutations (see section 4.2.7). Undigested bands were dissected and purified from the agarose and cloned into a pCR4-TOPO vector. Inserts were then sequenced using the M13R primer. HRM was also used to confirm the presence of mutations as described in section 2.4.1.3.

### 2.6 Cell culture techniques

#### 2.6.1 Routine cell maintenance

HUVECs (Promocell) were cultured on 0.1% gelatin-coated dishes in endothelial cell basal medium 2 (EBM-2, PromoCell) with an added supplement pack (Promocell), containing 5% FCS, epidermal growth factor (5 ng/ml), VEGF (0.5 ng/ml), FGF2 (10 ng/ml), long R3 insulin growth factor-1 (20 ng/ml), hydrocortisone (0.2 μg/ml), and
ascorbic acid (1 μg/ml). 50 mg/ml gentamycin (Sigma) and 250 μg/ml amphotericin (Sigma) were also added to each bottle of media. HUVECs were used at passages 3–6. Media was replaced every other day and cells were split when they reached 90% confluency.

When indicated, HUVECs were serum-starved and stimulated with VEGF. Media was removed from cells, which were then washed twice in PBS. PBS was removed and replaced with fresh M199 for 6 h to starve cells of serum and growth factors. Media was then removed from cells and replaced with either pre-warmed M199 containing either 50ng/ml VEGFA165 (Peprotech), 50 ng/ml VEGFC (Peprotech), or no supplement for the indicated times ranging from 0-30 min.

2.6.2 siRNA-mediated gene knockdown

For gene knockdown, HUVECs were seeded at 0.2 x 10⁶ cells/well in a 6-well plate and transfected with TM4SF1 siGENOME SMARTpool siRNA or Control siRNA (Thermo Scientific) using the GeneFECTOR reagent, (Venn-Nova), as per manufacturer’s instructions. TM4SF1 siRNA and control siRNA were diluted to a final concentration of 20 mM. For one well in a 6 well plate, 0.5 μl siRNA was diluted in 100 μl Opti-MEM siRNA (Thermo Scientific). In a separate tube, 6 μl geneFECTOR reagent was diluted in 100 μl Opti-MEM. The siRNA and geneFECTOR solutions were combined and incubated at room temperature for 5 min. Meanwhile, cells were washed four times in Opti-MEM and after the final wash, 1 ml Opti-MEM was added to the cells. The siRNA/geneFECTOR mix was then added to the cells and mixed by gentle shaking. The cells were incubated with the siRNA/geneFECTOR solution for three h at 37°C, 5% CO₂ before the solution was removed and replaced with complete medium. Cells were processed for RNA extraction or cell migration experiments after 48 h from transfection.

2.6.3 Lentivirus production and transduction

HEK293LTV cells were seeded at 5 x 10⁶ cells in a 10 cm dish. 30μg lentiviral transfer vector DNA, psPAX2 packaging and pMD2. G envelope plasmids were prepared at a ratio of 4:3:1 and added to 0.45 mL distilled H₂O and 50 μl 2.5M CaCl₂. Air was bubbled through the DNA mix whilst adding 0.5 mL 2x HEPES-buffered saline dropwise into the precipitate. Following a 30 min incubation, the precipitate was added dropwise to the cells and incubated at 5% CO₂ and incubate overnight. The media was replaced with 10 ml fresh growth medium and sodium butyrate was added to each dish (final concentration
1mM. Medium containing virus was collected after 24, 48 and 72 h and pooled prior to clearing by centrifugation at 1500 rpm for 5 min at 4°C. The medium was then filtered through a 0.45 μm pore PVDF Millex-HV filter (Millipore) and stored at -80°C until use.

For lentivirus transduction, HUVECs were seeded at 100,000 - 300,000 cells per 10 cm dish and 10 ml virus-containing medium was added to the cells 24 h after seeding. The lentivirus was incubated with the cells overnight and the media was replaced with complete media the following day. The cells were used for further experimentation 48 h after transduction.

2.6.4 Cell migration assay

HUVECs were pre-treated for 48 h before the experiment with control or TM4SF1 siRNA (as above), or transduced with control or TM4SF1 expressing lentivirus. Cells were grown to ~90% confluency and a 10 ml sterile pipette tip was used to introduce a ‘scratch’. The media was removed and replaced with M199 +/- 50ng/ml VEGFA165. HUVEC migration was continuously monitored over a period of 18 h using an IncuCyte instrument (ESSEN) and cell motility was quantified using imageJ.

2.7 Protein Manipulation Techniques

2.7.1 Cell lysis for immunoblotting

Cells were placed on ice and the media was discarded. Cells were washed twice with ice old PBS followed by addition of 50-250 μl RIPA lysis buffer (150mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0) inclusive of 20 μl protease inhibitor cocktail (1:250 Sigma p8340). The lysis buffer was incubated on cells for 10 min and then the cells were scraped off the dishes and transferred into pre-cooled microfuge tube. The lysates were centrifuged at 10,000 rpm 4°C for 10 min and the supernatant was removed and transferred into a fresh pre-cooled microcentrifuge tube. Protein concentrations were determined using the BCA protein assay (Pierce), as per manufacturer’s guidelines.

2.7.2 Immunoblotting

Protein lysates were prepared for immunoblotting upon addition of loading buffer comprising of laemmli buffer (8% SDS, 40% glycerol, 0.008% bromophenol blue, 0.25 M Tris HCL, pH 6.8) and 20% 2-mercaptoethanol. Protein preparations were either incubated at room temperature or boiled at 95°C for 5 min prior to loading. Proteins were separated
by SDS-PAGE using Biorad mini-protean gels and Biorad cassettes in 1x Tris/Glycine/SDS running buffer (25mM Tris, 190mM glycine, 0.1% SDS, pH8.3; Biorad). A prestained protein ladder (NEB) was run on each gel for estimation of band sizes. The gels were run at 120V – 200V for 40-90 min. Proteins were then transferred onto a nitrocellulose membrane using a Trans Blot Turbo RTA Transfer kit (Biorad) and 1x transfer buffer (5 x stock = 200 ml, 600 ml dH2O, 200 ml EtOH) in a Bio-Rad transfer cassette at 25V for 7 min. Membranes were briefly washed in 1x TBS (10x TBS: 24.23 g Trizma HCL, 80.06 g NaCl, mix in 800 ml dH2O, pH to 7.6 with HCl, up to 1 L) supplemented with 0.1% tween and then incubated in TBS blocking buffer containing 0.1% Tween 20 (TBS-T) and either 5% fat-free powdered milk (Marvel) or 5% BSA for 1 hour at room temperature. Membranes were then washed briefly in TBS-T and incubated with antibodies in the respective blocking buffers according the manufacturers’ recommendations of each antibody (table 2.10). Antibodies were incubated with the membranes for up to 3 h at room temperature or overnight at 4°C. Following three washes with TBS-T, horseradish peroxidise conjugated secondary antibodies (table 2.11) in 2.5% blocking buffer incubated with membranes for 1-2 h at room temperature. Following a further three washes in TBS-T, membrane bound antibodies were detected by ECL (ThermoScientific) according to manufacturers protocols and membranes were exposed to BioMax light films (Kodak). When indicated, membranes were treated with harsh stripping buffer (20 ml SDS 10%/12.5 ml 0.5M Tris HCl pH 6/67.5 ml ddH2O, 0.8 ml 14.3M 2-mercaptoethanol) and subsequently re-blocked and re-probed with additional antibodies.

**Table 2.10:** List of primary antibodies using for co-immunoprecipitation/immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Host</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag</td>
<td>Sigma F3165</td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td>L6 IgG</td>
<td>n/a</td>
<td>Mouse</td>
<td>1:500</td>
</tr>
<tr>
<td>L6 IgM</td>
<td>n/a</td>
<td>Mouse</td>
<td>1:500</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>Sigma T9026</td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td>TM4SF1</td>
<td>Abcam ab113504</td>
<td>Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>PTyr</td>
<td>Santa Cruz SC-7020</td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>R&amp;D Systems AF357</td>
<td>Goat</td>
<td>1:1000</td>
</tr>
<tr>
<td>VEGFR3</td>
<td>Santa Cruz SC-321</td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td>pP42/44 (pERK1/2)</td>
<td>Cell Signalling 197G2</td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td>P42/44 (ERK1/2)</td>
<td>Cell Signalling 137F5</td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Table 2.11: List of secondary antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Host</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Goat-HRP</td>
<td>Jackson</td>
<td>Rabbit</td>
<td>1:2000</td>
</tr>
<tr>
<td>Anti-Mouse-HRP</td>
<td>Dako</td>
<td>Goat</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-Rabbit-HRP</td>
<td>Dako</td>
<td>Goat</td>
<td>1:20,000</td>
</tr>
</tbody>
</table>

2.7.3 Co-immunoprecipitation

For co-immunoprecipitation of proteins, protein-G Dynabeads (Invitrogen) were labelled with antibodies at a concentration of 1-10 μg as per manufacturer’s protocol. Cells were placed on ice and the media was discarded prior to two washes with ice cold PBS. Cells were lysed in 0.25 - 0.5 ml NP40 lysis buffer (25mM Tris-HCL, pH 7.4, 0.5% (v/v) NP-40, 120mM NaCl, 2mM EDTA, 2mM EGTA) containing protease inhibitor cocktail (1:250, Sigma p8340) and phosphatase inhibitor cocktail (1:50, Calbiochem). The lysis buffer was added to the cells and incubated for 30 min at 4°C with a gentle agitation. The cells were scraped and transferred into pre-cooled microcentrifuge tubes. The lysates were centrifuged at 10,000 rpm 4°C for 10 min and the supernatant was removed and transferred into a fresh pre-cooled microcentrifuge tube. Protein concentrations were determined using the BCA protein (Pierce) assay as per manufacturer’s guidelines. Supernatants were incubated with antibody labelled Dynabeads at 4°C overnight with rotation. The dynabeads were isolated using a magnet following three washes in lysis buffer for 10 min with agitation. 30 μl of sample buffer was added to the beads and the samples were boiled for 5 min at 95°C. For antibodies targeting Tm4sf1 or Flag, protein was not eluted by boiling. Protein was eluted from the beads using a glycine solution (pH 2.8) prior to the addition of sample buffer. The samples were maintained at room temperature prior to loading. Following the elution of IP pull-down proteins from the Dynabeads, the eluates were subjected to immunoblot analysis as previously described in section 2.7.2.

2.8 Statistical analysis

Data analysis was performed using Microsoft Excel and the Student’s t-test was used to determine significance. P values of < 0.05 were considered to be statistically significant.
CHAPTER 3: Results

Characterisation of \( tm4sf1 \) function during angiogenesis \textit{in vivo} 

3.1 Introduction

The tetraspanin-like gene, \( tm4sf1 \), was identified as a potential TC-enriched gene by means of pharmacological manipulation of the two key signalling pathways that determine TC identity; the Vegfr and Notch signalling pathways. Previous \textit{in vitro} experimentation also suggests that \( tm4sf1 \) may play an important role in EC filopodia formation and motility (Zukauskas et al. 2011; Shih et al. 2009), key attributes of TC identity during blood vessel branching. Hence, we aimed to investigate the potential role of \( tm4sf1 \) in TC specification and function during angiogenesis \textit{in vivo} using live-imaging studies in the zebrafish embryo model system.

The zebrafish embryo model has been instrumental to our understanding of vertebrate biology and has clear advantages over other model systems of angiogenesis (Howe et al. 2013; Jao & Chen 2013). While \textit{in vitro} experimentation such as tube formation assays and scratch wound healing assays can be useful (Geudens & Gerhardt 2011), \textit{in vitro} models do not recapitulate the hierarchal organisation of ECs into leading TCs and trailing SCs. Hence, \textit{in vivo} experimentation is essential to develop a full understanding of cell dynamics during angiogenic sprouting. The mouse retina is a well-established model for the study of angiogenesis. Vascular sprouting occurs postnatally making the vessels accessible to manipulation, and the stages of angiogenesis are clearly identifiable due to the tightly regulated temporal and spatial pattern of sprouting vessels (Geudens & Gerhardt 2011; Norrby 2006). However, a major disadvantage to this model is the inability to investigate vessel sprouting in real-time. Thus, \textit{in vivo} dynamics of ECs that are essential to the development of new vessels can’t be assessed. \textit{Ex vivo} models are available for live imaging studies, such as aortic ring assays. These are useful since aortic rings can be dissected from transgenic animals for the study of a specific gene function on angiogenic sprouting (Geudens & Gerhardt 2011; Norrby 2006). However, the influence of other cells, organ systems, blood flow and inflammation are absent. Most importantly, supraphysiological levels of VEGFA are used and VEGF gradients are not established which are essential for coordinating the directional collective migration of ECs during new vessel formation (Gerhardt et al. 2003). Hence, \textit{in vitro} and \textit{ex vivo} models are not a true representation of \textit{in vivo} EC dynamics. In contrast, the zebrafish embryo model is an ideal
system for \textit{in vivo} live imaging of blood vessel sprouting due to their high transparency. Many transgenic zebrafish tools are also available for the study of angiogenesis and have been used to great effect (Hogan et al. 2009; Herbert et al. 2012; Siekmann & Lawson 2007). For example, transgenic lines driving fluorescent protein expression under the control of vascular specific promoters uniquely enable the visualisation of angiogenesis in real-time, by fluorescent imaging and time-lapse microscopy. Additional transgenic lines can also be established with ease using the \textit{tol2} transposon system (as reviewed in detail by Suster et al. (2009)). These transgenic lines, in addition to embryo transparency, make the zebrafish embryo ideal for the study of \textit{in vivo} dynamics of angiogenic sprouting. Hence, we planned to use these benefits to investigate \textit{tm4sf1} function \textit{in vivo} in real time at single cell resolution.

The \textit{Tg(kdrl:GFP)843} zebrafish transgenic line is a well-established model for the study of vascular development (Childs et al. 2002; Jin et al. 2005) and expresses GFP under the control of the EC-specific \textit{kdrl} promoter (Figure 3.1A). Hence, GFP is ubiquitously located throughout the cytoplasm of ECs, enabling the visualisation of blood vessel sprouting dynamics. The ISVs are the first vessels to sprout by the process of angiogenesis during development and are often used for live imaging studies (Figure 3.1A, yellow box). The ISVs branch from the first embryonic artery, the DA (Figure 3.1B, indicated), which is established prior to ISV sprouting by the process vasculogenesis at approximately 18-20 hpf (Lawson & Weinstein 2002). Once the DA has developed, the ISVs subsequently begin to sprout from the DA from 20-22 hpf (Figure 3.1 B-C). The ISVs sprout between the vertical somite boundaries on either side of the embryos and sprout towards a Vegfa gradient generated the medial regions of the somites to form two contralateral rows of vessels (Ellertsdóttir et al. 2010; Liang et al. 1998). Sprouting of the first ISVs initiates from the anterior side of the DA, with subsequent ISVs arising sequentially in an anterior to posterior wave across the DA, in a spatiotemporally conserved pattern. At 24 and 26 hpf, ISVs continues to grow as cell divisions and more SCs contribute to the ISV length. Moreover, ISV sprouting is highly directional, towards the dorsal edge of the embryos (Figure 3.1B-C). At 28 hpf, leading TCs begin to extend processes to their anterior and posterior, as they reach towards neighbouring ISVs to establish connections (Figure 3.1B-C). By approximately 30 hpf, ISVs have fully sprouted and fused with neighbouring ISVs by anastamosis, to form the DLAV (Figure 3.1B-C, indicated) (Ellertsdóttir et al. 2010; Lawson & Weinstein 2002).
Figure 3.1: The zebrafish embryo model for the study of angiogenesis. (A) A lateral view of a Tg(kdrl:GFP) embryos at 26 hpf with brightfield (left panel) and fluorescence (right panel) microscopy. The ISVs (yellow box) form in the embryo trunk, above the yolk extension are a well characterised model vascular bed for the study of angiogenesis. (B) A schematic to demonstrate the ISVs (black vertical lines) sprouting from the DA (red). CV (blue) indicates the cardinal vein. (C) Lateral still images of sprouting ISVs in a Tg(kdrl:GFP) embryos, from 22 hpf to 30 hpf. At 22 hpf, ISVs begin to sprout from the DA between somites. From 24-26 hpf, ISVs increase in length as more ECs contribute to the sprout or contributing ECs undergo mitosis. The sprouts reach towards the dorsal roof of the somites until at 28 hpf, the ISVs reach the dorsal side of the embryo and TCs extend towards adjacent ISVs to establish connections via anastamosis. At 30 hpf, adjacent ISVs have fused to form the (DLAV).
Consequently, this highly conserved pattern of ISV development in space and time facilitates the identification of gene knockdown-induced phenotypes that disrupt angiogenesis, as any changes in ISV patterning or delays in spouting can be easily identified. Hence, the zebrafish embryo is the ideal model system in which to assess the knockdown phenotype of tm4sf1.

When the zebrafish orthologue of human TM4SF1 was first identified, it was initially annotated as ‘tm4sf1’ due to its high orthology to TM4SF1, with 53% DNA sequence identity and 49% amino acid sequence homology (Figure 3.2A). Subsequently, zebrafish tm4sf1 was re-annotated to tm4sf18. However, zebrafish tm4sf18 remains more orthologous to human TM4SF1 than human TM4SF18 (Figure 3.2B). Moreover, in humans TM4SF1 and TM4SF18 reside side-by-side on chromosome 3 whereas zebrafish tm4sf18 resides as a single gene with no neighbouring tm4sf1 (Figure 3.3). Hence, human TM4SF18 likely arose via gene duplication of TM4SF1 in mammals (or visa versa) and tm4sf18 is the primary TM4SF1 orthologue in zebrafish. Consequently, for consistency and to avoid confusion, throughout this thesis, we refer to zebrafish tm4sf18 as tm4sf1.
Figure 3.2: Sequence similarity between zebrafish *tm4sf1* and human *TM4SF1/18*. (A) CLUSTAL sequence alignment between zebrafish and human Tm4sf1, and (B) Zebrafish and human Tm4sf18. (A-B) Zebrafish Tm4sf18 shares 49% amino acid sequence homology with human TM4SF1, but only 37% amino acid sequence homology to human TM4SF18. Identical (\*), highly conserved (:), and weakly conserved (.) amino acids are indicated. Small/hydrophobic residues (red), acidic residues (blue), basic residues (magenta), hydroxyl/amine/basic residues (green) are shown. h, human; z, zebrafish.
Figure 3.3: The genomic region of zebrafish tm4sf1/tm4sf18 and human TM4SF1. (A) Zebrafish tm4sf1 (‘tm4sf18’, highlighted in green) resides on chromosome 22 on the forward strand. Note the absence of a ‘tm4sf1’ gene; thus in zebrafish, tm4sf18 is the primary orthologue of human TM4SF1. (B) Human TM4SF1 resides on chromosome 3 on the reverse strand, directly adjacent to TM4SF18. Human TM4SF18 likely arose via gene duplication of TM4SF1 in mammals (or visa versa).
3.2 Results

3.2.1 Analysis of the spatio-temporal dynamics of \( tm4sf1 \) expression

TCs and SCs are ECs that exhibit different behavioural characteristics. TCs are predicted to have higher motility than SCs and extend more filopodial protrusions (Herbert and Stainier 2011). \( tm4sf1 \) was identified by previous investigations in our laboratory that manipulated the Vegfr-Notch signalling axis to select for TC-enriched genes (Herbert et al. 2012). Furthermore, in vitro experimentation from other laboratories has suggested that \( tm4sf1 \) may regulate filopodia formation and cell motility (Zukauskas et al. 2011; Shih et al. 2009), two key characteristics of TC identity. Hence, \( tm4sf1 \) may regulate key aspects of TC behaviour during angiogenesis.

To determine whether \( tm4sf1 \) expression is indeed associated with TC identity in vivo, we assessed the spatiotemporal localisation of \( tm4sf1 \) mRNA by whole-mount chromogenic ISH during zebrafish development. \( tm4sf1 \) expression was assessed from 22 hpf, when the first TCs are beginning to emerge from the DA, to 30 hpf, where ISVs have fully sprouted and are undergoing anastomosis with adjacent vessels to form the DLAV (Figure 3.1B) (Isogai 2003; Isogai et al. 2001). By imaging sprouting vessels in \( Tg(kdrl:GFP)^{s43} \) embryos at the indicated time points, we showed that ISVs sequentially sprouted from the DA, beginning from the anterior region of the DA (Figure 3.4A; 22 hpf - 26 hpf, blue brackets), as previously reported (Isogai et al. 2001). When ISVs were fully sprouted, they established connections with neighbouring ISVs by anastomosis to form the DLAV (Figure 3.4A; 28 hpf - 30 hpf, red brackets), while ISVs at the posterior region of the embryos continued to sprout (Figure 3.4A, 28 hpf - 30 hpf, blue brackets). At 22 hpf, when ISV sprouting commences, \( tm4sf1 \) expression was observed in ISVs that were beginning to protrude from the DA (Figure 3.4B, arrows) and also at punctate regions further along the DA, potentially corresponding to regions of future ISV sprouting. At 24 hpf, as more ISVs began to sprout, and \( tm4sf1 \) was also observed in nascent sprouting ISVs (Figure 3.4B, arrows). Interestingly, while \( tm4sf1 \) was strongly expressed in sprouting ISVs, \( tm4sf1 \) expression was excluded from the adjacent parental vessel, the DA (Figure 3.4B), which does not form by angiogenesis but the distinct process of vasculogenesis. At subsequent 2 h intervals, \( tm4sf1 \) expression was increasingly enriched in sprouting ISVs, with the greatest number of \( tm4sf1 \) positive vessels being observed at 26 hpf (Figure 3.4B, arrows). At this time point, the majority of ISVs are sprouting and have not fused with...
Figure 3.4: *tm4sf1* is transiently expressed in sprouting ISVs during angiogenesis. (A) Lateral views of *Tg(kdrl:GFP)* embryos and (B) whole-mount ISH analysis of *tm4sf1* mRNA expression in WT embryos, at the indicated time points (hpf). (A) Blue brackets represent sprouting ISVs; red brackets represent ISVs that have fully sprouted and anastomosed; white arrowheads indicate the progressive sprouting of ISVs at one position at the end of the yolk extension during embryo development. DA, indicated. (B) Black arrow heads indicate the expression of *tm4sf1* in ISVs at one position at the end of the yolk extension during ISV sprouting, black arrows identify sprouting ISVs that express *tm4sf1*. Red arrowhead highlights expression of *tm4sf1* in the venous region. (C) *tm4sf1* expression in the MCeV (black/white arrows), PMHB vessel (red bracket) and the PPrA (arrowheads) of the head. (D) Whole mount ISH for *tm4sf1* expression in *clo* mutants. (E) Quantification of *clo* mutants expressing *tm4sf1* in comparison to control (WT) embryos. Data represents at least four replicate experiments.
neighbouring ISVs by anastamosis; thus, the Vegfr signalling pathway that promotes 
*tm4sf1* expression and TC and SC identity is still active. At 26 hpf, in addition to 
expression in sprouting ISVs, *tm4sf1* expression also appeared to be present in venous 
tissue (Figure 3.4B, 26 hpf, red arrowhead). At this time point, primitive erythroid cells 
enter the circulation via the CV (Herbert et al. 2009; Bertrand et al. 2010). Moreover, at 
this timepoint, *tm4sf1* expression appears similar to ISH staining previously observed for 
gata1, a primitive erythroid marker (Long et al. 1997). Hence, *tm4sf1* expression in the 
CV may represent cells of the erythroid lineage or a subset of CV ECs; although further 
investigations would be required to determine this. Interestingly, from 28 to 30 hpf, once 
ISVs had fully sprouted and anastamosed with adjacent vessels (Figure 3.4A, red 
brackets), *tm4sf1* expression appeared to be repressed in regions of fused vessels (Figure 
3.4B). Repression of *tm4sf1* expression occurred in an anterior to posterior manner, 
consistent with repression upon anastamosis. Moreover, ISVs that continued to sprout at 
later time points (Figure 3.4A, 28-30 hpf; blue brackets) still expressed *tm4sf1* (Figure 
3.4B, 28-30 hpf, arrows). By focusing on one point along the DA at the end of the yolk 
extension from 22 to 30 hpf (Figure 3.4A and B, arrowheads), our data suggested that 
*tm4sf1* was expressed transiently for a period of 4 h during ISV sprouting. *tm4sf1* 
expression was then down regulated when the ISVs had fully sprouted, anastamosed and 
thus deactivated the signalling pathways that control TC behaviour (Figure 3.4B, 30 hpf, 
black arrowheads). Hence, *tm4sf1* expression was tightly spatiotemporally regulated *in vivo* and strongly linked to TC behaviour during ISV sprouting.

To determine whether *tm4sf1* expression was restricted only to sprouting ISVs, we 
also investigated the expression of *tm4sf1* by ISH in other vascular beds in the zebrafish 
embryo. *tm4sf1* expression was also present in a number of cranial vessels undergoing 
angiogenesis during early development. Specifically, *tm4sf1* was expressed in the mid-
cerebral vein (MCeV; figure 3.4C, arrows), primordial hindbrain channel (PHBC; figure 
3.4C, red brackets) and the primitive prosencephalic artery (PPrA; figure 3.4C, 
arrowheads). Since the MCeV, PMHB and PPrA also form by angiogenesis (Isogai et al. 
2001), these data suggested that *tm4sf1* expression may generally be associated with new 
blood vessel sprouting.

To determine whether *tm4sf1* expression was EC-specific, we also performed 
whole-mount chromogenic ISH staining for *tm4sf1* in *cloche* (*clo*) mutant embryos (Figure 
3.4D). In zebrafish *clo* mutants, blood vessel formation and haematopoiesis are severely 
disrupted due to the near complete ablation of *flk1*, *gata1* and *gata2* expressing cells,
including angioblasts (Stainier et al. 1995; Liao et al. 1997). tm4sf1 expression was detected in 80% of WT embryos; whereas tm4sf1 expression was not detected in any clo mutant embryos (Figure 3.4D-E). Hence, these data confirmed that tm4sf1 expression was observed in sprouting ECs.

### 3.2.2 tm4sf1 is dynamically regulated by the Vegfr and Notch signalling pathways

Our ISH expression analyses suggested that tm4sf1 may be expressed in sprouting TCs, consistent with its identification as a TC-associated gene. The expression of other TC-restricted genes, including flt4 and esm1, are tightly regulated by the Vegfr-Notch axis (Strasser et al. 2010; Hogan et al. 2009; Siekmann & Lawson 2007; Rocha et al. 2014). Thus, in order to investigate the potential molecular regulation of tm4sf1 expression in sprouting TCs, we manipulated the Vegfr and Notch signalling pathways. First we assessed whether tm4sf1 was regulated by Vegfr signalling upon chemical inhibition of Vegfr and qPCR quantification of tm4sf1 mRNA levels. We incubated embryos with a well characterised inhibitor of Vegfr tyrosine kinase activity, SU5416 (Sacilotto et al. 2013; Rocha et al. 2014), for 1, 2 and 3 h and compared tm4sf1, dll4 and flt4 and kdrl expression levels to DMSO-treated control embryos. After inhibiting Vegfr activity for 1 h, there was no significant difference in the expression levels of any of the genes investigated (Figure 3.5A). This was likely due to the time taken for the inhibitor to penetrate the tissues and inhibit Vegfr activity. However, after 2 or 3 h of incubation with SU5416, tm4sf1 expression was rapidly decreased by 61% (+/-0.07) and 63% (+/-0.05) versus DMSO-treated controls, respectively (Figure 3.5A). Genes known to be regulated by Vegfr signalling, such as dll4 and flt4 (Benedito et al. 2012; Lobov et al. 2007), were also down-regulated upon Vegfr inhibition, as expected (Figure 3.5A), although to varying extents. However, the primary vegfr2 paralogue in zebrafish, kdrl (Habeck et al. 2002; Bussmann et al. 2008), was not significantly affected by SU5416 treatment (Figure 3.5A). Hence, similar to known TC-associated genes, tm4sf1 expression appears to be positively regulated by Vegfr signalling.

While Vegfr signalling promotes TC specification, Notch signalling represses TC identity and angiogenic sprouting. TCs have high Vegfr signalling and low levels of Notch signalling whereas SCs have low levels of Vegfr signalling and high levels of Notch signalling (Hellström et al. 2007; Suchting et al. 2007; Siekmann & Lawson 2007). Therefore, in the absence of Notch signalling, TC numbers increase because TC identity is
Figure 3.5: *tm4sf1* expression is positively regulated by Vegfr signalling and negatively regulated by Notch signalling. (A) Relative quantification of *tm4sf1*, *kdrl*, *dll4*, and *flt4* expression at 26 hpf following incubation of embryos with the Vegfr inhibitor, SU5416, for the indicated length of time. (B) Relative quantification of *tm4sf1* and *kdrl* following *dll4* MO-mediated knockdown at 26 hpf. (C) Whole-mount chromogenic ISH for *tm4sf1* at 26 hpf in control embryos and following MO-mediated knockdown of *dll4*. In the absence of *dll4*, *tm4sf1* expression is ectopically expanded throughout the DA (red arrowhead). (D) Model to depict the potential molecular regulation of *tm4sf1* expression by the Vegfr-Notch axis during TC/SC sprouting. Error bars represent means from 3 biological replicates +/- SEM. *P<0.05, Student’s t test.
no longer repressed in the SC population (Siekmann & Lawson 2007). If tm4sf1 is associated with TC sprouting then its expression may also be negatively regulated by Notch. We therefore assessed the expression of tm4sf1 by qPCR following knockdown of the Notch ligand, dll4, using a well characterised MO (Siekmann & Lawson 2007). In the absence of dll4, tm4sf1 expression was increased by 59% (+/-0.15) relative to control MO-injected embryos (Figure 3.5B). In contrast, there was no significant change in kdr1 expression upon dll4 knockdown (Figure 3.5B). The increase in tm4sf1 expression observed in the absence of dll4 was also confirmed by whole-mount chromogenic ISH for tm4sf1 in dll4 morphants (Figure 3.5C). Compared to the expression pattern of tm4sf1 in control embryos at 26 hpf (Figure 3.5C, top panel), following MO-mediated knockdown of dll4, tm4sf1 expression was retained in sprouting ISVs but was also ectopically expanded to the DA (Figure 3.5C, arrowhead). Thus, Dll4-Notch signalling repressed tm4sf1 expression in non-angiogenic populations of cells. Consequently, we propose a model whereby tm4sf1 is positively regulated by Vegfr signalling in sprouting TCs, but is negatively regulated by Notch signalling in other ECs (Figure 3.5D).

3.2.3 tm4sf1 expression can be efficiently knocked down using a splice-site targeting MO

The above data demonstrated a highly specific expression pattern for tm4sf1 during ISV sprouting, which was regulated by the Vegfr-Notch axis and tightly associated with TC behaviour. We speculated that tm4sf1 may regulate TC behaviour, and thus aimed to assess the consequences of tm4sf1 loss of function in zebrafish by MO. Two types of MO are often used to block gene function in zebrafish; namely, translation-blocking MOs, that sterically block the translation initiation complex; and splice blocking MOs, that block pre-mRNA intron-exon splicing (Bill et al. 2009). Validation of gene knockdown using translation-blocking MOs requires validation via detection of resultant levels of gene-derived protein. However, there are currently no commercially available antibodies that recognise zebrafish Tm4sf1 protein. In contrast, splice blocking MOs can be validated by PCR since these MOs often modify normal mRNA splicing events, thereby generating alternative gene products that can be detected by PCR (Bill et al. 2009). Hence, we designed tm4sf1-targeted splice-blocking MOs to assess the tm4sf1 loss-of-function phenotype. Initially, we designed a splice blocking MO to the exon 1-intron 1 splice junction of tm4sf1 with the intention of blocking intron 1 splicing; however, the MO did not affect tm4sf1 splicing (data not shown). Consequently, we designed a second splice-MO designed to target the intron 2-exon 3 boundary. This second MO did promote intron
2-exon 3 mis-splicing; however toxicity at low concentrations prevented use of this MO (data not shown). Finally, we designed a splice-blocking MO targeted to the *tm4sf1* intron 1-exon 2 splice site junction (Figure 3.6A). To determine the concentration of MO required for disruption of *tm4sf1* intron 1-exon 2 splicing, we injected WT embryos with 2, 4 or 8 ng of MO or 8 ng of control MO at the single cell stage. The control MO targets a human β-globin intron mutation that causes β-thalassemia. Hence, this MO does not target any mRNA in zebrafish and is widely used as a standard control (Watson et al. 2013; Herbert et al. 2012). Following MO-injection, mRNA was extracted from embryos at 26 hpf prior to cDNA synthesis. 26 hpf was selected since at this time point *tm4sf1* expression levels were at their highest (Figure 3.4B). To detect *tm4sf1* splicing alterations by PCR, primers were used that amplified full length of WT *tm4sf1* cDNA (Tm4sf1 F1 and R1 in table 2.2; figure 3.6A-B, indicated). Additionally, primers that amplified between exon 1 and intron 1 (primers Tm4sf1 F1 and R2 in table 2.2; figure 3.6A-B, indicated) were also used, as increasing amounts of PCR product would be observed when intron 1 fails to be correctly spliced out. Compared to control MO-injected embryos, which expressed high levels of WT *tm4sf1*, embryos injected with 4 ng or 8 ng *tm4sf1* MO had no detectable WT transcript (Figure 3.6B). These findings were consistent with the MO-mediated disruption of intron 1-exon 2 splicing that would promote retention of the 5 kb intron 1. Hence, loss of WT transcript was likely due to the PCR conditions no longer being optimal for the resultant 5.6 kb amplicon. Indeed, mis-splicing of *tm4sf1* transcript was evident in embryos injected with 4 and 8 ng *tm4sf1* MO, as greatly increased levels of exon 1-intron1-derived PCR product were observed relative to controls, in a dose dependent manner (Figure 3.6B). This demonstrated atypical retention of the *tm4sf1* intron 1. The small amount of exon 1-intron1-derived PCR product observed in control embryos was likely due to the presence of small amounts of newly synthesised, un-spliced mRNA (Figure 3.6B). DNA sequencing of the PCR product derived from the F1/R2 amplicon from 4 ng MO-injected embryos confirmed that intron 1 sequence was present (Figure 3.6C). Most importantly, retention of the *tm4sf1* intron 1 resulted in the inclusion of multiple early stop codons, indicated (Figure 3.6C). Hence, 4 ng splice-targeting *tm4sf1* MO efficiently disrupted intron 1-exon 2 splicing, resulting in a transcript that likely generates truncated, non-functional protein.
Figure 3.6: Injection of a splice-site-targeting MO induces mis-splicing and inclusion of intronic sequence in *tm4sf1*. (A) An illustration to show the region of *tm4sf1* targeted by the splice MO. Arrows represent pairs of primers to show the regions amplified by PCR (B) Ethidium bromide stained agarose gel showing WT *tm4sf1* bands (F1/R1) and inclusion of intronic sequence (F1/R2). β-actin was used as a loading control. (C) DNA sequencing results following gel extraction and TOPO cloning of PCR product obtained using primers Tm4sf1 F1 and R2 and cDNA from control or 4 ng *tm4sf1* MO-injected embryos. ‘tm4sf1’ refers to control *tm4sf1* sequence whereas ‘seq1’ is the sequencing result obtained from MO-injected embryos. ‘STOP’ identifies in-frame STOP codons in the retained intronic sequence.
3.2.4 Analysis of ISV sprouting following MO-mediated tm4sf1 knockdown

Following validation of a tm4sf1 splice-blocking MO, we were able to assess the vascular phenotype of tm4sf1 loss-of-function in zebrafish embryos. To do this, we injected 4 ng tm4sf1 MO or 4 ng control MO into Tg(kdrl:GFP)843 zebrafish embryos at the one cell stage. In the first instance, fluorescent images of lateral views of the injected embryos were acquired at 26 hpf (Figure 3.7A). At this time point, the majority of ISVs in control embryos had fully sprouted and reached the dorsal roof of the somites (Figure 3.7Ai, dashed line). In contrast, the majority of ISVs in tm4sf1 morphant embryos had begun to sprout; however all ISVs appeared shorter than those in control embryos (Figure 3.7Aii). Interestingly, all ISVs in tm4sf1 morphant embryos appeared to be stunted and of the same length at all positions along the DA (Figure 3.7Aii), whereas ISVs in control embryos were longer at anterior positions and gradually shorter in length in posterior regions. Moreover, none of the ISVs in tm4sf1 morphant embryos had reached the dorsal roof of the somites by 26 hpf (Figure 3.7Aii, dashed line). Hence, knockdown of tm4sf1 appeared to delay the sprouting of ISVs, suggesting a key role for Tm4sf1 in TC migration and angiogenesis.

A primary advantage of the zebrafish embryo model system for studies of angiogenesis is the ability to visualise blood vessel sprouting in real-time in vivo. Hence, a direct comparison of ISV sprouting dynamics in control MO-injected versus tm4sf1 MO-injected embryos was performed upon imaging of ISV sprouting using time-lapse movies. Images were captured every 0.3 hours from 22 hpf to 25.6 hpf. Following maximum projection of confocal z-stacks, ISV sprouting in control and tm4sf1 morphant embryos was compared frame by frame. Initial observations confirmed a subtle, temporal delay in ISV sprouting in the tm4sf1 morphant embryos when compared with controls (Figure 3.7B), as previously indicated (Figure 3.7A). Unlike controls, sprouting of ISVs in tm4sf1 morphants stalled at the horizontal myoseptum (HM; figure 3.7B, indicated) for a short period of time before ISVs continued to sprout (Figure 3.7B). At each of the time points analysed, control ISVs appeared to have sprouted further than ISVs in stage-matched tm4sf1 morphant embryos (Figure 3.7B, yellow arrows). Additionally, by 25.6 hpf control ISVs had begun to fuse with neighbouring ISVs to form the DLAV (Figure 3.7B, indicated). In contrast, the majority of ISVs in tm4sf1 morphants did not reach the dorsal
Figure 3.7: *tm4sf1* knockdown disrupts ISV sprouting. (A) Lateral view of control MO- or *tm4sf1* MO-injected Tg(kdrl:GFP)<sup>s843</sup> embryos at 26 hpf. White dashed line indicates the expected position of the DLAV at the dorsal roof of the somites. ISV sprouting is delayed in *tm4sf1* MO-injected embryos. (B) Lateral views of control MO and *tm4sf1* MO-injected Tg(kdrl:GFP)<sup>s843</sup> embryo ISVs at the indicated time points. Images represent stills taken from time-lapse confocal movies. Yellow arrows identify the longest ISVs at each time point. Blue dashed line marks the horizontal myoseptum (HM). Note formation of the DLAV in control embryos but not *tm4sf1* morphant embryos at 25.6 hpf.
roof of the somites or form the DLAV at this time point (Figure 3.7B). Hence, MO-mediated knockdown of \textit{tm4sf1} subtly delayed ISV sprouting.

**3.2.5 Quantitative analysis of ISV sprouting upon \textit{tm4sf1} knockdown**

After observing a potential delay in ISV sprouting in \textit{tm4sf1} morphant embryos we decided to quantify ISV sprouting dynamics to determine whether the delay was significant. The lengths (µm) of ISVs were quantified from 22 hpf every 0.3 hours during zebrafish development by tracking the tip of sprouting ISVs (Figure 3.8A; yellow arrow). The positions of ISV tips were normalised relative to the base of the ISV (Figure 3.8A; red arrow), to take the growth and movement of the embryo into consideration throughout the period of imaging. From 22 hpf, control ISVs increased in length linearly, at a rate of approximately 20 µm per h until a maximal length of approximately 100 µm was reached (Figure 3.8C). At this point, the ISVs then fused to neighbouring ISVs by anastamosis to form the DLAV (Figure 3.8B-C). Between 22 hpf and 23.5 hpf, there were no significant differences in ISV length between control MO injected embryos and those of \textit{tm4sf1} morphant embryos. Further quantification of ISV lengths confirmed that sprouting was temporarily disrupted from 23.5 hpf (Figure 3.8B-C). As previously observed (Figure 3.7B), ISV sprouting stalled for around a 2 h period at the horizontal myoseptum (Figure 3.8B, blue line), when the ISV sprouts had grown to a length of 24.9 µm (+/- 2.5; figure 3.8C). However, at 25.3 hpf, ISV sprouting recovered in \textit{tm4sf1} morphant embryos and by 28.9 hpf, ISVs reached the DLAV, around 2 h after controls (Figure 3.8C). A temporal delay in ISV sprouting could be a consequence of degradation or dilution of the MO over time and thus recovery of \textit{tm4sf1} expression. To test this, we injected WT embryos with 4 ng of \textit{tm4sf1} MO or 4 ng of control MO and analysed \textit{tm4sf1} expression at 48 hpf. We used PCR primers Tm4sf1 F1 and R1 (table 2.2) to amplify full length \textit{tm4sf1}, or T4msf1 F1 and R2 (table 2.2) to amplify \textit{tm4sf1} cDNA between exon 1 and intron 1 (as previously described; figure 3.6). At 48 hpf, MO-mediated \textit{tm4sf1} knockdown was still efficient, as determined by loss of WT full length \textit{tm4sf1} transcript (Figure 3.8D). Moreover, inclusion of \textit{tm4sf1} intronic sequence was still observed at 48 hpf (Figure 3.8D). Hence, recovery of ISV sprouting was not due to the degradation or dilution of the MO. To summarise, the MO-mediated knockdown of \textit{tm4sf1} caused a temporal delay in ISV , suggesting that Tm4sf1 plays a key role in coordinating normal EC migration during ISV sprouting \textit{in vivo}. 
Figure 3.8: Knockdown of tm4sf1 caused a temporal delay to ISV sprouting. (A) A lateral view of an ISV in a Tg(kdrl:GFP)\textsuperscript{s843} embryo, indicating the tip (yellow arrow) and base (red arrow) positions used to determine the length of ISVs in µm. (B) Lateral images of ISVs in control MO- and tm4sf1 MO-injected Tg(kdrl:GFP)\textsuperscript{s843} embryos. Images represent stills taken from time-lapse movies recorded from 22 hpf. (C) Quantification of ISV lengths from 22 hpf (indicated) for 10.2 hours. Error bars represent mean +/- SEM; n = at least 36 ISVs from 12 embryos over three separate experiments. *p < 0.05 versus control, Student's t test. (D) Ethidium bromide stained agarose gel of full length tm4sf1 (Primers Tm4sf1 F1 and R1) or exon 1-intron 1 of tm4sf1 (Primers Tm4sf1 F1 and R2) PCR products from cDNA derived from 48 hpf control or tm4sf1 MO injected embryos. Lack of full length tm4sf1 and inclusion of intronic sequence confirmed the MO was effective at 48 hpf. β-actin was used as a loading control.
3.2.6 Evaluation of TC and SC motilities at single cell resolution

Our data showed that loss of *tm4sf1* caused a delay in ISV sprouting; however this does not inform us about the behaviours of individual TCs and SCs that contribute to nascent vessels. The transgenic zebrafish line, *Tg(kdrl:nlsEGFP)*^zf109^, expresses nuclear-targeted GFP under the control of the EC-specific promoter, *kdrl*. This line enables the visualisation and tracking of individual TC and SC nuclei motility during vessel sprouting. To facilitate image interpretation, we assigned specific nomenclature to ECs as they contributed to a new sprout. Cells were labelled according to their position in the sprouting ISV. Thus, the first cell to sprout from the DA, i.e. the TC, was denoted ‘cell 1’ (Figure 3.9A). The second cell to contribute to a sprouting ISV, i.e. the first SC, was denoted ‘cell 2’ (Figure 3.9A). Similarly, the third EC (second SC) to contribute to a sprouting vessel was denoted ‘cell 3’ (Figure 3.9B). If a TC were to divide (Figure 3.9A, asterisk), this would give rise to two daughter cells, with the dorsal-most daughter being denoted cell ‘1.1’ whereas the ventral daughter would be denoted cell ‘1.2’ (Figure 3.9A). Similarly, if the trailing SCs ‘2’ or ‘3’ were to divide (Figure 3.9B, asterisk), the two daughter cells derived from the divisions would be denoted cell ‘2.1’ and cell ‘2.2’ (Figure 3.9B) or cell ‘3.1’ and cell ‘3.2’, respectively. Hence, designation of cells with the above nomenclature enabled the in-depth characterisation of TC and SC movements during ISV sprouting.

To elucidate the effects of *tm4sf1* MO-mediated knockdown on TC/SC dynamics at the single cell resolution, we injected *Tg(kdrl:nlsEGFP)*^zf109^ embryos with 4 ng *tm4sf1* MO or 4 ng control MO at the one-cell stage prior to time-lapse confocal imaging. Images were captured every 0.3 h for 10.2 h until 32.2 hpf (Figure 3.10A-B). The positions of the leading TC (cell 1), and the first SC (cell 2), for both control MO-injected and *tm4sf1* MO-injected embryos were then recorded at each time point. Quantification revealed that the dorsal movement of control TCs was significantly faster than adjacent SCs (Figure 3.10A-B). While TCs are presumed to be more motile than SCs by much of the literature (Nakayama et al. 2013; Herbert & Stainier 2011; Eilken & Adams 2010), this data provided the first quantitative evidence that this is the case during blood vessel sprouting *in vivo*. As similarly observed during the quantification of ISV lengths (Figure 3.8), TC nuclei progressed dorsally at a steady rate of approximately 20 μm per h until gradually plateauing at around 120 μm when ISVs had sprouted to their full length (Figure 3.10A-B). Control SCs progressed dorsally more slowly and after the 10.2 hour duration of the movie analysis, SC on average reached a height of approximately 80 μm (Figure 3.10B).
Figure 3.9: Nomenclature of the labelling of individual ECs during ISV sprouting. (A) The first cell to contribute to a new sprout is the TC, labelled ‘1’, whereas the second cell is a SC, and labelled ‘2’. When a TC divided (yellow asterisk), the dorsal most daughter cell was labelled ‘1.1’ whereas the ventral daughter was labelled ‘1.2’. (B) Similarly, for a SC division, the dorsal most daughter cell was denoted ‘2.1’, whereas the ventral most daughter was denoted ‘2.2’. The next SC to enter the ISV was labelled cell ‘3’.
Similarly, in \textit{tm4sf1} morphant embryos, the dorsal nuclear progression of the second cell nuclei to contribute to the ISV, i.e. control SCs (cell 2), also reached a height of approximately 80 \( \mu m \) (Figure 3.10B). Hence, loss of \textit{tm4sf1} expression does not affect SC motility during ISV branching. In contrast, TCs in \textit{tm4sf1} morphant embryos progressed dorsally more slowly than control TC nuclei (Figure 3.10A-B). Similar to the imaging and quantification of ISV lengths (Figure 3.8), we observed a significant delay in TC nuclei motility after the nuclei had moved dorsally from the DA by approximately 20 \( \mu m \) (Figure 3.10B). In particular, our previous data analysis of ISV lengths demonstrated a delay in \textit{tm4sf1} morphants at approximately 25 \( \mu m \) (Figure 3.9B). A 5 \( \mu m \) difference in dorsal movement prior to delay may be accounted for since we tracked nuclei as opposed to the tip of the ISV, which would be extending dorsally towards the VEGFA gradient (Gerhardt et al. 2003). The period of delay continued for approximately a 2 hour period before TC nuclei continued to progress dorsally and eventually contributed to the DLAV at much later time points than controls (Figure 3.10A-B). Interestingly, between 22 hpf and 27.4 hpf, TCs in \textit{tm4sf1} morphant embryos displayed the same motility as SCs in control embryos (Figure 3.10B), due to the delayed TC sprouting observed in \textit{tm4sf1} morphant embryos. Therefore, differences observed in ISV sprout lengths between the control MO-injected and \textit{tm4sf1} morphant embryos (Figure 3.8) were potentially due to a selective decrease in TC dorsal movement. Hence, consistent with the association of \textit{tm4sf1} expression with TC identity (Figure 3.4 and 3.5), our findings suggested that \textit{tm4sf1} may function to specifically control TC motility during ISV sprouting \textit{in vivo}.

Although we observed that \textit{tm4sf1} expression specifically modulated TC motility during ISV sprouting, it was possible that Tm4sf1 may also regulate other aspects of EC behaviour. To test whether the delay observed in TC dorsal movement and ISV sprouting following by loss of \textit{tm4sf1} was due to alterations in EC proliferation, we calculated a number of additional parameters from the cell tracking dataset. Further detailed analysis of time-lapse movies allowed us to determine the number of ECs contributing to each sprouting vessel, the number of TC divisions, the number of SC divisions, and the total number of EC divisions occurring per ISV from 22 – 32.2 hpf. Additionally, the average distance TCs and SCs had migrated before divisions occurred and the time when divisions were observed was also determined. In agreement with previous reports (Blum et al. 2008; Siekmann & Lawson 2007), at 32.2 hpf approximately three ECs contributed
Figure 3.10: Knockdown of tm4sf1 disrupted TC motility. (A) Lateral images of ISVs in control MO- and tm4sf1 MO-injected Tg(kdrl:nlsEGFP)zf109 embryo. Images represent stills taken from movies recorded from 22 hpf (indicated) for 9.6 hours. Red arrows indicate TCs. Blue arrows indicate SCs. (B) Quantification of dorsal movement of TCs and SCs in control MO- and tm4sf1 MO-injected embryos for 10.2 hours starting from 22 hpf. Significant differences were observed between control and tm4sf1 morphant TC motilities. (C-H) Quantification of (C) TC, SC and total cell divisions, (D) total EC cell numbers, (E) the average position and (F) time (hpf) of TC divisions, and (G) the average position and (H) time (hpf) of a SC divisions following imaging of control MO- and tm4sf1 MO-injected embryos for 10.2 hours starting from 22 hpf. Error bars represent mean +/- SEM. n = at least 36 ISVs from 12 embryos over three separate experiments *p < 0.05 versus control, Student’s t test.
to each sprouting ISV in both control and tm4sf1 morphant embryos (Figure 3.10D). Moreover, when we quantified the number of TC divisions, SC divisions and total EC divisions per ISV for the duration of the movies (10.2 hours), there were no significant differences between control embryos and tm4sf1 morphants embryos (Figure 3.10C). This indicated that loss of tm4sf1 by MO-mediated knockdown does not affect EC proliferation. We also mapped the positions of TC divisions in the ISV of both control embryos and tm4sf1 morphant embryos to determine the distance TC nuclei had emerged from the DA before they underwent mitosis. The average position of TC divisions in control embryos and tm4sf1 morphant embryos were 21.1 µm (+/- 4.2) and 20.2 µm (+/- 4.1), respectively, and there was no significant difference between groups (Figure 3.10E). Similarly, there was no significant difference between the time when TC divisions were observed in control embryos and tm4sf1 morphant embryos (Figure 3.10F). The same analyses were applied to SC divisions and similarly no differences were found between the position of SC divisions (Figure 3.10G) and the time when SC divisions occur (Figure 3.10H) in control versus tm4sf1 morphant embryos. Hence, Tm4sf1 does not modulate the number and/or timing of TC/SC divisions.

Whilst differences in cell proliferation between control and tm4sf1 MO injected embryos were not observed, the analysis did bring the position of TC divisions to our attention. We noticed that delays to TCs during ISV sprouting in the tm4sf1 morphant embryos occurred after cells had migrated approximately 20 µm (Figure 3.10B), and this correlated with the average position at which TCs divided (Figure 3.10E). As Tm4sf1 is specifically required for TC motility, this observation raised an interesting question as to whether tm4sf1 may be important for re-acquisition of TC motility after TC mitosis.

We know from unpublished work in the laboratory that the majority of TCs (76%) divide during zebrafish ISV sprouting (Costa et al 2015; in revision, Nature). Hence, the rapid re-establishment of TC motility in one daughter cell of a TC division would be key to ensuring undisturbed ISV sprouting during proliferative growth. To determine whether tm4sf1 was important for TC motility following mitosis, we tracked the dorsal nuclear movement of both daughter cell nuclei after TC divisions. Tracking commenced when the two daughter cell nuclei had fully resolved and thus completed cytokinesis (Figure 3.11A, asterisk indicates dividing cell). For analysis, we adopted the same cell tracking approach to analyse TC daughter motilities in control and tm4sf1 morphant embryos, using the cell labelling nomenclature as described in section 3.7. Briefly, when a TC (cell 1) divided, the two resultant daughter cells were denoted cell 1.1 and cell 1.2 (Figure 3.9A.). When a SC
divided, the two resultant daughter cells were denoted 2.1 and 2.2 (Figure 3.9B). Tracking of single cells revealed that TC divisions in control embryos gave rise to two daughter cells that rapidly acquired differential motilities (Figure 3.11A-B). The dorsal daughter (cell 1.1) adopted parental TC-like motility promptly after the cell division, whereas the ventral daughter (cell 1.2) adopted SC-like motility (Figure 3.11A-B). Hence, the TC-SC hierarchy was re-established rapidly after TC mitosis. In contrast, the dorsal-most daughter (cell 1.1) derived from a TC divisions in tm4sf1 morphant embryos was significantly less motile than that of a control embryo (Figure 3.11A-B). Interestingly, for the first 4 h after TC division, there was no significant difference between the motility of the dorsal daughters (cell 1.1) in tm4sf1 morphant embryos, relative to the motility of the ventral daughters (cell 1.2) of control TC divisions (Figure 3.11B). Thus, during this period, the motility of the dorsal daughters of TC division (cell 1.1) in tm4sf1 morphant embryos more closely resembled the motility of a SC rather than a TC. We also compared the motility of ventral daughter cells (1.2) in control or tm4sf1 morphant embryos following TC mitosis, and there was also a significant difference in the dorsal nuclear movement of ventral daughters (1.2) in tm4sf1 morphants versus controls; albeit, for a very short period of time (Figure 3.11B). However, these differences are likely due to the slower motility of the leading TCs that potentially limit the dorsal movement of the ventral daughter. Overall, in the absence of tm4sf1, post-mitotic TC motility in the dorsal most daughter is disrupted, consistent with a key role for Tm4sf1 in determining TC motility and behaviour.

From our previous transcriptomic analysis (data not shown), whole mount ISH staining (Figure 3.4) and chemical treatments (Figure 3.5), we have shown that tm4sf1 is potentially enriched in sprouting TCs and its expression is positively controlled by Vegfr signalling. Hence, Tm4sf1 may specifically function to regulate TC behaviour and may not be required for SC motility. For this reason, we wanted to determine whether loss of tm4sf1 by MO also affected the behaviour of SC daughter cells after a SC division. In ISVs from control embryos, SCs divided to give rise to two daughter cells, cells 2.1 and 2.2. We manually tracked the movement of daughter cell nuclei following completion of SC cytokinesis in control embryos, as described above for TCs. Similar to TC divisions, the dorsal most daughter of control SC division (cell 2.1) exhibited higher motility than the ventral daughter (cell 2.2; Figure 3.11C). Hence, SC divisions also rapidly give rise to two daughters of differential motilities. Interestingly, we found that the motility of the dorsal-most daughter from a SC division, cell 2.1, was very similar to that of the parental SC, as well as the ventral most daughter of a TC division (Figure 3.11C, dashed line represents
Figure 3.11: *tm4sf1* promoted TC-like motility in the distal daughter of TC division. (A) Lateral images of ISVs in control MO- and *tm4sf1* MO-injected Tg(kdrl:nlsEGFP) embryos. Images represent stills from time-lapse movies acquired 0.3 h before the point of TC division. Dorsal-most cell (1.1) and ventral-most cell (1.2) from a TC division are indicated; yellow asterisk identifies dividing cells. (B-C) Quantification of the dorsal nuclear movement of TC daughters (B) or SC daughters (C) following division in control MO- and *tm4sf1* MO-injected embryos. Dashed line indicates cell 1.2. Error bars represent mean +/- SEM. n = at least 36 ISVs from 12 embryos over three separate experiments *p < 0.05 *tm4sf1* MO 1.1 versus control 1.1, **p < 0.05 *tm4sf1* MO 1.2 versus control MO 1.2. Student’s t test.
cell 1.2). Hence, like a TC division, SC divisions generated a dorsal daughter that rapidly re-established the motility of their parental cell. Furthermore, when we tracked the nuclei of SC daughters in the tm4sf1 morphant embryos, there was no significant difference in the motility of these cells relative to controls. Hence, loss of tm4sf1 expression specifically hinders re-acquisition of TC motility, whereas the motilities of SCs remain unaffected. These findings are consistent with the TC restricted expression of tm4sf1. Taken together, we identify tm4sf1 as a novel Vegfr-Notch-regulated determinant of TC motility.
3.3 Discussion

Prior to these studies, the physiological role of \textit{tm4sf1 in vivo} was yet to be determined and the current literature primarily focused on the role of TM4SF1 \textit{in vitro} (Lekishvili et al. 2008; Shih et al. 2009). Here, we have presented the first \textit{in vivo} data demonstrating that \textit{tm4sf1} is a novel TC-associated gene; and that the Vegfr and Notch signalling pathways tightly spatiotemporally regulate \textit{tm4sf1} expression in sprouting TCs. \textit{tm4sf1} was almost exclusively expressed in the tips of sprouting ISVs and was down-regulated when ISVs fused with neighbouring vessels by anastamosis. The expression pattern and transcriptional control of \textit{tm4sf1} in zebrafish ISVs is highly comparable to that of \textit{flt4} (Hogan et al. 2009; Siekmann & Lawson 2007) and \textit{esm1} (Siekmann & Lawson 2007; Strasser et al. 2010; Rocha et al. 2014), known markers of sprouting TCs during new blood vessel sprouting. The comparable expression patterns of \textit{tm4sf1} and known TC genes supports our idea that \textit{tm4sf1} is a new marker of TCs. \textit{flt4} and \textit{esm1} play important roles in regulating TC behaviour and TC identity, and are induced by Vegfa-Vegfr signalling (Benedito et al. 2012; Rennel et al. 2007). Similarly, \textit{tm4sf1} expression was positively regulated by Vegfr signalling, indicating that a similar transcriptional programme may regulate \textit{flt4, esm1} and \textit{tm4sf1} expression. Moreover, high levels of Notch signalling robustly repress Vegfr signalling and the TC potential of ECs during sprouting (Hellström et al. 2007; Suchting et al. 2007; Siekmann & Lawson 2007). Hence, in the absence of Notch signalling, increased numbers of sprouting ECs adopt TC-like behaviour due to enhanced Vegfr activity, and the increased expression of TC-associated genes such as \textit{flt4} and \textit{esm1} (Siekmann & Lawson 2007; del Toro et al. 2010). We showed that loss of Notch signalling enhances the expression of \textit{tm4sf1}, with expression ectopically expanded throughout the DA. This is similar to previous studies of the TC gene, \textit{Flt4/flt4}, whose expression is ectopically expanded in the mouse retina (Tammela et al. 2008) and zebrafish DA (Siekmann & Lawson 2007) whenDll4 or Notch signalling are disrupted. Similarly, \textit{esm1} is negatively regulated by Dll4-Notch signalling as its expression is increased in Dll4\textsuperscript{+/-} heterozygous mice (del Toro et al. 2010). Similarities in the regulation of TC gene expression are interesting and suggest a common signalling mechanism, which is induced in TCs but not SCs, controls their expression. Hence, multiple TC genes may be under the control of a common TC-specific transcriptional programme.

Efficient angiogenic sprouting requires the coordinated collective migration of ECs that maintain a hierarchal organisation of leading tip and trailing SCs (Herbert & Stainier 2011). The VEGFR and Notch pathways are central to the coordination of EC collective
migration and maintenance of the TC-SC hierarchy (Zarkada et al. 2015; Siekmann & Lawson 2007; Gerhardt et al. 2003; Hellström et al. 1988). The TC-SC hierarchy refers to differences in behaviour between TCs and SCs, specifically; that TCs lead a new blood vessel sprout and are highly motile, whereas SCs are less motile and trail behind TCs (Figure 3.12). While there is the general consensus in the current literature that TCs and more motile than trailing SCs (Nakayama et al. 2013; Herbert &. Stainier 2011; Eilken & Adams 2010); until now, the motilities of TCs and SCs have never been quantified at single cell level in vivo. Our quantifications of TCs and SCs motilities have confirmed that TCs have a characteristically higher motility than SCs (Figure 3.12). Furthermore, when a TC divides, only the dorsal-most daughter adopts parental TC motility, while the ventral daughter rapidly adopts SC motility (Figure 3.12). In addition to stereotyped TC and SC motilities, TC and SC division are also highly standardised; such that, the numbers of TC and SC divisions and the position of those divisions are confined to a narrow window of time and space. Hence, even when TCs divide, the TC-SC hierarchy is seamlessly maintained, enabling simultaneous cell migration and proliferation without delaying ISV sprouting. Hence, the integration of EC mitosis with re-establishment of differential TC/SC motility may play a key role in the coordination of collective migration of ECs during new vascular sprout formation.

Unpublished work from our laboratory has shown that post-mitotic TC-SC selection is independent of Dll4-Notch signalling (Costa et al 2015; in revision, Nature). In dll4 morphant embryos, the majority of ECs contributing to new vascular sprouts exhibit TC-like motilities, since Notch signalling is not activated in SCs and thus TC identity is not repressed. However, following a TC division in dll4 morphants, behavioural asymmetries are still evident between the two TC daughters. Hence, Dll4-Notch signalling is not responsible for immediately establishing differences in TC daughter motilities. Here we have shown that tm4sf1 confers higher motility to the dorsal daughter of TC mitosis (Figure 3.12). In the absence of tm4sf1, the dorsal-most daughter of TC division does not exhibit TC like motilities but progresses at a much slower rate, more comparable to that of a SC (Figure 3.12). In contrast the motility of SC daughters remains unaffected by the absence of tm4sf1 (Figure 3.12). Hence, these data imply that tm4sf1 may be responsible for asymmetries in TC behaviour observed after a TC division.
Figure 3.12: Model for the proposed role of tm4sf1 in determining TC behaviour. (A) In WT vessels when a TC undergoes mitosis, this gives rise to two daughter cells. The distal daughter cell adopts parental TC-like motility and thus, has a faster motility that the ventral daughter cell. Following a SC division, the dorsal most daughter has similar motility to the ventral TC daughter and the ventral SC daughter has a lesser motility than the dorsal daughter. Blue arrows indicate the relative motilities of cells before and after cell after division. (B) In the absence of tm4sf1, when a TC divides the motility of the distal daughter is hindered and thus the TC division initially gives rise to two daughter cells of similar motilities and the dorsal-most cell now displays behaviour more similar to that of a SC than a TC. SC divisions are unaffected by the loss of tm4sf1. Blue arrows indicate the relative motilities of each cell after division.
These findings raise the possibility that tm4sf1 may be asymmetrically acquired during a TC division; thus, the cell that acquires more tm4sf1 would display TC motility. Asymmetric cell division is known to be crucial for differential cell identity decisions throughout development, such as during neuronal differentiation (Matsuzaki 2000). As a general rule, asymmetry arises from polarised deposition of cell identity determinants prior to division (Medioni et al. 2012; Li et al. 2013). Thus it is possible that tm4sf1 mRNA or protein may be localised at the distal edge of TCs during division. Therefore when a TC divides, higher levels would be acquired by the dorsal TC daughter versus the ventral daughter. Previous experiments in our laboratory have shown that immediately following TC mitosis, the dorsal-most daughter cell has higher levels of Vegfr signalling compared to the ventral daughter cell (in revision, Nature). Hence, if tm4sf1/Tm4sf1 were to be asymmetrically acquired by the dorsal TC daughter, is it possible that this may be responsible for the higher levels of Vegfr signalling observed following division. Alternatively, since Vegfr signalling is higher in dorsal daughter cells following TC division, tm4sf1 may simply be rapidly upregulated by Vegfr signalling in the dorsal daughter. We know that Vegfr-mediated changes in tm4sf1 expression are rapid and highly dynamic from qPCR analysis of tm4sf1 expression upon Vegfr inhibition (Figure 3.5). Hence, the higher levels of Vegfr signalling in TC dorsal daughters following TC mitosis may rapidly establish the higher Tm4sf1 expression levels that subsequently promote TC motility. Thus, the temporal dynamics of tm4sf1 expression may enable rapid TC versus SC identity decisions following TC division.

Dynamic shuffling of TCs and SCs during blood vessel sprouting has been reported by multiple studies (Jakobsson et al. 2010; Arima et al. 2011). These shuffling events are thought to arise due to relative differences and fluctuations in key components of the Vegfr-Notch feedback loop between adjacent TC and SCs. Time-lapse studies suggest TCs are regularly challenged by adjacent SCs for the TC position and dynamic shuffling of ECs during sprout elongation is important for coordinating collective cell migration during angiogenesis (Arima et al. 2011; Jakobsson et al. 2010; Bentley et al. 2014). From our data tracking individual TCs and SCs during ISV sprouting and analysing multiple datasets, we rarely observed TC shuffling events during angiogenic sprouting. Much of the data published on EC shuffling has been derived from in vitro investigations, which are not a true representation of in vivo EC dynamics, primarily due to the supraphysiological levels of VEGF used and the absence of any VEGF gradient. Furthermore, on the rare occasions when shuffling was observed, the leading TC was often undergoing mitosis. During this
time, key signalling pathways that control TC and SC selection may temporarily cease to function, enabling SCs to overcome Dll4-Notch mediated repression. Overall, our detailed single cell analysis of TC/SC dynamics suggest that TC-SC shuffling is rare during ISV sprouting in zebrafish, indicating that the prevalence of shuffling events during angiogenesis in vivo is unclear and requires further investigation.

While the phenotype we observed upon the loss of tm4sf1 expression is highly specific, affecting the behaviour of only TCs (not SCs) and only one cell after a TC division, concerns have been raised over the validity of MO-induced phenotypes. It is widely documented that MOs can induce non-specific toxicity and thus false phenotypes in developing zebrafish embryos, largely due to activation of the tumour suppressor, p53, and consequential apoptosis (Robu et al. 2007; Nasevicius & Ekker 2000). These concerns were amplified when Kok et al (2015) reported a poor correlation between MO phenotypes and mutant phenotypes in many cases (Kok et al. 2015). As the phenotype we observe following the MO-induced knockdown of tm4sf1 is highly specific, only affecting TCs, it is highly unlikely that ubiquitous activation of p53 or any other off target effects would selectively induce a phenotype in such a small sub-population of ECs. Additionally, tm4sf1 MO does not induce phenotypes that are often associated with MO toxicity, such as curling of the tail or developmental delays. Thus, we are confident the phenotype we observed following tm4sf1 MO knockdown is not an artefact. However, it is still important to validate MO phenotypes with a mutant and generation of a mutant fish line would enable the assessment of the viability of tm4sf1 homozygous null fish. Hence, generation of a tm4sf1 mutant fish line would enable validation of the tm4sf1 loss-of-function phenotype and also provide an efficient tool for further functional investigations.
CHAPTER 4: Results

Generation and analysis of tm4sf1 mutant fish lines

4.1 Introduction

While MOs are good tools for the rapid assessment of loss of function phenotypes in vivo, due to the associated problems with MO toxicity as discussed in section 3.3, we opted to generate a tm4sf1 mutant fish line for the loss of Tm4sf1 function. Over the last few years, a variety of genome engineering techniques have been developed that enable the editing of virtually any genomic sequence; namely, zinc finger nucleases (ZFN), transcription activator-like effector nuclease systems (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR). These genome editing tools are comprised of a targeted DNA binding domain fused to a DNA nuclease that cooperatively enable specific genetic alterations with high efficiency (Jao & Chen 2013; Cermak et al. 2011). Here we discuss the principles of two techniques, TALENs and CRISPR, which were utilised for the generation of mutant zebrafish lines for this project.

TALENs are sequence-specific DNA-cleaving enzymes generated by the fusion of TAL effector arrays with a FokI nuclease domain. TAL effectors recognize DNA sequences in a modular fashion and consist of highly similar modular repeats of 34 amino acids that differ from each other at amino acid positions 12 and 13, specifically. These two residues, namely, the repeat-variable diresidue (RVD, figure 4.1, blue), determine the DNA-binding specificity of TAL effectors. There are four RVDs, HD, NG, NI, and NN, which bind to the corresponding nucleotide bases C, T, A, and G, respectively (Cermak et al. 2011). By fusing the TAL effectors to FokI nuclease, the sequence specificity of the TAL effectors guides the nuclease to the targeted region whereby FokI induces cleavage (Figure 4.1, red). Importantly, FokI nucleases function as a dimer. When the forward and reverse TAL effectors are in close proximity, separated by a spacer region of 13-20 nucleotides, the FokI domains induce double strand breaks (Figure 4.1). The need for FokI dimerisation for DNA cleavage reduces the chances of non-specificity and enables targeted gene editing in vivo (Cermak et al. 2011). Double strand breaks can be repaired by two distinct processes; non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is a double strand break DNA repair pathway that directly ligates DNA ends without the need for homologous template; however, this process is prone to errors and
Figure 4.1: Schematic diagram of TALEN induced mutagenesis. TALENs are composed of TAL effector repeats (blue) flanked by N-terminal and C-terminal domains (yellow) and a nuclease domain from the FokI endonuclease (red). The TAL effector repeats target a specific genomic region of interest and guides the FokI nuclease to the targeted region. TALENs bind and cleave as dimers on a target DNA site and cleavage by the FokI nuclease occurs in the spacer region between the forward and reverse TALENs. The FokI nuclease induces a double strand break in the DNA that is repaired by error-prone non-homologous end joining.
thus is exploited for the induction of mutations by genome editing techniques (Figure 4.1, indicated). Alternatively, double strand breaks can also be repaired by HR, another DNA repair process that involves the exchange of DNA nucleotides between two molecules of DNA that exhibit some degree of homology (Cermak et al. 2011; Christian et al. 2010; Miller et al. 2011). Thus by introduction of a carefully designed DNA donor with arms of homology, HR can be used for the insertion of DNA during genome editing.

Recently, CRISPR/Cas9 technology has also been introduced which provides a more simple method for the targeted modification of genes. CRISPR systems are naturally occurring immune mechanisms of many bacteria for protection against foreign DNA (Sander & Joung 2014). Their principles have been manipulated for the targeted editing of specific genomic DNA sites. In contrast to ZFNs and TALENs, which use protein-DNA interactions for DNA sequence targeting (Carlson et al. 2012), CRISPR/Cas9 technology utilises RNA-guided nucleases that target specific DNA sequences via complementary nucleotide base pairing (Sander & Joung 2014). The CRISPR system utilises a guide RNA (gRNA), which is complementary to and targets a specific genomic region of the gene of interest (Figure 4.2). The gRNA is a fusion between a CRISPR RNA (crRNA), which contains a variable sequence (which would usually be transcribed from invading DNA by the bacteria), and a fixed trans-activating CRISPR RNA (tracrRNA) (Figure 4.2). The gRNA forms a complex with the CRISPR-associated nuclease, Cas9. At the 5′ end of the gRNA are twenty nucleotides, which are altered to correspond to the target DNA recognition sequence. Immediately upstream of the target genomic DNA recognition sequence is a protospacer adjacent motif (PAM) sequence, 5′-NGG (Figure 4.2, indicated), essential for Cas9 cleavage. The PAM is present in the genomic DNA but not the gRNA. When the gRNA and Cas9 mRNA are expressed simultaneously inside a cell, the gRNA recognises the complementary target sequence and Cas9 is recruited to the region to induce a double stranded break, usually within 3-4 bps upstream of the PAM (Figure 4.2). Thus, Cas9 can be directed to any target sequence that fits the criteria, N_{20}-NGG, by altering the first twenty nucleotides of the gRNA. Similar to TALENs, the double strand break can then be repaired by NHEJ (Figure 4.2), which often introduces mutations (Sander & Joung 2014; Jao & Chen 2013). Hence, TALENs and CRISPR/Cas9 technology are effective and convenient tools for the targeted mutagenesis of any gene sequence.
Figure 4.2: Schematic diagram of CRISPR-induced mutagenesis. The CRISPR system gRNA is designed to target a specific genomic region of the gene of interest. The genomic target contains a PAM sequence (NGG) immediately after the guide target sequence. The gRNA forms a complex with the CRISPR-associated nuclease, Cas9, and guides the nuclease to the target site. Cleavage occurs within 3-4 bps upstream of the PAM and a double strand break is induced. The break may be repaired by non-homologous end joining which can introduce mutations.
4.2 Results

4.2.1 Generation of an exon 1 targeted TALEN for tm4sf1

Since there are problems associated with MO-mediated gene knockdown, we wanted to confirm the tm4sf1 morphant embryo phenotype and also validate the MO for further tm4sf1 functional studies. To do this, we used the genome engineering tool, TALENs, to generate a tm4sf1 mutant fish line. At the time the mutant was generated, CRISPR/Cas9 technology had not yet been established. When identifying a genomic region for mutagenesis, known regions of functional importance to the protein would usually be the primary target, such as catalytic domains or receptor binding sites. Unfortunately the function of various domains of Tm4f1 have not yet been characterised, thus this approach was not possible. However, previous work in human cancer cells suggests both the N- and C- termini and intracellular loop between the second and third transmembrane domain might be important for the recruitment of TM4SF1 to TEMs (Lekishvili et al. 2008). Due to the limited literature available for TM4SF1 function, we aimed to truncate the protein as early as possible to generate a complete tm4sf1 null fish line. Hence, a TALEN pair was designed to target exon 1. To design our TALEN, we used the TALE Effector Nucleotide Targeter (TALE-NT) design tool (Cermak et al. 2011) and input the tm4sf1 exon 1 sequence. The software examined the input sequence for sites 15-30 bp in length separated by a spacer region of 15 bp, which is optimal for FokI cleavage (Christian et al. 2010). Coordinates and identified sequences of possible TALEN targets were provided in table format which represented recognition sites for the forward and reverse TALEN monomers, in addition to the spacer region (table 4.1). For efficient TAL effector activity, it is important for recognition sites to be preceded by a thyamine (Moscou & Bogdanove 2009), thus the output target sequences matched these criteria. Importantly, the design tool also supplied RVD sequences required for the assembly of the TALENs (table 4.1). As discussed in section 4.1, there are four RVDs, HD, NG, NI, and NN, which bind to the nucleotide bases C, T, A, and G, respectively. Published data suggests that HD and NN bind their corresponding nucleotides, C and G, strongly, whereas binding of NI and NG to T and A, respectively, are relatively weaker (Streubel et al. 2012). For this reason, we selected a TALEN close to the ATG start site of exon 1 with a high ratio of HD/NNs relative to NI/NGs (table 4.1). The TAL effectors were then assembled following the Cermak et al (2011) guidelines, which takes advantage of Golden Gate cloning (Engler et al. 2009). Golden Gate cloning uses Type IIS restriction endonucleases (RE), which cleave outside their recognition sequences to create unique 4 bp overhangs (Engler et al.
Cloning was facilitated by simultaneous digestion-ligation reactions of plasmids containing RVDs within the same mixture, since enzyme recognition sites are disrupted when RVDs are ligated correctly (Cermak et al. 2011).

### Table 4.1: The parameters of our selected TALEN target site and corresponding RVDs

<table>
<thead>
<tr>
<th>TAL effector</th>
<th>Start position coordinate from ‘A’ of ATG</th>
<th>Target genomic sequence</th>
<th>Corresponding RVDs</th>
<th>HD/NN :NI/NG ratio</th>
<th>Spacer region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>2</td>
<td>TGTGCTCTAC AGGATTTGCC</td>
<td>NN NG NN HD NG HD</td>
<td>53%</td>
<td>AGGT CTCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NG NI HD NI NN NN NI NG NG NG NN HD HD</td>
<td></td>
<td>GGTCTT C</td>
</tr>
<tr>
<td>Reverse</td>
<td>53</td>
<td>GCCCTGGTCC CTCTCGCCA</td>
<td>NN NN HD NN NI NN NI NN NN NN NI HD HD NI NN NN NN NN HD</td>
<td>78%</td>
<td></td>
</tr>
</tbody>
</table>

A detailed description of the TALEN assembly is described in section 2.5.1. Briefly, for each forward and reverse TAL effector consisting of 19 and 18 RVDs, respectively, RVDs 1-10 and 11-18/19 were cloned from RVD module plasmids into the intermediary array plasmids, pFUS_A and pFUS_B, respectively (Figure 4.3). BsaI, a typeIIS RE, was used to generate unique sticky ends thus enabling Golden Gate cloning and ligation to occur in a single reaction (Figure 4.3); correct assembly of the RVDs in the pFUS plasmids was confirmed by DNA sequencing. Subsequently, the pFUS_A and pFUS_B array plasmids containing the RVD modules were subjected to a second Golden Gate cloning reaction. Esp3I, another type IIS RE was used to generate unique sticky ends so the pFUS array plasmids, last repeat plasmid (pLR) and the destination vector, RCIScript Goldy TALEN, could be digested and cloned in a single reaction (Figure 4.3). The RCIScript Goldy TALEN vector is derived from the pT3Ts vector, contains the catalytic domain of the FokI nuclease after the C-terminal and also enables the in vitro transcription of capped mRNA that can be injected directly into zebrafish embryos (Osborn et al. 2013; Carlson et al. 2012). We confirmed the correct assembly of the full RVD sequence corresponding to the target region of *tm4sf1* in the RCIScript Goldy TALEN vector. Hence, generation of the *tm4sf1* TALEN construct had been a success and subsequent steps could be taken to test the efficacy of the TALEN in vivo.
Figure 4.3: Construction of \textit{tm4sf1} TALEN constructs by Golden Gate cloning. (A) RVDs 1-10 and 11-19/18 were cleaved out of their module plasmids and cloned into the intermediary array plasmids, pFUS\textsubscript{A} and pFUS\textsubscript{B} (B) The second reaction contained the pFUS\textsubscript{A} and pFUS\textsubscript{B} array plasmids, the last repeat module and the destination vector, RCIScript Goldy TALEN. \textit{Esp}3I was used to generate unique sticky ends to enable digestion and ligation in a single reaction. The final construct consisted of the full sequence of RVD modules, flanked by N and C terminal domains and the \textit{FokI} endonuclease in the RCIScript Goldy TALEN vector; correct assembly was confirmed by DNA sequencing.
4.2.2 TALEN-mediated mutagenesis of tm4sf1 exon 1

Following generation of the TALEN constructs and confirmation of correct assembly by DNA sequencing, we were in a position to test the efficiency of the TALEN to induce target-specific mutations. The RCIScript Goldy TALEN forward and reverse constructs were subsequently linearised by *SacI* and mRNA encoding each TALEN arm (both forward and reverse) were *in vitro* transcribed. 100 pg of each forward and reverse TALEN RNA was then injected into the single cell of WT embryos. At 24-48 hpf, genomic DNA was extracted from individual embryos and the region targeted by the TALEN was amplified using primers TALEN I.D F and R (table 2.2; figure 4.4A). To determine whether the TALEN induced somatic lesions in *tm4sf1*, we used high-resolution melt (HRM) analysis. Since the thermal stability of a DNA duplex is defined by its nucleotide sequence, if the TALEN was to introduce mutations, either by single nucleotide polymorphisms (SNPs), insertions or deletions, the thermal stability of the duplex would change (Montgomery et al. 2007; Erali & Wittwer 2010). The resultant melting behaviour of the DNA duplex would also change which can be observed by HRM analysis. During HRM analysis, a fluorescent dye binds newly synthesised double stranded DNA as it is generated during PCR amplification. Post-PCR amplification, the fluorescent signal is detected and monitored by a specialised analyzer as the PCR product is exposed to increasing temperature. As the temperature is increased, the DNA duplex denatures and the fluorescence is released; thus, a melting profile characteristic of the DNA duplex stability can be observed (Montgomery et al. 2007; Erali & Wittwer 2010). Analysis of the HRM curves identified DNA fragments amplified from TALEN-injected embryos that displayed subtle shifts in their melt curve profiles, relative to fragments amplified from WT non-injected controls (Figure 4.4B–C). Sequencing of DNA products amplified from the targeted genomic region of TALEN-injected embryos confirmed the presence of mutations in *tm4sf1* at the TALEN target site. Moreover, injection of the TALEN RNA achieved an 80% somatic lesion frequency; with 4 out of the 5 sequenced clones from TALEN-injected embryos displaying detrimental frameshift mutations (Figure 4.4D). Two independent studies that determined the relationship between the somatic lesion frequency and germline transmission rate of ZFNs found that somatic lesion frequencies as low as 1% were sufficient for germline transmission, with 10–25% of screened adults identified as founders (Sood et al. 2013; Zhu et al. 2011). Hence, the high efficiency of our TALEN should increase our chances for germline transmission of *tm4sf1* mutations.
Figure 4.4: Design, validation and efficiency of a TALEN targeting exon 1 of \textit{tm4sf1}.

(A) An illustration to show the basic structure of the TALEN and its genomic target site at the start of exon 1 of \textit{tm4sf1}. Positions of Tm4sf1 TALEN ID F and R primers are indicated. (B-C) HRM of normalised fluorescence against increasing temperature, indicating WT product melting curves compared to PCR products with altered thermal stability due to the presence of somatic lesions. Red box indicates the region amplified in (C), comparing one WT product melting curve (blue, indicated) and one with altered thermal stability (yellow) to emphasise the difference in thermal stability of WT products and those containing somatic lesions. (D) Somatic lesions identified in one TALEN injected embryo upon TOPO cloning of PCR-amplified target regions and sequencing of resulting individual colonies. The WT (top) sequence shows the TALEN recognition sites (red) separated by the spacer region.
4.2.3 Establishment of an F₁ generation of TALEN-induced tm4sf1 mutant fish

Following confirmation that the exon 1 TALEN was able to induce somatic lesions in tm4sf1 at the TALEN target site at high efficiency, we performed the necessary steps to establish an F₁ tm4sf1 mutant fish line that could be used for phenotypic analysis. TALEN RNA-injected fish were raised to adulthood and subsequently crossed with WT embryos. The progeny were collected and genomic DNA was extracted from individual embryos at 24-48 hpf. Sequencing of DNA products amplified from the TALEN-targeted genomic region of tm4sf1 confirmed the presence of mutations. In particular, we aimed to identify an F₀ founder containing a deleterious germline mutation; preferably one that introduced an early stop codon. One F₀ founder fish we identified had four mutations in the germline (Figure 4.5A); all of which introduced a frame shift (Figure 4.5A) and an early stop codon. The tm4sf1 TALEN F₀ founder fish was out-crossed with Tg(kdrl:nlsEGFP)zf109 fish to generate a mutant carrier line that would enable us to visualise TC and SC dynamics. For selection of fish for our F₁ generation, we tail clipped adult fish and extracted their DNA for PCR amplification of the TALEN-targeted genomic region of tm4sf1 and subsequent sequencing. We selected fish carrying a 19 bp deletion (Figure 4.5A, boxed) for ease of identification, since 19 bp differences between WT and mutant PCR products could be resolved easily by agarose gel electrophoresis. This 19 bp deletion would be predicted to affect the translation of tm4sf1 such that the first 10 amino acids are correct (Figure 4.5B, underlined), but after this incorrect amino acids are inserted and an early stop codon is introduced after a further 6 amino acids (Figure 4.5B, arrow). Hence, the protein is truncated early and should lead to a complete null. F₁ founder fish carrying the Tg(kdrl:nlsEGFP)zf109 transgene were isolated and used for further experimentation.

4.2.4 Phenotypic analysis of tm4sf1 exon 1 mutant embryos

To determine whether our tm4sf1 TALEN mutant exhibited vascular defects, two heterozygous tm4sf1 TALEN fish in the Tg(kdrl:nlsEGFP)zf109 background were crossed and their progeny were collected. Eight nlsGFP-expressing embryos were selected at random and embedded in low melt agarose at 22 hpf, as the first ISVs were beginning to sprout from the DA (Figure 3.1A). Using multipoint visiting, images of sprouting ISVs were captured every 0.3 hours for 10.2 hours until the embryos were 32.2 hpf. Following maximum projection of these movies, individual TCs were tracked to determine their motility, as previously described in section 3.2.6. After imaging, embryos were retrieved
Figure 4.5: Identification of germ-line *tm4sf1* mutations. (A) Germline mutations identified in our F₀ founder fish, upon TOPO cloning of PCR-amplified target regions from embryos and sequencing of resultant individual colonies. The WT (top) sequence shows the TALEN recognition sites (red) separated by the spacer region and our selected 19 bp F₁ mutation is highlighted at the bottom (boxed). (B) A comparison of the amino acid sequences of WT Tm4sf1 verses mutant Tm4sf1 from an F₁ founder with a 19 bp deletion. Incorrect amino acids are inserted after position 10 and an early stop codon is introduced after position 16 (indicated). Identical (*), highly conserved (:), and weakly conserved (.) amino acids are indicated. Small/hydrophobic residues (red), acidic residues (blue), basic residues (magenta), hydroxyl/amine/basic residues (green) are shown.
from the agarose and genotyped by extraction of genomic DNA, PCR amplification of the TALEN targeted region and subsequent analysis on an ethidium bromide gel (Figure 4.6A). WT embryos were identified by one band at 185 bps whereas homozygous mutant embryos were identified by one band of 166 bp. Heterozygous embryos had a band at 185 and 166 bp, which often appeared as a smear (Figure 4.6A). Following analysis of cell motility, no significant differences were observed between the motility of WT, heterozygous and homozygous tm4sf1 mutant TCs (Figure 4.6B). We then went on to track the dorsal movement of TC daughters following a TC division, as previously described in section 3.2.6. Similarly, there was no significant difference in dorsal movement between the two daughter cells derived from a TC division between WT, heterozygous and homozygous tm4sf1 mutants (Figure 4.6C). We also compared the dorsal movement of the TCs and TC daughters of the control MO injected embryos from our previous experiments (section 3.2.6) with WT, heterozygous and homozygous tm4sf1 mutants. Similarly, there were no significant differences in dorsal movements between TCs and TC daughters after a TC division (data not shown). We can also confirm that fish homozygous for the tm4sf1 mutant allele survive to adulthood and are fertile. There were no gross morphological differences between WT and tm4sf1 homozygous mutant fish and no vascular or circulatory phenotypes were observed in the adult fish. Hence, unfortunately there was no detectable vascular phenotype in our tm4sf1 exon 1 TALEN mutants.

### 4.2.5 Identification of a second tm4sf1 isoform

The absence of a phenotype in our tm4sf1 homozygous mutant TALEN was unexpected since the phenotype we observed following MO-mediated knockdown of tm4sf1 was very specific, affecting TC behaviour but not SC behaviour. Thus, the MO phenotype was unlikely due to the effects of MO toxicity. Shortly after generating our tm4sf1 TALEN, the zebrafish genome was re-annotated in ensemble to reveal the presence of a shorter, second isoform of tm4sf1 in zebrafish (Figure 4.7A-C). Our tm4sf1 TALEN was targeted to the start of exon 1 which only mutates the long tm4sf1 isoform. The second, shorter isoform originates from an ATG start codon at the beginning of exon 2; thus, the resultant protein would lack the first 59 amino acids of the long isoform which are predicted to encode for the N-terminal, first transmembrane domain (TM1), short extracellular loop (EC1) and part of the second transmembrane domain, (TM2; Figure 4.7B). Hence, our mutant fish line may not have a phenotype because our TALEN mutation only targets one isoform (Figure 4.7A). The tm4sf1 MO utilised for experiments in chapter 3, blocks the intron 1-exon 2 splice junction. Consequently, the MO
Figure 4.6: Exon 1 of tm4sf1 is not required for TC motility. (A) Example genotyping results for individual embryos following PCR amplification of the TALEN targeted region from genomic DNA. PCR products were separated on an ethidium bromide agarose gel. WT embryos are indicated by a band of 185 bp; mutant embryos are indicated by a band of 166 bp; heterozygous embryos are indicated by two overlapping bands of 166 and 186 bps. (B-C) Quantification of dorsal nuclear movement of (B) TCs, and (C) TC daughters, in WT, heterozygous and homozygous tm4sf1 mutant embryos starting from 22 hpf (indicated) (B) or immediately after cell division (C) No significant differences were observed in the dorsal movement of TCs or TC daughters after division upon mutation of tm4sf1 exon 1.
Figure 4.7: The long and short isoforms of tm4sf1. (A) A diagrammatic representation of the exons and introns of the long and short isoforms of tm4sf1. The MO target site is indicated at the intron 1-exon 2 splice junction. (B) A diagrammatic representation of the predicted protein structures for the long and short tm4sf1 isoforms. (C) Direct comparison of the nucleotide sequences of the long and short isoforms of tm4sf1. The tm4sf1 TALEN lesion is indicated in red and MO target site is shown in blue. ATG start sites are indicated in yellow. (C) Direct comparison of the amino acid sequences of the long and short isoforms of tm4sf1. The peptide sequence that the Tm4sf1-targetting antibody was raised against (highlighted) is present in both isoforms.
also blocks the exon 2 ATG start site. Thus, to MO may additionally act like an ATG translation-blocking MO, or inclusion of intron-1 may disrupt subsequent transcription, which may explain why we see a phenotype in our tm4sf1 morphant embryos. Hence, the shorter isoform of Tm4sf1 may compensate for mutated long isoform in our tm4sf1 TALEN mutants.

To confirm whether our tm4sf1 TALEN mutant lacked the long isoform of tm4sf1 but still retained expresses the short isoform, we raised an antibody to zebrafish tm4sf1 using the peptide sequence YPFEQDEGRYLFARERWSEC (Figure 4.7C, highlighted). This antibody should recognise both the long and short isoform as this C-terminal sequence is conserved between both isoforms. To detect Tm4sf1 we extracted protein from WT and tm4sf1 homozygous TALEN mutant zebrafish and performed an immunoblot. Using a protein molecular weight prediction tool, the long and short isoforms of tm4sf1 were predicted to have a molecular weight of approximately 22 kDa and 15 kDa, respectively. Unfortunately, the antibody that we raised didn’t work by immunoblot, likely due to the antibody not recognising the denatured Tm4sf1 protein. This is a common feature for Tm4sf1 antibodies and a recognised problem in the field (Shih et al. 2009). Thus, we were unable to determine whether our mutant lacked Tm4sf1 protein (data not shown).

4.2.6 Design and assembly of a tm4sf1 exon 2 targeting CRISPR

As our tm4sf1 TALEN mutant fish line did not exhibit any detectable vascular defects, potentially due to the presence of a shorter isoform of tm4sf1 that escaped mutation, we decided to generate a second tm4sf1 mutant fish line that targeted exon 2. To do this, we took advantage of the recently developed CRISPR/Cas9 technology (Ran et al. 2013; Jao & Chen 2013) (described in section 4.1). By targeting exon 2, both the long and short tm4sf1 isoforms would be mutated. Additionally, we decided to induce the mutation in the background of our already established homozygous TALEN, to prevent the possibility of exon skipping to generate a protein that lacks exon 2 but contains exon 1. The resultant fish line would therefore contain two lesions, one after each potential translational start site. To select a target site for our CRISPR guide, we used the ‘Optimized CRISPR Design Tool’ by the Feng Zhang group (Ran et al. 2013). The tool enables the input of a target region of a gene and potential CRISPR gRNAs are identified. We input the sequence for tm4sf1 exon 2 and the sequence was scanned for CRISPR guides, which are comprised of 20 nucleotides proceeded by the PAM sequence, NGG.
Figure 4.8: Generation of CRISPR gRNA. Two complementary oligos with additional nucleotide overhangs added were designed to target a specific genomic region within exon 2 of *tm4sf1*. The oligos were annealed and subsequently cloned into a vector containing the T7 promoter for RNA transcription and the gRNA backbone required for targeted mutagenesis. The vector was linearised using *BamHI* and the gRNA was *in vitro* transcribed from the T7 promotor. The RNA was purified ready for injection into zebrafish embryos.
The CRISPR guide selected was 5’-CCTGTGTGTTCCTGGGAATG-3’ (Figure 4.8), and a PAM sequence of ‘GGG’ followed after the guide sequence. The sequence was also scanned for possible off-target matches throughout the zebrafish genome. The guide had 71 potential off-target binding sites, although none of these were within genes. While the number of off-targets was relatively high, most gRNAs have many off-target hits. Importantly, all off-targets identified by the design tool had three or more adjacent mismatches. Investigations performed by two independent studies suggest that two or less mismatches on the gRNA can be tolerated by Cas9, but three or more adjacent mismatches severely affects the activity of Cas9 (Jao & Chen 2013; Fu et al. 2013). Hence, these gRNAs are considered good quality and this is reflected in its quality score of 90/100.

Following design and selection of the CRISPR guide, we used the Chen & Wente protocol for gRNA assembly (Jao & Chen 2013), as described in detail in section 2.5.2. Briefly, complementary primers containing the gRNA target sequence were designed and nucleotides ‘TAGG’ and ‘AAAC’ were added to the 3’ end of the forward and reverse primers (Jao and Chen, 2013), respectively (Figure 4.8). The added nucleotides provide complementary overhang for the cloning of the guide into its destination vector, T7cas9sgRNA2. The two primers were annealed and subsequently cloned into the T7cas9sgRNA2 vector in a one-step digestion and ligation reaction (Jao and Chen, 2013) (Figure 4.8). Correct clones were identified by DNA sequencing and the gRNA was in vitro transcribed (Figure 4.8) and purified, ready for injection. Similarly, capped nls-zCas9-nls RNA was synthesised following linearization of the pT3TS-nls-zCas9-nls plasmid and purified ready for injection (Jao and Chen, 2013).

4.2.7 CRISPR-mediated mutagenesis of tm4sf1 exon 2

Following generation of the CRISPR gRNA we aimed to generate a mutant zebrafish line containing lesions in exon 1 and exon 2 of tm4sf1. To do this, 30 pg tm4sf1 gRNA and 100 pg nls-zCas9-nls RNA were injected into the single cell of embryos homozygous for the TALEN lesion and also carrying the Tg(kdrl:nlsEGFP)zf109 transgene. At 24-48 hpf, genomic DNA was extracted from individual injected embryos and the region targeted by the gRNA was amplified using primers CRISPR ID F and CRISPR ID R (table 2.2; figure 4.9A). Importantly, our CRISPR guide was selected so that the genomic region it targeted contained a unique RE site. The presence of a unique RE site facilitated in the identification of potential mutations, since mutations induced by the gRNA/Cas9 complex would disrupt the RE recognition site. Our gRNA contained the unique RE, BsII, which recognises and cleaves the sequence CCNNNNN▽NNGG, where
Figure 4.9: Exon 2-targeting CRISPR efficiently induces tm4sf1 mutations. (A) An illustration to show the design of the tm4sf1 exon 2-targeted CRISPR gRNA. The guide is shown in red and the PAM is shown in blue. BslI RE recognises the region as indicated. (B) Ethidium bromide agarose gel to show PCR products derived from amplification of the CRISPR target region from un-injected control embryos and tm4sf1 gRNA injected embryos following BslI digestion. PCR products derived from control embryos were fully BslI digested resulting in two bands of 65 and 138 bp. PCR products derived from gRNA injected embryos remain either undigested or partially digested by BslI, resulting in one band of 203 bp or three bands of 65, 138 and 203 bp. (C) Somatic lesions identified in one CRISPR gRNA injected embryo upon TOPO cloning of PCR-amplified target regions and sequencing of resulting individual colonies. The WT (top) sequence shows the CRISPR guide recognition site (red) and the PAM (blue). The numbers in brackets indicate the frequency of each lesion.
‘N’ can be any nucleotide (Figure 4.9A). Hence, mutations in the CRISPR target region would protect the PCR product from digestion; thus undigested bands were indicative of possible mutations. Following amplification of the genomic region targeted by the CRISPR, PCR products from individual embryos were subjected to BsslI digestion (Figure 4.9B). We also amplified the same region from two non-injected embryos as comparative controls, since we know that 100% of PCR product amplified from these embryos should be digested. The genomic region amplified was 203 bp in length and when cleaved by BsslI, resulted in two cleavage products of sizes 65 and 138 bps. If the PCR product was protected by mutations, one band of 203 bps or all three possible band sizes would be observed. Indeed, PCR products derived from control embryos were 100% digested, resulting in two bands of 65 and 138 bps (Figure 4.9B), as expected. In contrast, embryos injected with the tm4sf1 gRNA and nls-Cas9-nls remained predominantly undigested to generate a large band of 203 bp and fainter bands of 65 and 138 bps (Figure 4.9B). The undigested bands were gel extracted, purified and sequenced to confirm the presence of mutations. Of 16 colonies generated from one embryo, 14 contained lesions, thus achieving an 87.5% somatic lesion frequency (Figure 4.9C). However, only one of those lesions induced a frameshift mutation. As previously discussed, a somatic lesion frequency as low as 1% is sufficient to obtain germline transmission (Sood et al. 2013; Zhu et al. 2011). Hence, once the ability of the gRNA to induce somatic mutations was confirmed, we injected approximately 150 embryos and raised them to adulthood to generate a mutant line.

4.2.8 Establishment of an F1 generation of tm4sf1 double mutant fish

Following confirmation that our tm4sf1 CRISPR guide was able to induce somatic lesions in tm4sf1 at the gRNA target site, we aimed to establish an F1 double mutant fish line that could be used for phenotypic analysis. Thus, when the CRISPR gRNA injected fish reached adulthood, we crossed them with a Tg(kdrl:nlsEGFP)zf109 fish and collected the progeny to screen for mutations. Genomic DNA was extracted from individual embryos between 24-48 hpf and the CRISPR guide targeted region was subjected to HRM analysis to, as previously described in section 4.2.2. We identified an F0 founder fish whose progeny displayed five very specific HRM curves, presumably due to different lesions generated by CRISPR-mediated mutagenesis of the germ line (Figure 4.10A). Sequencing of DNA products amplified from the tm4sf1 gRNA targeted genomic region confirmed the presence of mutations in individual embryos. One HRM curve represented
Figure 4.10: Identification of an F₀ tm4sf1 double mutant founder by HRM and sequencing. (A) HRM plot of normalised fluorescence against increasing temperature to show the thermal stability of PCR products. PCR products were amplified from the CRISPR targeted tm4sf1 genomic regions derived from individual embryos of F₀ and Tg(kdrl:nlsEGFP)zf109 outcrosses. Five distinct products of differing thermal stabilities were observed, and (B-E) demonstrates individual melting profiles of the five PCR products. The WT melting profile is shown in blue. Four germline mutations were identified (orange) consisting of (B) a 6 bp deletion, (C) a 3 bp insertion, (D) a 27 bp deletion, and (E) a 16 bp deletion/2 bp insertion. (F) Sequencing results from genomic DNA extracted from embryos exhibiting specific melting profiles relating to the four germline mutations identified. (G) Comparison of the WT tm4sf1 amino acid sequence and the amino acid sequence of the F₁ founder (-16/+2 bp) for the short isoform of tm4sf1. Incorrect amino acids occur after position 5 and an early stop codon is introduced after position 53. Identical (‘*’), highly conserved (‘:’) and weakly conserved (‘.’) amino acids are indicated. Small/hydrophobic residues (red), acidic residues (blue), basic residues (magenta), hydroxyl/amine/basic residues (green) are shown.
WT sequence (Figure 4.10B-4.10E, blue line) whereas the four others represented a 6 bp deletion (Figure 4.10B), 3 bp insertion (Figure 4.10C), a 27 bp deletion and a 16 bp deletion/2 bp insertion (Figure 4.10E). Since three lesions were in frame deletions, the only deleterious frameshift mutation was the 16 bp deletion/2 bp insertion. Hence, this lesion was selected for our F<sub>1</sub> generation. The F<sub>0</sub> founder was out-crossed with Tg(kdrl:nlsEGFP)<sup>zf109</sup> fish to generate a line that enabled the visualisation of TC and SC dynamics. When the fish reached adulthood, their tails were clipped for genotyping as previously described. Approximately 1 in 5 fish were found to carry the +16/-2 bp mutation and these fish were isolated and used for further experimentation. To summarise, we generated a fish line using both TALENs and CRISPR/Cas9 technology that mutated both the long and short tm4sf1 isoforms.

### 4.2.9 TC motility is disrupted in tm4sf1 double mutants

We have previously shown that mutation of the long isoform of tm4sf1 does not display a detectable angiogenic phenotype (section 4.2.4). To determine whether TC behaviour was affected by mutation of both tm4sf1 isoforms, we performed time-lapse confocal imaging of ISVs during sprouting. Images were captured every 0.3 h for 10.2 h, and the positions of TCs (cell 1) and stalk cells (cell 2) were recorded at each time point, as previously described (section 3.2.6). Firstly, we compared TC (cell 1) dorsal movement between WT and tm4sf1 double mutant embryos. Initially, TC nuclei in tm4sf1 mutants progressed at a similar rate as TC nuclei in WT embryos, and there was no significant difference in TC dorsal movement from 22 hpf until 27.4 hpf (Figure 4.11A). From this point, TCs in tm4sf1 double mutants progressed dorsally more slowly than TC nuclei in WT embryos. This reduced rate of TC dorsal movement continued until around 30.4 hpf, and during this three hour period, significant differences in TC motility were observed (Figure 4.11A). The delay in TC dorsal movement in tm4sf1 double mutants was in agreement with the tm4sf1 morphant phenotype, albeit, more subtle. Embryos heterozygous for the exon 1 and exon 2 lesions also displayed a very mild delay in TC dorsal movement (Figure 4.11B), suggesting the effects of Tm4sf1 may be dose-dependent. To determine whether mutation of both tm4sf1 isoforms specifically affected TC (cell 1) motility, but not SC (cell 2) motility, we also assessed the motility of SCs (cell 2). Indeed, no significant differences were observed in the dorsal movement of SCs between WT, heterozygous and tm4sf1 double mutant embryos (Figure 4.11A-B). Hence, consistent with the tm4sf1 morphant embryo phenotype, mutation of both the long and
Figure 4.11: Mutation of both tm4sf1 isoforms disrupts TC motility. (A-B) Quantification of dorsal movement of TCs (cell 1) and SCs (cell 2) in (A) WT and tm4sf1 double mutants, and (B) WT and heterozygous embryos, for 10.2 hours starting from 22 hpf (indicated). Significant differences were observed between WT and mutant/heterozygous TC motilities, but not SC motilities. Error bars represent mean +/- SEM. n = at least 32 ISVs for TCs and 13 ISVs for SCs from 5 separate experiments *p < 0.05 versus control, Student’s t test.
short isoforms of *tm4sf1* specifically provoked a reduction in TC motility of *tm4sf1* double mutants, whereas SC motility was unaffected.

### 4.2.10 *tm4sf1* influences post-mitotic TC motility

Our MO data revealed a subtle difference between the motility of dorsal TC daughters in control versus *tm4sf1* morphant embryos. Although the double mutant phenotype was somewhat weaker, we observed a significant difference between the motile behaviour of TCs in WT embryos compared to TCs in double mutants. Hence, we wanted to see whether mutation of both the long and short isoforms of *tm4sf1* could replicate the post-mitotic motility phenotypes we observed in the *tm4sf1* morphant embryos following TC division. To do this, we tracked the dorsal nuclear movement of both daughter cell nuclei after a TC division. Tracking commenced when the two daughter cell nuclei had completed cytokinesis. For analysis, we adopted the same cell tracking approach using the nomenclature described in figure 3.9, whereby the dorsal/ventral daughters derived from TC and SC divisions were denoted 1.1/1.2 and 2.1/2.2, respectively. Tracking of single cells revealed that after a TC division (cell 1), the dorsal-most daughter (cell 1.1) of a TC division in WT embryos was significantly more motile than the dorsal-most daughter (cell 1.1) in *tm4sf1* double mutants (Figure 4.12A-B). Significant differences in TC dorsal daughter (cell 1.1) motility were observed almost immediately following TC division, from 0.9 h to 6 h (Figure 4.12A-B). As previously observed for heterozygous TCs (cell 1), a very mild phenotype in TC dorsal daughter motility was observed in heterozygous embryos compared to WT embryos (Figure 4.12C), again suggesting a potential dose-dependent effect of Tm4sf1 function. Interestingly, for 3.6 hours after TC division, TC dorsal daughters from *tm4sf1* double mutants displayed motilities more similar to ventral daughters (cell 1.2) derived from a WT TC division (Figure 4.12B). Hence, the TC dorsal-daughter (cell 1.1) phenotype observed in our double mutant is consistent with the phenotype observed upon MO-mediated *tm4sf1* knockdown, indicating that Tm4sf1 modulates post-mitotic acquisition of TC motility. We have previously shown that MO-mediated loss of *tm4sf1* only affects the dorsal daughter cell from a TC division; however the motilities of the two daughter cells from a SC division were unchanged (section 3.2.6). To determine whether our *tm4sf1* double mutant agreed with these findings, we tracked the dorsal nuclear movement of the two daughter cell nuclei after a SC division. No significant differences were observed in the dorsal movement cells 2.1 and 2.2 derived from a SC division between WT and *tm4sf1* double mutants (Figure 4.12D). Hence, consistent with the MO phenotype, *tm4sf1* is specifically required to promote TC motility.
Figure 4.12: Mutation of both tm4sf1 isoforms specifically disrupts post-mitotic TC behaviour. (A) Lateral images of ISVs from WT and tm4sf1 double mutant embryos in the Tg(kdrl:nlsEGFP)zf109 transgenic background. Images represent stills taken from movies from 0.3 h prior to TC division. (B-C) Quantification of the dorsal movement of TC daughters, 1.1 and 1.2, following a TC division for the comparison of (B) WT and tm4sf1 double mutants and (C) WT and tm4sf1 heterozygous embryos. (D) Quantification of the dorsal movement of SC daughters, 2.1 and 2.2, from WT, heterozygous tm4sf1 mutant and homozygous tm4sf1 mutant embryos. Error bars represent mean +/- SEM. n = at least 19 ISVs for TCs and 5 ISVs for SCs from 5 separate experiments *p < 0.05 versus control, Student’s t test.
4.2.11 *tm4sf4* mRNA expression is upregulated upon *tm4sf1* mutation

We noticed that the *tm4sf1* double mutant phenotypes were milder than the *tm4sf1* morphant embryo phenotypes. Additionally, the *tm4sf1* TALEN lacked any detectable vascular phenotype. However, it has recently been reported that upregulation of genes to compensate for loss-of-function occurs in mutants but not in morphants (Rossi et al. 2015). Additionally, studies have shown that loss of a specific tetraspanin can be compensated for by an increase in surface expression of other tetraspanin family members (Fradkin et al. 2002; Lekishvili et al. 2008; Hemler 2005). It is therefore possible that other genes may compensate for *tm4sf1* loss of function in mutants and we decided to screen for such genes. We identified potential L6 family members in thezebrafish genome and found five genes that exhibited homology with *tm4sf1*, namely; *si:ch211137i24.10*, *zgc:172079*, *LOC100002960*, *tm4sf4*, and *tm4sf5*. Additionally, due to previous reports of enhanced expression of specific tetraspanins in cancer cells lacking *tm4sf1*, we also assessed the mRNA expression of tetraspanins *CD63*, *CD81*, and *CD151* (Lekishvili et al. 2008). We compared the relative expression levels of these *tm4sf*/tetraspanin family members between age-matched WT and *tm4sf1* TALEN homozygous mutant embryos at 26 hpf, the time point at which *tm4sf1* expression is at its highest (Figure 3.4, section 3.2.1). We used the exon 1 TALEN mutants as opposed to the *tm4sf1* double mutant since the homozygous double mutant line was not established. Of the eight genes investigated, only one gene was found to have significantly increased expression levels in the *tm4sf1* TALEN mutants compared to the WT. *tm4sf4*, the most closely related paralogue to *tm4sf1* had a 4.0-fold (+/- 0.55) increase in expression in *tm4sf1* TALEN mutants when compared to WT controls (Figure 4.13A). Hence, loss of the long isoform of *tm4sf1* may be compensated for by overexpression of another L6 family member, *tm4sf4*.

Considering that *tm4sf4* expression was upregulated in *tm4sf1* exon 1 mutants, we wanted to see whether *tm4sf4* was similarly expressed in the ISVs as observed with *tm4sf1*, since this may indicate potential functional compensation by *tm4sf4* in the absence of *tm4sf1*. To do this, we performed whole mount chromogenic ISH for *tm4sf4* in WT and *tm4sf1* TALEN mutants at 26 hpf. In WT embryos, *tm4sf4* expression was not detectable in the vasculature. However, in *tm4sf1* TALEN mutants, *tm4sf4* mRNA expression was detected in what appeared to be the CV region (Figure 4.13B; red bracket). This was interesting since our ISH staining pattern for *tm4sf1* also displayed staining within the
Figure 4.13: tm4sf4 expression is enhanced in tm4sf1 TALEN mutants, but not in ECs. (A) Relative quantification of L6 family members and closely related tetraspanins by qPCR. Significant Error bars represent mean +/- SEM. n = 4 separate experiments *p < 0.05 versus control, Student’s t test. (B) Chromogenic ISH for tm4sf4 RNA in WT and tm4sf1 homozygous TALEN embryos. Red lines indicate the venous staining. (C) Double fluorescent ISH for tm4sf4 (red) and gfp (green) in WT and tm4sf1 homozygous TALEN embryos, both of which carry the Tg(kdrl:nlsEGFP)^{109} transgene. Images represent maximum intensity z-projections, individual slices through the centre of the embryos of tm4sf4 (red), gfp (green), and a merge of tm4sf4 and gfp (red and green).
same region at 26 hpf (Figure 3.4B). However, tm4sf1 expression was strongest in the ISVs, particularly at 26 hpf when the majority of ISVs are sprouting from the DA (Figure 3.4). In contrast, there was no evidence of tm4sf4 mRNA expression in ISVs (Figure 4.13B). However, the chromogenic ISH may not be sensitive enough to see low levels of tm4sf4 mRNA expression. Additionally, it is also difficult to see staining at cellular resolution by chromogenic ISH. Hence, we decided to look at the expression of tm4sf4 by fluorescent ISH (FISH). We performed a double FISH for both tm4sf4 mRNA (Figure 4.13C, red) and gfp mRNA (Figure 4.13C, green) in WT or tm4sf1 TALEN embryos expressing kdrl:nlsEGFP. Since nls:EGFP is under the control of the EC-specific promoter, kdrl, the gfp mRNA was used to identify ECs. Lateral images of ISVs were captured and confocal z-stacks were maximum projected (Figure 4.13Ci). Additionally, individual slices through the centre of the embryo for tm4sf4 (Figure 4.13Cii, red), gfp (Figure 4.13Ciii, green), and a merge of tm4sf4 and gfp (Figure 4.13Civ, red and green) are represented. In WT embryos, tm4sf4 expression was minimal (Figure 4.13Cii, red), in agreement with the result from the chromogenic ISH. In contrast, in tm4sf1 TALEN mutant embryos, FISH revealed that tm4sf4 was highly upregulated but not expressed in the ECs of the vein, since the cells that were positive for gfp (Figure 4.13Ciii, green) were negative for tm4sf4 (Figure 4.13Cii, red). tm4sf4 staining appeared to be in the cells that reside within the vessel (Figure 4.13Civ, red and green), and thus presumably primitive erythroid cells, that entering the circulation via the CV at 26 hpf (Bertrand et al. 2010). To conclude, these data show that tm4sf4 expression was enhanced when exon 1 of tm4sf1 is mutated. However, the increase in expression was not in ECs, but in cells that reside inside the vessel, which are likely putative primitive erythroid cells.
4.3 Discussion

Data presented in this chapter shows the successful generation of two mutant fish lines; one potentially eliminating the long isoform of \textit{tm4sf1}, and the other potentially eliminating both the long and short isoforms of \textit{tm4sf1}. Following the tracking of individual TCs and SCs to determine their motile behaviour during ISV sprouting, we did not identify any defects in blood vessel sprouting in the exon 1 \textit{tm4sf1} TALEN mutant. Hence, mutagenesis of the long \textit{tm4sf1} isoform had no effect, suggesting it may be redundant, at least in angiogenic vessels. In contrast, when both the long and short isoforms of \textit{tm4sf1} were mutated, embryos exhibited a mild vascular phenotype, in agreement with the \textit{tm4sf1} morphant embryo phenotype. This suggests exon 1 is not essential for the angiogenic function of \textit{tm4sf1}. In support of this, the key functional region of L6 family member, TM4SF5, has been mapped to the C-terminal tail in exon 4, whereby interactions with c-Src are important for the activation of epidermal growth factor receptor (EGFR) (Jung et al. 2013). The C-terminal tail is also reported to play key roles in the function of tetraspanins. For example, the C-terminal Gly-Tyr-Glu-Val-Met (GYEVM) motif of CD63 is essential for the targeting of tetraspanin CD63 to intracellular compartments via interactions with the μ3A subunit of adaptor protein 3 (AP-3)(Rous et al. 2002). Additionally, CD63 contains another C-terminal motif, Tyr-Xaa-Xaa-∅ (YXX∅) which, when mutated, disrupts the endosomal localisation of CD63, redirecting it to the cell surface (Bonifacino & Dell'Angelica 1999; Rous et al. 2002). Moreover, we already know that \textit{tm4sf1} contains a PDZ-binding motif located at its C-terminal tail (Borrell et al. 2000). PDZ domains are key elements in the construction of functional protein complexes, known to facilitate protein-protein interactions and thus may be important for \textit{tm4sf1} function (Nourry et al. 2003). Hence, the redundancy of exon 1 in the angiogenic function of \textit{tm4sf1}, in combination with the presence of important motifs in the C-terminal tail of \textit{tm4sf1} suggests the C-terminus, as opposed to the N-terminus, may be of functional importance for \textit{tm4sf1}. This may explain why mutation of exon 1 of \textit{tm4sf1} does not recapitulate MO-mediated disruption of TC sprouting.

While mutation of exon 1 of \textit{tm4sf1} does not appear to have functional importance in ECs, we have shown that \textit{tm4sf4} mRNA was upregulated. However, the enhanced expression of \textit{tm4sf4} was not detected in ECs by ISH and FISH, but most likely erythroid cells that reside within the CV. However, to fully confirm this possibility we would need to perform a double FISH for \textit{tm4sf4} and a primitive erythroid marker, such as \textit{gata1}. The absence of \textit{tm4sf4} expression in ECs raised a number of questions. Firstly, is \textit{tm4sf4}
expressed in ECs but at undetectable levels? This would suggest that \textit{tm4sf4} could be compensating for the loss of \textit{tm4sf1} and may provide an explanation as to the lack of an angiogenic phenotype in the \textit{tm4sf1} TALEN. To test this, we could knock down \textit{tm4sf4} in the \textit{tm4sf1} TALEN mutant fish to see whether we observe a vascular phenotype. If a phenotype is observed, this could be indicative of \textit{tm4sf4} compensation of Tm4sf1 loss of function. Additionally, we could inject the \textit{tm4sf1} MO into the \textit{tm4sf1} homozygous TALEN fish. If the long isoform is redundant in ECs and not compensated for by \textit{tm4sf4}, we would expect to see a phenotype comparable to that we observe in the \textit{tm4sf1} morphants. Hence, while our data suggests Tm4sf4 does not compensate for loss of Tm4sf1 function in ECs, further experimentation is required to confirm this.

We have shown that mutation of both the long and short isoforms of \textit{tm4sf1} caused loss of TC, but not SC motility, and also dampened the motility of dorsal TC daughters following TC mitosis. This is in agreement with the TC restricted expression of \textit{tm4sf1}, as indicated by ISH and qPCR following manipulation of the VEGFR and Notch pathways (chapter 3). However, the \textit{tm4sf1} double mutant phenotype was milder than the phenotype observed by MO knockdown of \textit{tm4sf1}. Specifically, when comparing control embryos with \textit{tm4sf1} morphants, significant differences in TC motility (cell 1) were observed from 23.5 hpf until 32.2 hpf, i.e. for the duration of the movie. The delay occurred at when the ISVs had sprouted approximately 20 \textmu m, and this was found to be the position when TCs most often divide. While these parameters have not yet been determined for the \textit{tm4sf1} double mutant, significant differences in TC (cell 1) motility between WT and \textit{tm4sf1} double mutants were not observed until 27.4 hpf and an ISV height of approximately 75 \textmu m. Hence, this could possibly suggest TC divisions are occurring later in the \textit{tm4sf1} double mutant embryos, compared to the \textit{tm4sf1} morphants. Additionally, following a TC division and tracking the dorsal-most daughter for 7.5 hours, dorsal daughters of TC divisions in \textit{tm4sf1} morphant embryos (cell 1.1) almost fully recover and reach the DLAV. In contrast, double mutant dorsal daughters (cell 1.1) do not reach the DLAV during this time. Moreover, differences were observed between \textit{tm4sf1} morphants and \textit{tm4sf1} double mutants in the motility of the ventral TC daughter (cell 1.2). While both \textit{tm4sf1} morphants and \textit{tm4sf1} double mutants show differences in the motility of ventral daughter cells (1.2) when compared with control MO injected embryos and WT embryos, respectively, the \textit{tm4sf1} double mutants exhibit greater differences in motility. Hence, there are subtle differences in the dynamics of TCs and SCs between \textit{tm4sf1} morphants and \textit{tm4sf1} double mutants but mechanistically, the reason why is unclear.
A recent publication in Nature may shed light on potential reasons why \textit{tm4sf1} double mutants exhibit a milder phenotype compared to morphants (Rossi et al. 2015). Rossi et al (2015) generated a mutant for \textit{egfl7}, an endothelial ECM gene that when knocked down by MO, resulted in severe vascular defects. However, \textit{egfl7} homozygous mutant embryos had no detectable vascular phenotype and survived to adulthood. Investigations into MO specificity and toxicity confirmed that the morphant phenotype observed was not due to off target effects or p53 induced toxicity. It was also shown that \textit{egfl7} mutants were less sensitive to \textit{egfl7} MO injections compared to WT and \textit{egfl7} heterozygotes, confirming the specificity of the \textit{egfl7} MO. Interestingly, in the \textit{egfl7} mutant embryos, but not \textit{egfl7} morphant embryos, Emilin3a protein expression was found to be increased. Furthermore, additional Emilins were enhanced at the RNA level and all the proteins identified contain one of the key functional domains of Egfl7 and can regulate the same process of elastinogenesis. Moreover, \textit{emilin2} or \textit{emilin3} mRNA could rescue the \textit{egfl7} morphant phenotype. Thus, in the absence of \textit{egfl7}, other proteins were able to compensate for \textit{egfl7} function. This phenomenon was not specific to \textit{egfl7}. A similar occurrence was noted in \textit{vegfaa} mutants whereby \textit{vegfab} was upregulated in mutants but not morphants (Rossi et al. 2015). These findings highlight the importance of the MO since MOs may reveal phenotypes that give clues as to the functional importance of some proteins that would otherwise appear redundant due to compensation. Due to the milder phenotype observed in our \textit{tm4sf1} double mutants compared to the \textit{tm4sf1} morphant embryo phenotype, it is possible that loss of \textit{tm4sf1} may be partially compensated for by the upregulation of structurally similar proteins. Potential genetic compensation of \textit{tm4sf1} by tetraspanins has already been documented in human breast cancer cells, where loss of \textit{tm4sf1} enhanced the expression of a number of other tetraspanins, namely CD63, CD81 and CD151 (Lekishvili et al. 2008). Hence, proteomic and transcriptomic analyses are planned to identify potential proteins, likely other L6 family members or tetraspanins, which might be compensating for loss of \textit{tm4sf1}. If such proteins are identified, we will check if knockdown of compensating proteins in \textit{tm4sf1} double mutants specifically induces a stronger TC phenotype. Additionally, we will attempt to rescue the \textit{tm4sf1} morphant embryo phenotype with RNA injections. In summary, the \textit{tm4sf1} double mutant phenotype is milder than the \textit{tm4sf1} morphant phenotype, and this may be due to genetic compensation.

Essentially, both \textit{tm4sf1} morphants and \textit{tm4sf1} double mutants display similar TC phenotypes. Specifically, \textit{tm4sf1} is important for the post-mitotic motility of the dorsal
daughter cell following a TC division. The phenotype is highly TC-specific since we see no differences in the motility of the two daughters derived from SC divisions. However, further investigations are required to determine the mechanisms by which Tm4sf1 function affects TC motility. The zebrafish embryo model system has proven essential for many crucial aspects of tm4sf1 characterisation, including its dynamic expression pattern, tight spatiotemporal regulation, and involvement in TC behaviour, specifically. However, the lack of zebrafish-specific antibodies limits the mechanistic investigations that can be performed in vivo. Hence, to determine the mechanisms by which Tm4sf1 promotes TC motility, further investigations would need to be performed in vitro.
CHAPTER 5: Results

The functional role of TM4SF1 in ECs

5.1 Introduction

Human TM4SF1 is located on chromosome 3 between coordinates 149,369,022 and 149,377,865, (figure 5.1, indicated). The gene spans approximately 8.84 kb and consists of five exons, as opposed to zebrafish tm4sf1 which consists of four. Like zebrafish tm4sf1, human TM4SF1 has a number of potential protein coding transcripts (Figure 5.1, as identified using ensemble); however, only one transcript (Figure 5.1, top) has been referred to in the literature (Lin et al., 2014). As previously described, TM4SF1 is a tetraspanin-like protein of the L6 family. Tetraspanins have long been implicated in regulating cell motility, fusion and signalling events, often attributed to their association with integrins in TEMs (Berditchevski 2001; Sawada et al. 2003; Shigeta et al. 2003; Takeda et al. 2007). Members of the tetraspanin superfamily are the primary structures that contribute to TEMs, also described as a tetraspanin web. They form a complex network and associate with various transmembrane and intracellular molecules in addition to integrins, such as receptors tyrosine kinases, protein and lipid kinases (Hemler 2005; Latysheva et al. 2006). Hence, although often overlooked, tetraspanins and their multi-molecular organisation in TEMs at the plasma membrane are of considerable functional importance to regulating cell behaviour.

L6 family members are distinct from tetraspanins, due to the absence of a highly conserved CCG motif in their second extracellular loop (Wright et al. 2000), L6 family member functions described to date suggest that they play similar roles to conventional tetraspanins in regulating cellular processes (Jung et al. 2013; Hemler 2005; Miao et al. 2013). Additionally, multiple studies have localised human TM4SF1 to TEMs and its absence from these structures is thought to hinder cell motility in vitro (Zukauskas et al. 2011; Lekishvili et al. 2008; Shih et al. 2009). Investigations into the functions of other L6 family members, as with TM4SF1, are still in their infancy. However, in vitro studies have revealed that the L6 family members, TM4SF4 and TM4SF5, both function to modulate the activity of tyrosine kinase receptors. Like TM4SF1, TM4SF5 was identified as a tumour associated antigen expressed on the surface of multiple cancers of different origins (Lee et al. 2008; Muller-Pillasch et al. 1998; Kaneko et al. 2001). TM4SF5 has 49%
Figure 5.1: The structure of human TM4SF1 and known protein coding transcripts. Human TM4SF1 is located on chromosome 3 (coordinates 149,369,022 – 149,377,865, indicated) and spans approximately 8.84 kb. Three protein coding transcripts have been identified for human TM4SF1 and the resultant proteins consist of 202, 117 and 232 amino acids respectively. The transcript which encodes for a 202 amino acid TM4SF1 protein (top) is described in the literature. White box indicated untranslated regions (UTR); grey box indicated exons; horizontal lines indicate introns.
homology with TM4SF1 (Figure 5.2A) and is the best characterised member of the L6 family. Importantly, TM4SF5 plays an essential role in cell migration, in addition to cell proliferation, and invasion; likely driven by its association with numerous integrins; namely α2, β1, and α5 (Lee et al. 2009; Lee 2011; Choi et al. 2008; Lee et al. 2006). Mechanistically, TM4SF5 is thought to regulate cell motility and invasive protrusions via c-Src, which interacts with TM4SF5 via its C-terminal cytosolic tail. Activation of c-Src by TM4SF5 is essential for the phosphorylation of Tyr845 and subsequent activation of epidermal growth factor receptor (EGFR) (Jung et al. 2013). Hence, L6 family members have previously been implicated in the indirect activation of receptor tyrosine kinase activity.

Similar to the role of TM4SF5, TM4SF4 also regulates cell migration and filopodial protrusion formation. Human TM4SF4 consists of 202-amino acids and is the most homologous family member to TM4SF1 with 50% sequence homology (Wright et al. 2000)(Figure 5.2B). Expression has been localised to plasma membranes and perinuclear vesicles, similar to the expression described for TM4SF1 in ECs (Shih et al. 2009). Moreover, consistent with previous reports for TM4SF1 and TM4SF5, knockdown of TM4SF4 in adenocarcinoma cells hindered cell growth and rendered cells less migratory. Interestingly, TM4SF4 is thought to indirectly promote the activation of another tyrosine kinase receptor IGFR1 (Choi et al. 2014). Hence, the current literature for TM4SF4 and TM4SF5 suggests L6 family members may influence the activity of receptor tyrosine kinases for the regulation of cell motility. These observations suggested that TM4SF1 could potentially regulate TC motility by influencing VEGFR activity in ECs.
Figure 5.2: Structural similarities between L6 family members. (A) CLUSTAL amino acid alignment between human TM4SF1 and TM4SF5 showing 49% amino acid sequence homology, and (B) human TM4SF1 and human TM4SF4 showing 50% amino acid sequence homology. Identical (*), highly conserved (:), and weakly conserved (;) amino acids are indicated. Small/hydrophobic residues (red), acidic residues (blue), basic residues (magenta), hydroxyl/amine/basic residues (green) are shown.
5.2 Results

5.2.1 siRNA-mediated knockdown of TM4SF1 disrupts endothelial gene expression

In order to investigate the mechanisms by which TM4SF1 promotes TC-like behaviour, our first priority was to establish an efficient system for TM4SF1 knockdown in ECs. Hence, we opted for the highly characterised and widely studied EC culture model, HUVECs, for the study of TM4SF1 function. For the siRNA-mediated knockdown of TM4SF1, we used SMARTpool siRNA which pools 3-4 siRNAs that target different regions of the TM4SF1 RNA transcript. This approach has been shown to enhance knockdown efficiency and phenotypic penetrance (Parsons et al. 2009). For transfection of siRNA into HUVECs, we used geneFECTOR, a liposomal formulation optimised for use with primary cell lines, with recent success in HUVECs (Abraham et al. 2015). HUVECs were transfected with TM4SF1 siRNA or control siRNA using geneFECTOR and after 48 hours, RNA was extracted from the cells and converted to cDNA. The relative expression of TM4SF1 in control or TM4SF1 siRNA-treated cells was then determined by qPCR. siRNA-mediated knockdown of TM4SF1 achieved an 81% (+/- 0.84) reduction in transcript levels compared to controls after 48 hours of treatment (Figure 5.3A). We also wanted to show loss of TM4SF1 at the level of the protein. We transfected HUVECs with control siRNA or TM4SF1 siRNA and processed the cell lysates for immunoblotting after 48 hours. We tested three different antibodies for TM4SF1 including anti-TM4SF1, L6 IgM and L6 IgG (Kao et al. 2003); however, non-specific binding of TM4SF1 antibodies was a problem and we were unable to detect a definitive band for TM4SF1 in the region of 22-28 kDa. Difficulties detecting endogenous TM4SF1 have been previously documented (Zukauskas et al. 2011; Shih et al. 2009). Despite this, we established an efficient system for TM4SF1 mRNA knockdown in HUVECs.

Following the establishment of TM4SF1 knockdown reagents, we wanted to assess the effects of siRNA-mediated loss of TM4SF1 on the expression of components of the VEGFR-NOTCH signalling axis. We assessed the mRNA expression levels of the endothelial marker CDH5, the VEGFRs, FLT1, KDR and FLT4, as well as the Notch ligands DLL4 and JAG1, and the NOTCH target gene HEY1 in migrating sub-confluent HUVECs. Compared to control siRNA transfected cells, HUVECs deficient for TM4SF1 mRNA showed a reduction in KDR mRNA and DLL4 mRNA levels by 43% (+/- 1.0) and 64% (+/- 0.06), respectively. VEGFR2/KDR and DLL4 are two critical components that
Figure 5.3: Loss of *TM4SF1* mRNA diminishes mRNA expression of crucial members of the VEGFR-NOTCH signalling axis. (A) Relative quantification of *TM4SF1* RNA by qPCR. RNA was extracted from cells transfected with control siRNA or *TM4SF1* siRNA. (B) Fold changes in *FLT1, KDR, FLT4, DLL4, HEY1, JAG1, CDH5* transcript levels following *TM4SF1* siRNA-mediated knockdown in sub-confluent HUVECs. Error bars represent means from 4 biological replicates +/- SEM. *P<0.05, Student’s t test.
modulate the TC phenotype (Zarkada et al. 2015; Gerhardt et al. 2003; Lobov et al. 2007). Hence, our data suggests that loss of TM4SF1 mRNA might affect the signalling of the VEGFR2/KDR. We also observed a significant reduction in HEY1 mRNA expression by 7.8% (± 0.09) (Figure 5.3B), consistent with reduced DLL4-NOTCH signalling upon loss of TM4SF1. Altogether, these findings suggest that in the absence of TM4SF1 mRNA, the transcriptional regulation of key signalling components important for TC behaviour in vivo are disrupted.

5.2.2 Generation of a flag-tagged hTM4SF1 overexpression construct for TM4SF1

Detection of endogenous TM4SF1 protein is extremely challenging (Shih et al. 2009; Zukauskas et al. 2011) and antibody specificity is a major problem for immunoblot experiments; possibly due to the highly conserved tertiary structures of tetraspanins and tetraspanin-like proteins (Wright et al. 2000). After numerous failed attempts at detecting endogenous TM4SF1, we decided to generate a TM4SF1 tagged construct for ease of detection. We cloned 3xflag_hTM4SF1 into a pCS2 expression vector, and for optimisation of expression, we used HEK293LTV cells due to their ease of transfection. Protein lysates derived from HEK293LTV cells transfected with the pCS2_3xflag_hTM4SF1 overexpression plasmid were prepared for immunoblotting. The expected molecular size of TM4SF1 is approximately 28 kDa, however it has previously been shown that to overexpression of TM4SF1 generates three bands of 22, 25 and 28 kDa (Zukauskas et al. 2011). Initially, we did not see a band at the expected size but a strong signal at the top of the well was observed (Figure 5.4A). We then considered the wells may be overloaded due to the highly efficient overexpression of TM4SF1; thus, we diluted the protein lysate and loaded 0 to 30 µg protein per well, as indicated (Figure 5.4A). Unexpectedly, the signal for flag-tagged TM4SF1 remained in the stacking gel at all concentrations (Figure 5.4A). In the Western blot procedure, heat denaturation is a common step prior to loading protein sample onto a gel. However, particularly for transmembrane proteins, high temperatures can cause the proteins to aggregate, resulting in a high molecular weight smear at the top of the Western blot (Lee et al. 2005; McLane et al. 2007). Thus, to test if this were the case for TM4SF1, after addition of laemmli sample buffer to the protein lysates derived from pCS2_3xflag_TM4SF1 transfected cells, we mixed the lysates thoroughly and incubated them at a number of temperatures ranging from room temperature up to 65°C for 5 minutes prior to loading. When the protein was denatured at higher temperatures, including 42°C and 65°C, we did not observe a signal
Figure 5.4: TM4SF1 aggregates at high denaturing temperatures. (A) Immunoblot for flag loaded with 0-30 µg protein lysate for the detection of TM4SF1. Protein lysates were denatured at 95°C prior to loading (B) Immunoblot for flag loaded with 30 µg protein lysate per lane for the detection of TM4SF1. Lysates were subjected to increasing temperatures prior to loading. ‘control’ indicates an empty pCS2 expression vector and ‘flag_TM4SF1’ indicates a pCS2 expression vector containing 3xflag tagged human TM4SF1. Data represents one of two independent experiments.
for flag-tagged TM4SF1 on the membrane (Figure 5.4B), presumably due to its retention in the stacking gel beneath the wells due to aggregation as previously described (Figure 5.4A). However when lysates were incubated at room temperature or 37°C prior to loading, flag-tagged TM4SF1 did not aggregate and was able to migrate through the gel and a signal for TM4SF1 was detected at approximately 20 kDa and 25 kDa when we blotted with anti-FLAG (Figure 5.4B). This raised the question as to whether we were previously unable to detect endogenous TM4SF1 because we were denaturing the samples at 95°C prior to loading. However, when we repeated the immunoblots for TM4SF1 in HUVECs and maintained the samples at room temperature prior to loading on the gel, endogenous TM4SF1 protein remained undetected (data not shown). To summarise, we generated a flag-tagged version of human TM4SF1 and optimised molecular techniques required for detection of expression of this construct.

5.2.3 Generation of lentivirus for over-expressing TM4SF1 in HUVECs

Transfection of plasmid DNA into HUVECs is notoriously difficult due to their slow growing nature, and primary cell lines are often difficult to transfect. Additionally, HUVECs are also highly susceptible to the toxic effects of transfection reagents (Hunt et al. 2010). Hence, we made the decision to generate a lentivirus for the transduction of flag-tagged TM4SF1 in HUVECs. Lentiviruses are versatile gene delivery systems based on HIV-1 that can transduce many ‘difficult-to-transfect’ cell lines. Lentiviruses can infect and replicate in both mitotic and non-mitotic cells and also integrate into the host genome for long-term stable expression (Tiscornia et al. 2006). To do this, we used the Trono lab’s vectors, which are based on a second generation lentivirus system that comprises three plasmids. The plasmids encode the proteins essential for the infectious cycle of the lentivirus, excluding those that enable the virus to replicate. The three plasmids included: (i) psPAX2 (Figure 5.5), a single packaging plasmid encoding the Gag, Pol, Rev, and Tat genes, driven by the CMV promoter; (ii) pMD2.G (Figure 5.5), a separate envelope plasmid encoding the Env gene driven by the CMV promoter; and (iii) pXLG3 (Figure 5.5), the lentiviral transfer vector which contains the viral LTRs flanking the gene of interest, 3xflag_hTM4SF1. The three plasmids were co-transfected intro HEK293LTV cells using a calcium chloride based method. The HEK239LTV cells were the ‘producer cells’ that express the lentiviral proteins for the production of lentivirus particles (Figure 5.5) (Tiscornia et al. 2006). Since our system was a second generation lentivirus, it utilised the 5’ LTR to drive gene expression, thus integration of our gene of interest into the
Figure 5.5: A schematic to demonstrate the steps taken to generate the lentivirus construct. 3xflag_hTM4SF1 cDNA was cloned into the lentiviral transfer vector and co-transfected into 293LTV producer cells with the envelope and packaging plasmids. The lentivirus particles produced by the 293LTV cells were collected, sterile filtered and transferred to HUVECs in the indicated volumes to determine the efficiency of the virus and required volumes for efficient expression of 3xflag_hTM4SF1 (Figure 5.6).
HEK293LTV genome was required for the production of viral particles. The 5’ LTR is a weak promoter and requires the presence of Tat to activate expression, which was provided by the psPAX2 packaging plasmid (Tiscornia et al. 2006). The viral particles produced by the HEK293LTV cells accumulated in the supernatant. Following purification of the supernatant containing the lentiviral particles, the supernatant was used to transduce HUVECs directly (Figure 5.5).

5.2.4 Optimisation of the hTM4SF1 lentivirus for efficient transduction of HUVECs

To determine the appropriate volume of virus-containing media we should add to HUVECs for efficient transduction, we added increasing volumes (2-10 ml) of the 3xflag_hTM4SF1 virus-containing media or 10 ml control ‘empty’ virus to 70% confluent HUVECs. The virus-containing media was incubated with the cells overnight and before the cells were harvested for lysis. The amount of flag-tagged TM4SF1 expression was assessed by immunoblot after 48 h (Figure 5.6A). Flag-tagged TM4SF1 expression increased in a dose dependent manner, as expected, with the greatest amount of flag-tagged TM4SF1 expression being achieved in cells treated with 10 ml of virus-containing media (Figure 5.6B). Minimal toxicity was observed across all treatments, regardless of the volume of virus-containing media added. Hence, for our experiments, 100% virus-containing media was used for HUVEC transduction in all further experiments.

5.2.5 TM4SF1 mediates VEGFA-induced ERK activation

We wanted to determine whether TM4SF1 modulates VEGFR-mediated signalling. To do this we first wanted to optimise protocols for the in vitro VEGF-mediated activation of VEGFR signalling in HUVECs. To do this, we assessed VEGFR2 and VEGFR3 phosphorylation following stimulation of HUVECs with VEGFA and VEGFC, respectively. HUVECs were washed in M199 to remove any serum or growth factors and then serum starved for 6 hours. Subsequently, we stimulated HUVECs with either 50 ng/ml VEGFA_{165} or 50 ng/ml VEGFC for 0-30 minutes, as indicated. The HUVECs were then harvested and processed for immunoprecipitation of VEGFR2 and VEGFR3, respectively. Immunoprecipitates were separated by gel electrophoresis and immunoblotted for phosphotyrosine (pTyr) to determine the phosphorylation status of the VEGFRs at each of the time points. As expected, VEGFR2 (Figure 5.7A) and VEGFR3 (Figure 5.7B) phosphorylation was absent in unstimulated cells. We found that VEGFR2...
Figure 5.6: Assessment of the transduction efficiency of 3xflag_hTM4SF1 lentivirus. (A) Immunoblot for flag to show expression of TM4SF1 protein in HUVECs. α-tubulin was used as a loading control. (B) Relative quantification of TM4SF1 expression with increasing volumes of 3xflag_hTM4SF1 lentivirus.
Phosphorylation was highest following 10 minutes of stimulation with VEGFA (Figure 5.7A). After this time point, VEGFR2 phosphorylation decreases over time (Figure 5.7A). For VEGFR3, phosphorylation reached its highest after 15 minutes of stimulation with VEGFC (Figure 5.7B). Hence, maximal activation of VEGFR2 or VEGFR3 signalling occurs after stimulation with VEGFA for 10 min or VEGFC for 15 min, respectively.

VEGFR2-induced ERK signalling is central to new blood vessel formation and influences multiple processes during angiogenesis including EC proliferation, migration, survival and contractility (Gourlaouen et al. 2013). Since ERK is a key downstream component of the VEGFR signalling pathway (Yu & Sato 1999; Rousseau et al. 1997), we wanted to investigate whether loss of TM4SF1 affected the levels of VEGF-induced ERK signalling. To assess ERK activation, cells were transfected with either control or TM4SF1 siRNA. After 48 hours, HUVECs were serum starved for 6 hours prior to stimulation with 50 ng/ml VEGFA165 for 10 minutes (Figure 5.7C). The cells were harvested and processed for immunoblotting. For unstimulated cells, basal levels of phosphorylated ERK were low and we found no significant difference in ERK phosphorylation between unstimulated HUVECs treated with control siRNA or TM4SF1 siRNA. In contrast, VEGFA-stimulated cells treated with control siRNA had much higher ERK activation than unstimulated cells, as expected. Interestingly, VEGF-induced ERK phosphorylation was significantly lower in HUVECs following the siRNA-mediated knockdown of TM4SF1 (Figure 5.7C). Hence, these data are the first to describe a functional role for TM4SF1 in the regulation of VEGF-induced signalling, providing a mechanistic basis for the reduced cell migration observed upon TM4SF1/tm4sf1 loss-of-function both in vitro and in vivo.
Figure 5.7: siRNA-mediated knockdown of TM4SF1 significantly disrupts VEGFA-dependent ERK1/2 activation. (A-B) IP for VEGFR-2 (A) or VEGFR-3 (B) and immunoblot for pTyr at the indicated time points following (A) VEGFA or (B) VEGFC stimulation, respectively. IgG was used to control for loading. (C) Immunoblot for pERK1/2 comparing control and TM4SF1 siRNA treated HUVECs with or without VEGFA stimulation. Total ERK was used to control for loading. Graphs are a quantification of band intensities. Error bars represent means +/- SEM from averages of 3 independent experiments. *P<0.05, Student’s t test.
5.3 Discussion

VEGFR2-driven ERK activation is essential for new blood vessel formation (Wang et al. 2010; Gourlaouen et al. 2013). siRNA-mediated knockdown of TM4SF1 caused a significant reduction in the phosphorylation of ERK, indicative of reduced VEGFR2 signalling. Furthermore, the loss of TM4SF1 selectively disrupted VEGFA-dependent ERK activation. Hence, our results suggest that TM4SF1 might be modulating the signalling of VEGFR2; however, the mechanisms by which this occurs are yet to be determined.

Unpublished data from our laboratory has shown that TCs exhibit higher levels of pERK compared to adjacent SCs. Additionally, ERK signalling is higher in the dorsal-most daughter cell compared to the proximal daughter cell immediately following a TC division (unpublished data). Our tm4sf1 morphant and double mutant embryo data shows that in the absence of tm4sf1, TC behaviour is reduced. In WT embryos following a TC division, the dorsal daughter adopts the motility of the parent cell; hence, the cell with higher levels of pERK signalling. However, in the absence of tm4sf1, the dorsal cell loses its characteristically faster motility. Our data suggests this might be due to a decrease in pERK in the TC in the absence of tm4sf1. Hence, TM4SF1 modulation of VEGFR2 signalling and subsequent pERK activation may be responsible for the extra 'boost' in motility observed in the dorsal daughter of TC division. However, activation of ERK is most often associated with EC proliferation as opposed to migration (Meadows et al. 2004; Pedram et al. 1998). Nevertheless, in-depth analysis of TC and SC divisions following MO-mediated knockdown of tm4sf1 suggests that cell proliferation is unaffected by loss of tm4sf1. There are examples in the literature whereby activation of ERK promotes EC migration. For example, the phosphatase PTP1b is a negative regulator of VEGFR2 phosphorylation following VEGFR2 internalisation. Loss of PTP1b in ECs resulted in enhanced activation of VEGF-dependant ERK signalling which subsequently increased both proliferation and migration (Lanahan et al. 2014). Furthermore, overexpression of Ephrin-B2 induced VEGFR endocytosis and ERK activation, which subsequently enhanced EC migration and filopodial protrusions. Interestingly, both of these examples are related to VEGFR endocytosis and in the absence of VEGFR2 endocytosis, ERK activation is almost completely ablated (Nakayama et al. 2013; Sawamiphak et al. 2010; Wang, et al. 2010). This suggests ERK-mediated EC migration could potentially be dependent on VEGFR endocytosis and TM4SF1 may be involved in this process.
While TM4SF1 modulates the activation of ERK signalling in vitro, we want to determine whether loss of tm4sf1 affects ERK signalling in vivo. Live cell imaging experiments coupled with immunofluorescent staining are planned to determine whether ERK activation is impaired in the TCs/TC dorsal daughters of tm4sf1 mutants. Furthermore, to determine whether TM4SF1 is a global determinant of VEGFR signalling, we will also assess whether tm4sf1 also affects the activation of other downstream components of the VEGFR signalling pathway, such as pPLCγ, pAKT and PI3K (Olsson et al. 2006). For our experiments to date, we have only assessed pERK following 10 minute stimulation with VEGFA. This timepoint was selected since VEGFR2 phosphorylation was at its highest. This is not necessarily optimal for the activation of downstream components of VEGFR signalling since signalling pathways downstream of VEGFR take different lengths of time to become activated following VEGFR activation. Thus, it is also important to look at the temporal dynamics of VEGFR signalling, to determine whether the activation of VEGFR pathway components are longer lived or shorter lived upon TM4SF1 loss-of-function.

In addition to a decrease in pERK signalling, loss of TM4SF1 also affected the mRNA expression of important TC determinants. VEGFR2 and DLL4 mRNA expression were significantly reduced when cells were deficient for TM4SF1. In order for the TC-SC hierarchy to be maintained, DLL4 levels in the TC need to be higher than those in the adjacent SC (Jakobsson et al. 2010). Additionally, subsequent repression of VEGFR expression in SCs by Dll4-Notch signalling prevents the SC from becoming a TC (Hellström et al. 2007; Suchting et al. 2007; Siekmann & Lawson 2007). Hence, a reduction in VEGFR2 and DLL4 in the TC may dampen the effects of Notch lateral inhibition. This may in turn disrupt the TC-SC hierarchy and create a more homogeneous population of sprouting ECs. This is in agreement with our in vivo findings, since knockdown or mutation of tm4sf1 causes TCs to exhibit motilities more similar to that of SCs. Again, this suggests TM4SF1 may function to induce extra VEGFR signalling in the TC. It is possible that the decreased expression of DLL4 and VEGFR2 are due to a reduction in ERK activation. ERK signalling has previously been shown to correlate with the induction of DLL4 expression at both the mRNA and protein level (Deng et al. 2013). Interestingly, Vegf induced ERK signalling regulates ETS factor-mediated activation of Dll4 expression via an intronic Dll4 F2 enhancer (Wythe et al. 2013). Hence, this could explain the reduction in DLL4 mRNA expression in the absence of TM4SF1; due to loss of TM4SF1-mediated activation of ERK signalling. On the other hand, since MO-mediated
knockdown of *vegfa* does not significantly alter the expression levels of *kdrl* in zebrafish (Wythe et al. 2013), the mechanisms underlying VEGFR2 regulation and how loss of TM4SF1 causes a reduction in VEGFR2 mRNA remain unclear.

Altogether, these data presented in this chapter and others suggest a novel role for TM4SF1 in the VEGFA-dependent modulation of VEGF2 signalling (Figure 5.9). Modulation of VEGFR2 signalling by TM4SF1 enhances downstream activation of ERK, since lower levels of ERK activation are observed with loss of TM4SF1 (Figure 5.9). ERK activation promotes the subsequent upregulation of the TC gene, *DLL4* (Figure 5.9), and characteristically high rates of TC verses SC motility, as determined by both *in vitro* and *in vivo* investigations. Additionally, TM4SF1 is negatively regulated by Dll4-Notch lateral inhibition, hence TM4SF1 is presumably downregulated in SCs (Figure 5.9), consistent with reduced motility in SCs compared to TCs. Overall, our results suggest that *tm4sf1* is a novel TC determinant and modulator of the TC-SC hierarchy, essential for the coordinated, collective migration of ECs during ISV sprouting. Further experiments are needed to determine the molecular interactions by which TM4SF1 functions, and these will be discussed in detail in Chapter 6.
Figure 5.9: The hypothesised role of TM4SF1 in the TC. TM4SF1 expression is induced in the TC by VEGFR signalling (left). By an unknown mechanism, TM4SF1 modulates the signalling of VEGFR2, which in turn activates downstream signalling via pERK. DLL4 mRNA expression is induced by pERK signalling which activates Notch in the adjacent SC. TM4SF1 expression is downregulated in the SC by Notch signalling. In the absence of TM4SF1 (right), VEGFR signalling is attenuated and subsequent downstream signalling via ERK is reduced. This causes a reduction in DLL4 mRNA expression levels and loss of TC motility. Loss of TM4SF1 does not affect SC behaviour since in normal WT conditions; TM4SF1 is downregulated by NOTCH signalling, as previously mentioned.
CHAPTER 6: General Discussion and Future Directions

6.1 Discussion

Our data suggests that TM4SF1 may be modulating VEGFR signalling in a VEGFA-dependent manner. This is consistent with the loss of TC motility in the absence of tm4sf1 in vivo. As discussed in section 5.3, investigations in our laboratory have recently shown that TCs exhibit more pERK compared to SCs. Additionally, following a TC division, the dorsal-most daughter cell exhibits more pERK compared to the ventral-most daughter cell. Taken together, these findings suggest that the TC-restricted expression of tm4sf1 could be responsible for the extra boost in TC versus SC motility seen in one daughter cell following division. While investigations into the potential function of TM4SF1 are still ongoing, our current knowledge raises a number of questions as to the mechanisms by which TM4SF1 regulates VEGFR2 signalling.

6.1.1 Does TM4SF1 promote VEGFR endocytosis?

Endocytic internalisation and subsequent trafficking of transmembrane receptors upon ligand binding is essential to maintain a balance between receptor degradation and recycling. In turn, this homeostasis between receptor degradation/recycling ensures the tight control of receptor-mediated signalling (Sadowski et al. 2008). Several publications highlight the importance of VEGFR2 endocytosis for the amplitude and duration of VEGFR2 signalling (Sawamiphak et al. 2010; Lampugnani et al. 2006; Lanahan et al. 2010). When VEGFA binds VEGFR2, clathrin and dynamin mediate internalisation of VEGFR2 (Lampugnani et al. 2006). Once internalised, VEGFR2 continues to signal within endosomal compartments; hence, the duration and amplitude of VEGFR2 signalling are enhanced by VEGFR internalisation. Additionally, some signalling events specifically require VEGFR2 endocytosis. For example, VEGFR2 internalisation is essential for the activation of ERK signalling (Gourlaouen et al. 2013). Gourlaouen et al (2013) found that the majority of ERK phosphorylation was attributed to VEGFR2 signalling within endosomal compartments and others have shown that in the absence of VEGFR2 endocytosis, ERK activation is almost completely lost (Nakayama et al. 2013; Sawamiphak et al. 2010; Wang et al. 2010). Furthermore, TM4SF1 has been localised to endocytic vesicles (Shih et al. 2009) and a recent study highlighted the internalisation of TM4SF1 within vesicles from the plasma membrane in a dynamin-dependent manner (Sciuto et al. 2015). Interestingly, structurally similar tetraspanins already have known roles in intracellular trafficking. For example, the C-terminal domain of tetraspanin CD151
associates with integrins α3β1, α5β1, and α6β1 to facilitate their internalisation to promote cell migration (Liu et al. 2007). Similarly, tetraspanin CD63 also plays a role in intracellular trafficking, interacts with adaptor protein complexes via C-terminal interactions, and has been linked to the clathrin-dependent pathways (Rous et al. 2002). Taken together, these reports and our findings that TM4SF1 is important for VEGFA-mediated pERK signalling suggests TM4SF1 might be functioning to facilitate VEGFR2 endocytosis.

PDZ domains are abundant interaction modules that associate with short amino acid PDZ binding motifs at the C-termini of proteins (Lee & Zheng 2010). Many biological processes, in particular those that involve signal transduction complexes, are facilitated by PDZ domain-mediated interactions (Lee & Zheng 2010). The last four amino acids located at the C-terminal cytoplasmic tail of human TM4SF1 form the PDZ binding motif, X-Y-X-C, that may provide a mechanism for interaction with multiple PDZ domain containing proteins involved in endocytosis. Syntenin-1, a protein involved in endocytic trafficking, is essential for Vegfr2 trafficking in mice (Lanahan et al. 2014). As discussed in section 1.10, in the absence of Syntenin-1, Vegfa-mediated internalisation of Vegfr2 occurs, however phosphorylation of Tyr 1175 of Vegfr2 and subsequent pErk and pP/γ signalling was decreased (Lanahan et al. 2014). This was due to delayed entry into early endosomes and thus Vegfr2 was exposed to phosphatases such as Ptp1b, that dephosphorylate Vegfr2 Tyr1175 (Lanahan et al. 2014). As a result, Vegfr2 downstream signalling is dampened. Interestingly, Syntenin-1 is PDZ domain containing protein (Grootjans et al. 1998). Hence, the PDZ binding motif located at the C-terminal of TM4SF1 may interact with the Syntenin-1 PDZ domain. Furthermore, Syntenin-1 has been identified as a component of TEMs and associates with tetraspanin CD63. Interactions between Syntenin-1 and CD63 occurs via the PDZ domain of Syntenin-1 and the C-terminal cytoplasmic tail of CD63 (Latysheva et al. 2006). Within TEMs, interactions between CD63 and TM4SF1 have already been reported (Lekishvili et al. 2008) and tetraspansins are known to interact with each other, forming a lateral web that facilitates receptor interactions and cross-talk with intracellular signalling structures (Levy & Shoham 2005; Hemler 2005). Hence, TM4SF1 may form a functional complex with Syntenin-1 and CD63 for the control of Vegfr2 endocytosis, which could explain the reduction in pERK signalling in the absence of TM4SF1.

Ephrin-B2 is a transmembrane ligand for Eph family receptor tyrosine kinases and conventionally, ligand-receptor interactions promote bi-directional signalling between both
ligand expressing and receptor expressing cells (Pasquale, 2005). In ECs, it has also been postulated that Ephrin-B2 may also possess cell-cell contact independent roles (Bochenek et al. 2010; Foo et al. 2006). Ephrin-B2 also contains a PDZ binding domain and Ephrin-B2 PDZ-mediated interactions have already been shown to be essential for development of the lymphatic system (Adams et al. 2005). Hence, the PDZ domain of Ephrin-B2 could potentially provide a means of interaction with the TM4SF1 PDZ binding motif. In support of this, Ephrin-B2 promotes the endocytosis of VEGFR2 and VEGFR3 (Sawamiphak et al. 2010; Wang et al. 2010). In both mouse and zebrafish, loss of Ephrin-B2/ephrin-b2 causes defective Vegfr internalisation and subsequently abrogates downstream signalling through Rac1, Akt and Erk1/2 (Sawamiphak et al. 2010; Wang et al. 2010). Interestingly, overexpression of Ephrin-B2 enhances filopodial extensions (Bochenek et al. 2010; Zukauskas et al. 2011; Sawamiphak et al. 2010), and this is in agreement with TM4SF1 overexpression in human cells (Shih et al. 2009). Furthermore, the C-terminal PDZ domain of Ephrin-B2 is essential for the morphological changes associated with Ephrin-B2 overexpression (Bochenek et al. 2010). Hence, TM4SF1 could potentially interact with Ephrin-B2 to modulate Vegfr signalling by facilitating Vegfr endocytosis.

6.1.2 Does Tm4sf1 activate VEGFR signalling via a Src-mediated mechanism?

Our data show that zebrafish tm4sf1 is essential for TC motility in vivo and data presented by others suggest human TM4SF1 also promotes the formation of filopodia (Zukauskas et al. 2011; Shih et al. 2009). Like TM4SF1, the closely related family member TM4SF5 has also been shown to promote cell migration and facilitate the formation of invasive protrusions. Hence, there appears to be similarities in the functions of these two L6 family members. The functional characterisation of TM4SF5 suggests avenues that should be addressed for the characterisation of TM4SF1. Recent evidence shows that the C-terminus of TM4SF5 binds c-Src, and that cell migration and the formation of invasive protrusions are dependent on this interaction. Additionally, activation of c-Src was found to correlate with the phosphorylation of the receptor tyrosine kinase, EGFR. This might suggest a role for TM4SF1 in the c-Src mediated activation of VEGFR2 and/or VEGFR3, both of which are known to be activated by c-Src (Jin et al. 2003; Galvagni et al. 2010). It has also been demonstrated that c-Src may play a role in the cross-talk between integrin αvβ3 and VEGFR2 (Mahabeleshwar et al. 2007). VEGF-induced complex formation of VEGFR2 and αvβ3 and subsequent VEGFR2 phosphorylation was dampened by c-Src inhibition (Mahabeleshwar et al. 2007). Interestingly, TM4SF1 also associates with integrin αvβ3 in a VEGFA-dependent manner (Shweiki et al. 1992; Shih et al. 2009; Pugh
Hence, VEGFA-mediated TM4SF1-αvβ3 interaction may function to promote activation of c-Src which subsequently modulates the activity if VEGFR2 signalling. In support of this, VEGFA-dependent c-Src-mediated activation of VEGFR2 at Tyr951 has been associated with EC actin reorganisation and migration *in vitro* (Ruan & Kazlauskas 2012; Sun et al. 2012; Matsumoto et al. 2005). Hence, TM4SF1 may function to promote c-Src activation, which in turn promotes VEGFR2-mediated EC migration following VEGFA ligand binding. Furthermore, evidence also suggests that c-Src mediated VEGFR2 activation can also occur independent of VEGF ligand (Jin et al. 2003). While our data suggests TM4SF1 modulation of VEGFR2 signalling is largely VEGFA-dependent, ligand-independent roles for TM4SF1 should not be dismissed since TM4SF5 potentially modulates EGFR activity in a ligand-independent manner (Jung et al. 2013). However, the mechanism by which this occurs is poorly understood. The potential for TM4SF1 to promote c-Src-mediated VEGFR activation requires further investigation.

### 6.1.3 Does TM4SF1 promote facilitate interactions between NRP-1 and VEGFR2?

NRP-1 and NRP-2 are co-receptors for VEGF ligand (Soker et al. 1998), expressed in arterial and venous tissue, respectively (Herzog et al. 2001). NRP-1 enhances the affinity of VEGFA$_{165}$ for VEGFR2 (Soker et al. 1998) which subsequently increase VEGFR2 phosphorylation and thus downstream signalling activation (Becker et al. 2005). Like TM4SF1, NRP-1 has also been associated with filopodia formation in TCs (Fantin et al. 2013; Fantin et al. 2015). Initially, it was thought that VEGFA$_{165}$ simultaneously binds to VEGFR2 and NRP-1, thereby forming a bridge which enables interaction and complex formation between the extracellular portions of VEGFR2 and NRP-1 (Soker et al 2002). However, more recent investigations suggest VEGFA$_{165}$ bridging of VEGFR2 and NRP-1 does not fully explain the enhanced downstream signalling achieved in the presence of NRP-1 (Prahst et al. 2008). Hence, intracellular interactions between NRP-1 and VEGFR2 may be important. In agreement with this, Prahst et al (2008) found that the C-terminal cytosolic PDZ domain of NRP-1 was required for VEGFR2-NRP-1 complex formation and the efficiency of this interaction was also dependent on the presence of synectin (Prahst et al. 2008). A subsequent study found VEGFR2-NRP-1 complex formation was critical for FAK phosphorylation and VEGFA-dependent HUVEC migration (Herzog & Pellet 2011). Hence, it is possible that TM4SF1 may facilitate the formation of VEGFR2-NRP-1 complexes.
6.2 Tm4sf1 expression is spatiotemporally restricted to TCs

During angiogenic sprouting, ECs can adopt two distinct identities, the TC and SC, which are essential for the coordinated collective migration of ECs to form new blood vessels (Herbert & Stainier 2011). As discussed in section 1.12, the specific molecular mechanisms that confer TC verses SC identities have not been fully delineated. We have identified a novel TC-associated gene, tm4sf1, which encodes for a tetraspanin-like protein with as yet, unknown function. tm4sf1 expression was restricted to sprouting ISVs, and when ISVs had fully sprouted and fused with neighbouring ISVs by anastamosis, tm4sf1 expression was down-regulated. Significantly, the Vegfr and Notch signalling pathways regulate the expression of tm4sf1 in a spatiotemporally conserved pattern, such that tm4sf1 expression is potentially restricted to the tips of sprouting ISVs. Consequently, Notch-dependent down-regulation of tm4sf1 may be responsible for inhibition of VEGFR signalling in SCs, thereby reinforcing the SC phenotype. Moreover, inhibition ofDll4-Notch signalling causes hypersprouting and an increase in TCs (Herbert et al. 2012; Siekmann & Lawson 2007). Our data shows that in the absence of Dll4-Notch signalling, tm4sf1 expression is ectopically expanded. Taken together, our data implies that the spatiotemporal expression of tm4sf1 may potentially drive TC and SC selection. This would be in agreement with the roles of other known TC associated genes, such as flt4, and Esm1 (Strasser et al. 2010; Hogan et al. 2009; Siekmann & Lawson 2007; Rocha et al. 2014). Vegfr2 induces Flt4/Vegfr3 expression in the TC to further enhance Vegfr signalling (Tammela et al. 2008). Similarly, Esm1 expression is also induced by Vegfr2 signalling and enhances Vegfr signalling by increasing the bioavailability of Vegfa (Rocha et al. 2014). This suggests that the proposed TC transcriptional programme, that upregulates the expression of tm4sf1, flt4, and esm1, may be established to induce extra Vegfr activity in the TC verses the SC. In doing so, this could speed up lateral inhibition decisions to facilitate the immediate selection of TCs during sprouting and following TC mitosis. Alternatively, it is possible that tm4sf1 could be functioning upstream of Notch. This phenomenon was recently described as an additional role for Nrp-1, whereby Nrp-1 is actively repressed by Notch signalling to promote SC identity via Tgfb/Bmp-mediated Smad activation (Aspalter et al. 2015). In summary, the spatiotemporally conserved expression of tm4sf1 may be responsible for driving TC selection and further experiments are warranted to address this.
6.3 Preliminary Data

In section 5, we described and optimised efficient systems for both TM4SF1 siRNA-mediated knockdown and overexpression of a flag-tagged TM4SF1 construct in human cells. Here we present preliminary data using these systems.

6.3.1 siRNA-mediated knockdown of TM4SF1 disrupts EC migration in vitro

We previously demonstrated that loss of tm4sf1 in zebrafish disrupted TC motility in vivo (Figure 3.10). Hence, we wanted to determine whether similar motility defects were observed in human cells. Experiments assessing HUVEC migration in scratch wound healing assays in the absence of TM4SF1 have already been performed and suggest that TM4SF1 regulates HUVEC migration (Shih et al. 2009). We wanted to replicate these experiments but compare the effects of VEGFA stimulation on HUVEC migration in the presence or absence of TM4SF1. HUVECs were transfected with control siRNA or TM4SF1 siRNA and the cells were allowed to grow for 48 h until they reached approximately 90% confluency. We induced a ‘scratch’ with a sterile pipette tip and the media was subsequently changed to M199 (without serum or supplements) or M199 plus 50 ng/ml VEGFA165. HUVEC migration was continuously monitored over a period of 12 h. The percentage of area covered by the HUVECs after 12 h was quantified compared to 0 h controls using ImageJ. Unstimulated HUVECs transfected with control siRNA migrated minimally over the 12 hour period (Figure 6.1A), covering just 33.1 % (+/- 0.8) of the original scratch area (Figure 6.1C). Similarly, migration of unstimulated HUVECs subjected to siRNA-mediated knockdown of TM4SF1 was also poor, covering 24.0 (+/- 1.6). However, there was a significant difference between control and TM4SF1 knockdown, thus loss of TM4SF1 affects migration of HUVECs, even in the absence of VEGFA (Figure 6.1A, C). Migration of VEGFA-stimulated HUVECs transfected with control siRNA was much more efficient, covering 83.9% (+/- 2.2) of the original scratch area after 12 hours. This was expected considering the key role played by VEGFR signalling in EC migration (Olsson et al., 2006). Interestingly, migration of VEGFA-stimulated HUVECs transfected with TM4SF1 siRNA was greatly reduced compared to controls, covering just 38.6% (+/- 3.5) of the original scratch area after 12 hours. Control cells migrated 45% more than those deficient for TM4SF1 when stimulated with VEGFA and the difference in migration was highly significant (Figure 6.1B-C). Hence, VEGFA-induced HUVEC migration appears to be TM4SF1-dependent. In summary, these
Figure 6.1: TM4SF1 is essential for efficient VEGFA-dependent migration of HUVECs. (A) Unstimulated and (B) VEGFA stimulated HUVECs transfected with control or TM4SF1 siRNA. Images represent stills taken at 0 and 12 h from time-lapse movies to show the migration of HUVECs from the scratch boundaries (yellow). (C) Quantification of percentage area closure from 0 hours to 12 h. Error bars represent means +/- SEM from averages of 6 fields of view from one experiment, *P<0.05, Student’s t test. Scale bar = 200 μm.
preliminary data show that \textit{TM4SF1} may be important in human ECs for efficient migration and that the mechanism by which TM4SF1 promotes EC migration is largely VEGFA-dependent.

\subsection*{6.3.2 TM4SF1 overexpression hinders VEGFA-dependent EC migration}

We have shown that siRNA-mediated depletion of \textit{TM4SF1} hinders VEGFA-dependent migration of ECs \textit{in vitro}. Loss of \textit{tm4sf1} \textit{in vivo} by MO or deleterious mutation also affects TC motility. Hence, we wanted to determine whether overexpression of TM4SF1 in HUVECs would promote EC migration, to give the opposite results to those obtained for loss of TM4SF1. To do this, we used our of flag-tagged TM4SF1 expressing lentiviral construct for the transduction of HUVECs. HUVECs were transduced with control virus or flag-tagged TM4SF1 lentivirus and the cells were allowed to grow for 48 h until they reached approximately 90\% confluency, as previously described (section 5.2.2). We induced a ‘scratch’ with a sterile pipette tip and the media was subsequently changed to M199 or M199 plus 50 ng/ml VEGFA$_{165}$. HUVEC migration was continuously monitored over a period of 12 hours. The percentage of area covered by the HUVECs after 12 h was quantified compared to 0 h controls. Unstimulated HUVECs treated with the control lentivirus migrated minimally after 12 hours, covering 30.1 \% (+/- 1.7) of the original scratch area (Figure 6.2A-C). This is similar to our previous data for unstimulated HUVECs transfected with control siRNA (Figure 6.2). Similarly, VEGFA stimulated HUVEC migration was much more extensive, covering 84.8\% (+/- 2.3), confirming that efficient HUVEC migration is VEGFA-dependent (Figure 6.2B-C). Unstimulated HUVECs overexpressing TM4SF1 also migrated minimally, covering 29.5 (+/- 2.2) of the original scratch area. There was no significant difference observed between unstimulated controls and unstimulated TM4SF1 overexpressing cells. Interestingly, there was also no significant difference observed between unstimulated and VEGFA stimulated TM4SF1 overexpressing HUVECs, which migrated 30.7\% (+/- 3.1). Thus, overexpression of TM4SF1 appeared to hinder the migration of HUVECs when stimulated with VEGFA and our data is similar to findings previously published (Zukauskas et al. 2011). Hence, preliminary findings suggests that both knockdown and overexpression of TM4SF1 expression may disrupt HUVEC migration \textit{in vitro}, suggesting that fine control of TM4SF1 expression levels may be important for controlling EC migration.
Figure 6.2: TM4SF1 gain of function hinders EC migration. (A) Unstimulated and (B) VEGFA stimulated HUVECs expressing pXLG3_control virus and pXLG3_3xflag_hTM4SF1 virus. Images represent stills taken at 0 and 12 h from time-lapse movies. (C) Quantification of percentage of scratch area closure from 0 -12 h in unstimulated and VEGFA stimulated HUVECs. Significant differences were observed between VEGFA-stimulated HUVECs expressing pXLG3_control and pXLG3_3xflag_hTM4SF1 viruses. Error bars represent means +/- SEM from averages of 6 fields of view from one experiment, *P<0.05, Student’s t test. Scale bar = 200 µm.
6.4 Future Directions

Our data suggests that TM4SF1 could be facilitating VEGFR2 endocytosis to enhance VEGFR2 signalling within endosomal compartments. A number of experiments could be performed in order to test this hypothesis. For example, we could use antibody feeding assays for the interrogation of VEGFR internalisation. By doing this in the context of TM4SF1 loss and gain of function, using our already established methods (as validated in chapter 5), alterations in VEGFR internalisation would suggest a role for TM4SF1 in endocytosis. Should TM4SF1 affect VEGFR2 internalisation, we could also investigate potential molecular and functional interactions of TM4SF1. Due to the presence of PDZ-binding domains (Adams et al. 2005; Grootjans et al. 1998), Syntenin-1 and Ephrin-B2 are potential TM4SF1 interaction partners and this could be tested by co-immunoprecipitation. Similarly, TM4SF1 could directly phosphorylate VEGFRs; hence, the levels of VEGFR phosphorylation following TM4SF1 loss and gain of function could be assessed, in addition to co-immunoprecipitation experiments to detect physical interactions of TM4SF1 with VEGFRs. Should TM4SF1 physically interact with endocytic-associated proteins or VEGFRs, the specific region of TM4SF1 that is essential for potential interactions and modulation of VEGFR2 could be identified by the generation of TM4SF1 truncation mutants. Due to the known PDZ-binding motif at the C-terminal tail of TM4SF1 (Borrell 2000), a mutant that lacks the PDZ-binding motif would be a good starting point. In summary, our findings indicate that TM4SF1 might play a role in VEGFR endocytosis. Hence, further investigations may reveal TM4SF1 as a novel TC-specific determinant of VEGFR endocytosis and subsequent signalling via ERK.

Published data regarding the function of TM4SF5 in the c-Src-mediated activation of EGFR suggests TM4SF1 could potentially modulate VEGFR signalling via interactions with c-Src. Co-immunoprecipitation, in combination with TM4SF1 loss- or gain-of-function experiments, could determine whether TM4SF1 physically interacts with c-Src. c-Src mediated signalling is often associated with phosphorylation of FAK and PI3K (Basile et al. 2005; Abu-Ghazaleh et al. 2001). Thus assessment of changes in VEGFR signalling following TM4SF1 loss- or gain-of-function would provide further insight into this potential interaction. Similar investigations could also be performed to investigate potential TM4SF1-NRP-1 interactions and modulation of downstream signalling pathways.

A significant question that has arisen from our investigations is whether the spatiotemporal expression of TM4SF1 during angiogenesis is able to drive TC and SC
selection. One way that this could be tested is by assessing the ability of \textit{tm4sf1} mutants to recover \textit{dll4} loss-of-function induced hypersprouting. Using techniques already described in section 3.2.6, the motility of TCs and SCs in \textit{tm4sf1} mutants injected with \textit{dll4} MO could be assessed to specific define the position of \textit{tm4sf1} in the Notch-Vegfr signalling pathway. Additionally, we could perform experiments whereby \textit{tm4sf1} mutant ECs are transplanted into WT embryos (or vice versa) to determine which cells assume the TC position. This would determine the ability of \textit{tm4sf1} to cell autonomously regulate TC selection. Alternatively, it is possible that \textit{tm4sf1} could be functioning upstream of Notch. Hence, experiments could be performed to determine whether TM4SF1 is able to modify Notch signalling by assessing NICD activation and induction of Notch target genes such as HEY1/2 and NRARPa/b by qPCR and Immunoblot, since we already know TM4SF1 influences the expression of \textit{DLL4} and \textit{HEY1}. Immunoprecipitation experiments as described above would also identify whether TM4SF1 physically interacts with Nrp-1. If this was the case, the ability of TM4SF1 to modulate TGFβ/BMP-mediated Smad activation could be tested.

In summary, the specific function of TM4SF1 in the modulation of VEGFR signalling, and ultimately TC behaviour, is currently unknown. The recent establishment of \textit{in vitro} tools and protocols will allow the in depth interrogation of TM4SF1 protein function and the experiments discussed above are already in progress.

\textbf{6.5 Future Perspectives}

\textbf{6.5.1 TM4SF1: a potential therapeutic target for the future?}

Hypoxia is the biggest driver of angiogenesis in both physiology and pathologies such as cancer. For example, in order for a tumour to grow, tumour cells require as supply of oxygen and nutrients and thus, secrete growth factors to facilitate the formation of new blood vessels. In particular, many tumour cells overexpress VEGFA in response to hypoxia. Like physiological angiogenesis, VEGFA acts as a paracrine mediator to generate ligand gradients to which new blood vessels are attracted (Carmeliet 2005; Gerhardt et al. 2003; Chung et al. 2010). However, VEGFA also function as an autocrine mediator to encourage tumour cell survival and invasiveness (Carmeliet et al. 2005; Chung et al. 2010). Hence, VEGFA and VEGFR2 have been targeted by anti-angiogenic agents that aim to suppress tumour growth directly by limiting their blood supply. Some approved anti-angiogenic therapies such as the VEGFA-specific antibody, Bevacizumab, and tyrosine kinase inhibitors, sorafenib and sunitinib, have had some success in the clinic (Chung et al.
However, due to severe side effects and drug resistance, new drug targets for the prevention of tumour angiogenesis and thus, tumour growth, are in high demand.

TM4SF1 was initially discovered as a tumour associated antigen, L6, that was overexpressed on the surface of many tumours (Zheng et al. 2015; Hellstrom et al. 1986; Allioli et al. 2011; Kao et al. 2003). Expression correlated with enhanced tumour cell motility, invasive properties, metastatic properties and poor prognosis (Gordon et al. 2011; Allioli et al. 2011; Miao et al. 2013). Initial experiments found that targeting L6 with a murine monoclonal antibody induced antibody-dependent cellular cytotoxicity (ADCC) (I. Hellström et al. 1988). Hence, the L6 antigen was quickly considered as a potential therapeutic target for the treatment of cancer. Subsequent phase I human trials in patients with advanced primary and secondary tumours that expressed high levels of L6, found the antibody localised well with tumours and treatment was well tolerated with limited side effects. Variable responses were recorded between trials with up to a third of patients achieving partial remission in some cases (Ziegler et al. 1992; Goodman et al. 1990; Goodman et al. 1993). Better responses were achieved when the L6 antibody was radiolabelled with up to a 50% of patients achieving partial remission (Richman et al. 1995). However, it remained unclear whether the L6 antibody was able to induce ADCC responses in humans. During these trials, the full extent of TM4SF1 expression was not known; it was only recently found that TM4SF1 is also expressed on blood vessels that supply tumours (Shih et al. 2009). In addition, our in vivo experimentation has shown that expression of tm4sf1 is not ubiquitously expressed in all ECs, but is specifically expressed in ECs that are contributing to the formation of new blood vessel sprouts. Furthermore, tm4sf1 expression is restricted to the TCs at the leading edge of new vessels. Hence, anti-TM4SF1 therapies may be useful for the prevention of angiogenesis in the earlier stages of cancer, when the tumours are recruiting a blood supply. Altogether, our increasing knowledge of the TM4SF1 localisation and function could prove invaluable for the development of new anti-TM4SF1 treatments.

A recent study has shown promise for the use of TM4SF1 antibodies as anti-cancer agents. Human blood vessels were engineered by mixing together endothelial colony-forming cells (ECFCs) and MSCs in Matrigel plugs that were subsequently implanted subcutaneously in immunodeficient nude mice (Lin et al. 2014). This is an established model for growing human blood vessels in mice to enable the testing of human antibodies. An anti-TM4SF1 antibody was found to target the large EC2 loop of TM4SF1 and in doing
so, was able to destroy human blood vessels in the Matrigel implants (Lin et al. 2014). Interestingly, images of the matrigel plugs suggest that the majority of blood vessels that were ablated by TM4SF1 antibody treatment appear to be small, microvessels that are sprouting from parental vessels. We know from our in vivo data that tm4sf1 is expressed only at the tips of new vessels that are sprouting. However, when the vessels fuse with their target vessel, tm4sf1 is rapidly downregulated. Hence, the vessels that were ablated by treatment with the TM4SF1 antibody are likely positive for TM4SF1 expression, whereas the adjacent parental vessels that remain intact following TM4SF1 antibody treatment presumably lack TM4SF1 expression (Lin et al. 2014) consistent with our ISH expression analysis. The large decrease in vascularisation following antibody administration shows great potential for anti-TM4SF1 as a therapeutic agent. Interestingly, when TM4SF1-positive PC3 prostate cancer cells were also added to the Matrigel, both blood vessels and PC3 cells were targeted and lysed by the TM4SF1 antibody (Lin et al. 2014). By targeting TM4SF1, the tumour is subjected to a ‘double hit’ that targets both the tumour mass and the supporting vasculature (Lin et al. 2014). Hence, anti-TM4SF1 has the potential to offer exceptional therapeutic benefit, particularly if combined with other drugs. The potential of anti-TM4SF1 as part of an antibody drug conjugate is already being investigated (Visintin et al. 2015). ADCs are monoclonal antibodies (mAbs) conjugated to biologically active drugs that thus enable the directed targeting of drugs to diseased tissues such as tumours. Early investigations into the efficacy of anti-L6 treatment suggested that following administration, the antibody co-localised efficiently with L6 antigen positive tumours (Visintin et al. 2015). Recent investigations have shown promise for TM4SF1 targeting of ADCs to both ECs in culture and tumour cells (Visintin et al. 2015). Importantly, when the TM4SF1 antibody reacted with the antigen at the plasma membrane it was internalised; this is an essential for the efficacy of ADCs (Visintin et al. 2015). Furthermore, when the TM4SF1 antibody was conjugated with a tubulin inhibitor, EC and tumour cell cytotoxicity was observed (Visintin et al. 2015). Hence, TM4SF1 ADCs provide promise for a novel cancer therapeutic in the future.

In addition to cancer, many other conditions are facilitated by excessive or uncontrolled angiogenesis that could also potentially benefit from targeted anti-TM4SF1 therapy, such as diabetic retinopathy, rheumatoid arthritis and skin conditions such as psoriasis (Carmeliet 2003). The main concern for therapies targeting TM4SF1 is potential reactivity with normal ECs; a concern also apparent for the approved VEGFA/VEGFR2 targeted therapies. In adults, the majority of blood vessels remain quiescent; however,
TM4SF1 is expressed weakly in these vessels in humans and also at low levels in a number of other tissues (Denardo et al. 1991; Marken et al. 1992; Storim et al. 2001). Data from early trials suggested targeting of TM4SF1 was well tolerated and toxicity and side effects were low (Ziegler et al. 1992; Goodman et al. 1990; Goodman et al. 1993). Further investigations are required to ensure the safety of such therapy, particularly for the use of TM4SF1 ADCs. These investigations will be facilitated with our increasing knowledge regarding TM4SF1 expression and function. Hence, TM4SF1 has the potential to be a promising therapeutic target for many angiogenesis-driven diseases in the future.
6.6 Final remarks

Sprouting angiogenesis is a crucial component of development, tissue regeneration and repair and also many pathological conditions. The last decade of research has identified some of the fundamental mechanisms underlying angiogenesis; most significantly, the VEGFR-Notch signalling axis for the control of TC and SC specification. This breakthrough was invaluable to the field of angiogenesis, and progress in the identification of therapeutic targets for the manipulation of angiogenesis will undoubtedly follow. Our findings contribute to the current understanding of TC specification *in vivo* and tm4sf1 can be described as a novel TC behaviour determinant. TM4SF1 is also an attractive candidate for anti-angiogenesis therapies. Investigations into the potential of TM4SF1 antibodies for the dual targeting of both tumours and their associated vasculature are already underway. Further functional characterization of TM4SF1 will certainly help to understand its role in development and potential as a drug target. Overall, the vital and vibrant research area of angiogenesis will continue to expand our current knowledge and understanding of human physiology and also contribute to our ultimate goal of fighting devastating diseases.
References


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