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ARHGAP18: A regulator of sprouting angiogenesis and junctional integrity

Garry Hoi-Kai Chang

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

Faculty of Medicine
The University of Sydney

2015
DECLARATION

The work described in this thesis was performed in the Vascular Biology Program at the Centenary Institute, Sydney, Australia between August 2009 and June 2015, under the supervision of Professor Jennifer Gamble. This work is original and has not been previously submitted for the purpose of obtaining any other degree at any other institution. All work presented herein is that of the candidate alone except where acknowledged in the text, also as stated below:

- Zebrafish experiments (Fig 4.7 and 4.8) were performed by Dr. Ka Ka Ting (Vascular Biology Program, Centenary Institute, Sydney, Australia).
- Aortic ring experiments (Fig 4.10) were performed in collaboration with Dr. Paul Coleman and Dr. Angelina Lay. GHKC analysed the images and generated the graphs.
- Miles assays (Fig 5.13) were performed by Ying Lu (Vascular Biology Program, Centenary Institute, Sydney, Australia).
- Tumour experiments (Fig 5.15) were performed by Yang Zhao (Vascular Biology Program, Centenary Institute, Sydney, Australia).

Garry Hoi-Kai Chang

BMedSc (Hons)

June 2015
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PUBLICATIONS

Publications arising from this thesis


Other relevant publications


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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>CT</td>
<td>Cycle threshold</td>
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<tr>
<td>DLL4</td>
<td>Delta-like ligand 4</td>
</tr>
<tr>
<td>DA</td>
<td>Dorsal Aorta</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GAP18</td>
<td>ARHGAP18</td>
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<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>FSP1</td>
<td>Fibroblast specific protein 1</td>
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<tr>
<td>hpf</td>
<td>Hours post fertilisation</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
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<tr>
<td>ISV</td>
<td>Intersegmental vessel</td>
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<tr>
<td>IB4</td>
<td>Isolectin B4</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MO</td>
<td>Morpholino</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
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<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PECAM1</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase PCR</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SASP</td>
<td>Senescence-associated secretory phenotype</td>
</tr>
<tr>
<td>SA-β-gal</td>
<td>Senescence-associated β-galactosidase</td>
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<tr>
<td>SL</td>
<td>Senescent-like</td>
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<td>Sp</td>
<td>Splice</td>
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<td>SC</td>
<td>Stalk cell</td>
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<td>TJ</td>
<td>Tight junctions</td>
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<td>TC</td>
<td>Tip cell</td>
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<td>TL</td>
<td>Transformed-like</td>
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<td>Tr</td>
<td>Translational</td>
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<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial cadherin</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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<tr>
<td>WT</td>
<td>Wildtype</td>
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<tr>
<td>α-SMA</td>
<td>α smooth muscle cell actin</td>
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ABSTRACT

The formation of the vascular network requires a tightly controlled regulation of pro-angiogenic and stabilising signals, including VEGF and the endothelial junctions respectively. Perturbation of this balance can result in dysregulated blood vessel morphogenesis and drive pathologies, including cancer. The Rho GTPases are major signaling regulators of the cytoskeleton and control functions such as cell migration and adhesion.

We have identified that ARHGAP18 controls RhoC activity and functions as an endogenous negative regulator of angiogenesis by limiting pro-angiogenic signaling and promoting vascular stability. Loss of ARHGAP18 promotes EC hypersprouting during zebrafish and murine retinal vessel development and enhances tumour vascularization and growth. Endogenous ARHGAP18 acts specifically via RhoC and relocalizes to the angiogenic and destabilized EC junctions in a ROCK dependent manner, where it is important in reaffirming stable EC junctions and suppressing tip cell behavior, at least partially through regulation of tip cell genes, Dll4, Flk-1 and Flt-4. The molecular mechanism governing the activation and translocation of ARHGAP18 to the cell periphery is at its infancy but phosphorylation and association with cortactin are involved.

Aberrant Ras signaling is associated with cancer and vascular pathologies, such as angiosarcomas. Chronic HRas activation induces a transformed-like phenotype, with fibroblastic cell phenotype, increased cell migration and sprouting, dysregulated endothelial junctions and a partial alteration of endothelial-to-mesenchymal markers. This is associated with but not dependent on downregulation of ARHGAP18 expression.
These findings highlight ARHGAP18 as a specific RhoGAP to fine-tune vascular morphogenesis, acting as a negative regulator to limit tip cell formation and promote junctional integrity to stabilize the angiogenic architecture.
CHAPTER 1

INTRODUCTION
1.1. THE VASCULAR SYSTEM

The vascular system is a complex network of vessels that functions to transport oxygenated blood, nutrients and circulating cells between tissues and organs. It is also a critical regulator of inflammation, regulating the efflux of blood borne factors and by providing the mechanisms for trafficking of circulating immune cells into the tissues. Dysregulation contributes to pathologies including cancer and ischemia (Adams and Alitalo, 2007; Potente et al., 2011) (Fig 1.1). Further, the function of the vasculature is impacted upon by the process of ageing (Kovacic et al., 2011).

The vascular system is organized into a hierarchical structure of large and small vessels that facilitate the circulation of blood between the heart, lungs and tissues (Fig 1.2). Blood circulation progresses from the heart through arteries to smaller arterioles then finally to capillary beds. Capillaries form extensive networks through tissues and organs to facilitate the exchange of gases and nutrients. The blood is returned though venules and veins to the heart and then to the lungs to be replenished with oxygen. Despite this complex organization, virtually all blood vessels arise by the sprouting from pre-existing blood vessels, a process termed angiogenesis (Herbert and Stainier, 2011). This process is driven by coordinated changes in the behavior of cells that line blood vessels, the endothelial cells (ECs).

1.1.1. DEVELOPMENT OF THE VASCULAR SYSTEM

1.1.1.1. Vasculogenesis

Vasculogenesis, the de novo formation of blood vessels, occurs during the embryonic development of the circulatory system (Fig 1.2) (Goldie et al., 2008; Herbert and Stainier, 2011; Patan, 2004). Endothelial progenitors (angioblasts) differentiate from mesodermal cells and may acquire arterial or venous fates to generate the first embryonic blood
Chapter 1. Introduction

vessels: the dorsal aorta and the cardinal vein, respectively (Coultas et al., 2005; Herbert and Stainier, 2011). Angioblasts also aggregate to form blood islands, which fuse and remodel to generate the primary capillary plexus (Adams and Alitalo, 2007; Carmeliet and Jain, 2011; Herbert and Stainier, 2011). Vasculogenesis also initiates heart development (Luttun and Carmeliet, 2003; Patan, 2004) and following the establishment of the blood circulation, the primary plexi are remodeled into the hierarchical structure of arteries, arterioles, capillaries, veins and venules (Adams and Alitalo, 2007).

1.1.1.2. Angiogenesis

Following vasculogenesis, the expansion of the vascular network predominantly occurs via angiogenesis. Continuous angiogenic remodeling and extensive expansion of the arterial, venous and capillary blood vessels lead to the formation of the complex and functional vascular network (Herbert and Stainier, 2011). After birth and the establishment of the vascular network, most blood vessels remain quiescent and angiogenesis only occurs during ovulation and pregnancy (Carmeliet, 2005; Robinson et al., 2009). However the ECs that line the blood vessels retain the ability to respond to physiological stimuli in order to divide and form new vascular sprouts (Carmeliet, 2005; Tonnesen et al., 2000). The cellular and molecular mechanisms regulating angiogenesis are reviewed in the sections below.

1.1.1.3. Lymphangiogenesis

The early embryonic veins also give rise to the lymphatic vessels to form the lymphatic system. Lymphangiogenesis is a specialized form of angiogenesis and refers to the growth and expansion of lymphatic vessels. The lymphatic system is a unidirectional network that runs parallel to the vascular system and regulates tissue fluid homeostasis, immune function and fat metabolism (Adams and Alitalo, 2007; Alitalo et al., 2005; Norrmen et al., 2011; Tammela and Alitalo, 2010). The blind-ended capillaries collect fluid,
macromolecules and cells from the interstitial space and drain through larger collecting lymphatic vessels into the venous circulation (Adams and Alitalo, 2007; Alitalo et al., 2005; Norrmen et al., 2011; Tammela and Alitalo, 2010). Lymphatic dysfunction may lead to lymphedema, a condition characterized by tissue oedema, immune deficiency and accumulation of subcutaneous fat (Alitalo et al., 2005; Norrmen et al., 2011). Despite the similarities between vascular angiogenesis and lymphangiogenesis, the mechanisms regulating these are strikingly different (Adams and Alitalo, 2007). An in depth description of these mechanisms in lymphangiogenesis is beyond the scope of this thesis.

1.2. CELLULAR MECHANISMS OF ANGIOGENESIS

The process of angiogenesis is initiated following the sensing of pro-angiogenic growth factors (GFs) and involves fundamental changes in the behavior of ECs and mural cells such as pericytes. The key events include ECs sprouting, adhesion and fusion, perfusion, stabilization, and maturation to form the new vessel (Fig 1.3). These new vessels are further remodeled into specific tissue and vessel types in order to fulfill the physiological functions.

1.2.1. STRUCTURE OF BLOOD VESSELS

In stable vessels, ECs typically form a monolayer of quiescent cells that line the luminal surface of blood vessels (Herbert and Stainier, 2011). To optimize blood flow, these vessels adjust the shape of endothelial cells and range from the highly aligned elongated cells in the arteries to the cobblestone-like shape in the quiescent capillary ECs (Carmeliet and Jain, 2011; Mazzone et al., 2009; Potente et al., 2011). The long lived ECs are attached to the laminin and collagen-rich basement membrane and are ensheathed by mural cells, including vascular smooth muscle cells (arteries and veins) and pericytes (capillaries), on the outer wall (Fig 1.3 A) (Adams and Alitalo, 2007; Carmeliet and Jain,
These mural cells stabilise the vessel by suppressing EC proliferation and promoting EC survival (Carmeliet and Jain, 2011). The ECs are tightly interconnected by junctional molecules, which regulate the passage of fluids, cells and macromolecules into the tissues (Dejana et al., 2009). Although quiescent, these ECs dynamically respond to signals from the nearby microenvironment. For example, during inflammation, the ECs are activated and the cell-cell junctions altered to allow infiltration of immune cells and mediators (Dejana et al., 2009).

1.2.2. Angiogenic Sprouting

The inactive phenotype of the quiescent ‘phalanx’ ECs is maintained until the ECs sense an angiogenic signal, such as VEGF (Potente et al., 2011). These angiogenic signals are released in the microenvironment by other cells, such as inflammatory or tumour cells during pathological angiogenesis or neuronal cells during development (Herbert and Stainier, 2011; Potente et al., 2011). These factors initiate and act to guide the process of angiogenesis (Fig 1.3 B) (Carmeliet and Jain, 2011; Herbert and Stainier, 2011; Potente et al., 2011). The pericytes are first detached from the vessel wall in response to Angiopoietin-2, a pro-angiogenic growth factor released by ECs (Augustin et al., 2009; Huang et al., 2010). ECs then loosen their cell-cell contacts, and liberate themselves from the basement membrane by proteolytic degradation by matrix metalloproteinases (MMPs) (Carmeliet and Jain, 2011; Herbert and Stainier, 2011; Potente et al., 2011). This is mediated by MT1-MMP, which is enriched in the sprouting endothelial cell (Potente et al., 2011; Sounni et al., 2002). The nascent vessel dilates and plasma proteins extravasate to form a provisional extracellular matrix (ECM) scaffold on which the ECs migrate (Carmeliet and Jain, 2011). The MMPs also liberate angiogenic growth factors from the
matrix and remodel the ECM into an angiogenic environment (Carmeliet and Jain, 2011; Potente et al., 2011).

Of the ECs that respond to angiogenic stimuli, only a small proportion become selected to lead the formation of the new sprout. The leading endothelial cell, the ‘tip cells’ (TCs), are highly motile and extend numerous dynamic filopodia that sense the attractive and repulsive guidance signals within the immediate microenvironment (De Smet et al., 2009; Gerhardt et al., 2003). These TCs are trailed by the ‘stalk’ cells (SCs), which are less motile but responsible for elongating and supporting the extension of the sprouting vessel, maintaining connectivity with the parent vessel and for the formation of the vascular lumen (Gerhardt et al., 2003; Herbert and Stainier, 2011; Phng and Gerhardt, 2009). The expansion of the vascular network requires the coordination of both the TC and SC responses. Importantly though, the TC and SC fates are transient phenotypes and a dynamic reshuffling of TCs and SCs occurs within the emerging sprout to promote vascular patterning and expansion (Bentley et al., 2014; Jakobsson et al., 2010). This regulation of TC and SC selection is mediated by VEGF-Notch signaling and is described in detail in section 1.4.1.

1.2.3. Vessel fusion and perfusion

EC sprouting occurs in a highly directional manner to guidance cues until TCs contact other vessels, undergo anastomosis and fuse with the connecting vessels (Fig 1.3 C, Fig 1.4 A) (Adams and Alitalo, 2007; Herbert and Stainier, 2011). Upon contact with other TCs or capillaries, TC behavior is repressed, with loss of their motile phenotype (Adams and Alitalo, 2007; Herbert and Stainier, 2011; Potente et al., 2011). Macrophages support vessel anastomosis by accumulating at the bridge sites and facilitating TC interaction with neighbouring ECs (Fantin et al., 2010). Once contact is established between ECs, strong EC-EC junctions containing the endothelial-specific cadherin, VE-cadherin, consolidate
the connection (Potente et al., 2011). The importance of the EC junctions is described in
detail in section 1.4.2.

The establishment of a functional perfused blood vessel requires the formation of a
vascular lumen, which may occur within growing endothelial sprouts or following vessel
fusion (Adams and Alitalo, 2007). The process of lumen formation is complex and not
well understood but thought to occur by different mechanisms, including cell hollowing
and cord hollowing (Potente et al., 2011). In cell hollowing, ECs form a lumen by the
fusion of intracellular pinocytic vacuoles and subsequently intercellular fusion of
vacuoles from neighbouring cells (Fig 1.4 B) (Gamble et al., 1999; Iruela-Arispe and
Davis, 2009; Kamei et al., 2006; Meyer et al., 1997). In cord hollowing, ECs adjust their
shape and rearrange their junctions to open up a lumen (Potente et al., 2011) (Fig 1.4 C).
Prior to lumen formation, nascent vessels consist of multicellular rods of ECs
interconnected by uniform EC-EC junctions (Herbert and Stainier, 2011). The first phase
involves lateral redistribution of junctional proteins from the apical EC surface to the
vessel periphery (Xu et al., 2011; Zovein et al., 2010). Thereafter negatively charged
glycoproteins are recruited to the apical membranes, which confer a repulsive signal to
open up a lumen, and is followed by cytoskeletal retraction to expand the lumen (Strilic et
al., 2009; Wang et al., 2010). However, the molecular events that initiate and manifest in
lumen formation are yet to be completely elucidated (Herbert and Stainier, 2011; Iruela-
Arispe and Davis, 2009; Potente et al., 2011).

1.2.4. VESSEL STABILIZATION, MATURATION AND REMODELLING

The newly formed immature vessels must be stabilized in order to be incorporated into
the functional vascular network (Potente et al., 2011). This process of stabilization
includes the recruitment of supporting pericytes, deposition of ECM into the basement
membrane, the strengthening of EC junctions and suppression of endothelial proliferation and sprouting (Fig 1.3 D, Fig 1.5 A) (Adams and Alitalo, 2007; Potente et al., 2011). Pericytes function to stabilize vessels by suppressing EC sprouting and proliferation and promoting EC survival (Gaengel et al., 2009; Hellstrom et al., 2001). Pericytes ensheath and establish direct contacts with ECs from capillaries and are recruited to immature sprouts in the growing blood vessel (Adams and Alitalo, 2007). There are a few signaling mechanisms of different origins that are known to regulate pericyte recruitment (Jain, 2003). Platelet derived growth factor B (PDGFB) is produced by endothelial cells, in particular the TCs, in response to VEGF, and signals to the PDGF receptor (PDGFR)-β expressed on pericytes to control their proliferation and migration (Abramsson et al., 2003; Hellstrom et al., 2001; Lindblom et al., 2003). Genetic knockout of PDGFB or PDGFR-β results in the lack of pericyte-EC attachment and mice display abnormal vascular morphogenesis, endothelial hyperplasia and embryonic lethality due to vascular oedema (Hellstrom et al., 2001; Lindahl et al., 1997). Further, angiopoietin-1 is produced by pericytes and activates the endothelial receptor TIE2 to promote pericyte adhesion and reduce vascular leak by signaling to tighten endothelial junctions (Augustin et al., 2009; Gamble et al., 2000; Uemura et al., 2002). Another GF, transforming growth factor β (TGF-β) triggers the differentiation, proliferation, migration and maturation of mural cells from progenitor cells (Chambers et al., 2003; Pardali et al., 2010). TGF-β also promotes vascular stabilization by stimulating ECM production (Jain, 2003). This is mediated by inducing the expressing of plasminogen activator inhibitor 1 in endothelial cells to prevent the degradation of the provisional ECM scaffold around the nascent vessel (Jain, 2003). Finally, sphingosine-1-phosphate (S1P) is a circulating ligand that binds to the S1PR1 receptor expressed on endothelial cells (Gaengel et al., 2012; Liu et al., 2000).
S1PR1 signaling regulates the trafficking of N-cadherin to the surface of ECs, which establishes and strengthens EC-pericyte interactions (Liu et al., 2000; Paik et al., 2004). In addition to circulating mediators, it has also been speculated that the onset of blood flow following lumen formation helps to stabilize new vessel connections (Fig 1.5 B) (Adams and Alitalo, 2007; Potente et al., 2011). Blood flow remodels the EC shape and contacts in the nascent vessel. Induction of the shear stress-responsive transcription factor, Krüppel-like factor 2 (KLF2) acts to modulate MAPK and PI3K to induce vessel remodelling (Nicoli et al., 2010). Moreover, oxygen delivery to the perfused vessel inactivates oxygen sensors to suppress hypoxia-driven VEGF expression, thereby shifting the ECs towards a quiescent phenotype (Adams and Alitalo, 2007; Potente et al., 2011).

Vascular regression is the pruning of vessel connections and may occur in vessels that have failed to become perfused (Fig 1.5 C) (Potente et al., 2011). Vascular regression is thought to occur by a reverse migrating mechanism by which endothelial cells retract from the vessel connection (Chen et al. 2012; Franco et al. 2015) resulting in the formation of empty basement membrane sleeves (Inai et al. 2004; Potente et al., 2011; Phng et al., 2007). Another mechanism regulating vascular regression involves the activation of apoptotic pathways (Dimmeler and Zeiher, 2000), triggered by the absence of pro-survival signals including pericyte-EC interactions, EC-EC contacts, integrin-matrix interactions (Jain, 2003) and VEGF activation of the PI3K/Akt survival pathway (Carmeliet et al., 1996; Hlushchuk et al., 2011).

1.2.5. EC specialization

ECs display remarkable heterogeneity in structure and function within the vascular system. This occurs not only at the macrovasculature level with respect to arteries, veins and lymphatic vessels, but also among the microvascular capillary beds of different organs (Aird, 2007). The differentiation of ECs into arteries or veins occurs during the
early embryo remodeling of the primary capillary plexi and continues throughout
development (Adams and Alitalo, 2007; Torres-Vazquez et al., 2003). This process is
termed arteriovenous differentiation and reflects the response to distinct haemodynamic
environments within the vascular network (Fig 1.6) (Adams and Alitalo, 2007). Arteries
form a high pressure and pulsatile flow system to transport blood to the capillaries
whereas the veins are a low-pressure system (Torres-Vazquez et al., 2003). Structurally,
arteries resist the high pressure shear stress by being surrounded in layers of vSMCs and a
specialized matrix of elastin fibres (Adams and Alitalo, 2007). Meanwhile veins are
thinner, enveloped by fewer vSMCs and possess flap-like structures to prevent the
backflow of blood (Torres-Vazquez et al., 2003). The regulation of arterial and venous
differentiation is controlled by several ligand-receptor signaling pathways including
Notch, ephrin and neurophilins (Torres-Vazquez et al., 2003). Blood flow dynamics also
regulate arteriovenous differentiation; changing arterial to venous flow in the embryonic
chick yolk sac suppresses the arterial phenotype (Kwei et al., 2004; le Noble et al., 2004).
Furthermore, engraftment of veins into arterial vasculature results in the development of
an arterial wall structure (Jain, 2003) and suggests that ECs possess a plasticity to adapt to
the local environmental cues. ECs from arterial and venous vessels show functional
differences in the regulation of endothelial permeability, leukocyte recruitment and in
haemostasis (Aird, 2007).

EC heterogeneity and adaption to microenvironmental cues is further demonstrated by the
functional differences capillary beds from different organs (Fig 1.7 A) (Adams and
Alitalo, 2007). For example, ECs in the central nervous system form the blood brain
barrier that tightly restricts the passage between the tissue and the circulating blood and is
in stark contrast to the highly permeable fenestrae-containing ECs in the endocrine glands
(Rocha and Adams, 2009). The mechanisms regulating tissue specificity are poorly
understood, but are believed to be due to specific molecular signatures in the microvascular ECs in response to the tissue microenvironment (Nolan et al., 2013). The ECs also play fundamental roles in regulating organogenesis. The ECs respond to the tissue specific microenvironment and secrete factors such as PDGF-B and Notch to regulate the development of organs including the kidney and liver (Ramasamy et al., 2015). In addition, the tissue specific ECs have critical roles in organ regeneration. These ECs support the homeostasis and regeneration of progenitor cells following tissue injury by secreting trophic GFs (angiocrine factors) (Nolan et al., 2013). Following partial liver hepatectomy, pro-angiogenic growth factors such as VEGF trigger liver sinusoidal EC proliferation and expression of Wnt2, hepatocyte growth factor (Ding et al., 2010) and CXCR7 (Ding et al., 2014) which drive liver regeneration. However, the liver sinusoidal ECs when transplantation into injured lungs are unable to promote lung regeneration (Fig 1.7 B) (Ding et al., 2011). Thus, ECs have unique signatures within each organ and are programmed to satisfy the functions of each particular organ (Ramasamy et al., 2015).

1.2.5.1. **Endothelial-to-mesenchymal transition**

In addition to displaying tissue and vessel heterogeneity, ECs possess the ability to differentiate into mesenchymal cells in a process known as Endothelial-to-Mesenchymal Transition (EndMT). EndMT is a specialized and related form of Epithelial-to-Mesenchymal Transition (EMT) that occurs in ECs rather than epithelial cells (Kovacic et al., 2012). This results in the progressive loss of endothelial and the gain of mesenchymal characteristics, including changes in cell polarity, adhesion and migration and is accompanied by a change in the expression of endothelial (e.g. VE-cadherin, PECAM-1) and mesenchymal (e.g. α-SMA, FSP-1) markers (Fig 1.8) (Medici and Kalluri, 2012). Many of the signaling pathways regulating EMT are also regulators of EndMT. EndMT can be initiated following the activation of a variety of signaling pathways, most notably
TGF-β 1 or 2, but also others including Notch, Wnt and PDGF (Kovacic et al., 2012; Medici and Kalluri, 2012). These act to activate the Snail family of transcription factors which act to coordinate the change from endothelial to mesenchymal marker expression (Medici and Kalluri, 2012). EndMT is crucial in the development of the embryonic heart, where the ECs contribute to the mesenchymal cells that form the atrioventricular cushion, valve and septa (Liebner et al., 2004). Further, EndMT derived cells also have stem-like properties and can differentiate into osteoclasts and chondrocytes (Medici et al., 2010). However, EndMT also contributes to pathology in kidney fibrosis (Zeisberg et al., 2008) and myocardial infarction and ischemia, where it contributes to cardiac fibrosis (Zeisberg et al., 2007b). EndMT also accounts for approximately 40% of the cancer-associated fibroblasts that form the tumour stroma (Zeisberg et al., 2007a). These fibroblasts can act to promote tumour formation through the secretion of factors that can promote tumour growth and/or angiogenesis. It has also been thought that the TC, which are highly migratory and have no lumen, may in fact be ECs undergoing EndMT (Gerhardt et al., 2003; Potenta et al., 2008).

### 1.2.6. **Other forms of vessel formation**

There are several other modes of vessel formation that have been identified. As previously mentioned, the developing vasculature is formed from progenitors in a process known vasculogenesis, while the expansion of the vascular network occurs by angiogenesis, in the cellular mechanisms detailed above. Arteriogenesis refers to the growth of pre-existent collateral arterioles into large functional arteries in response to occluded arteries (Fig 1.9 A) (Carmeliet, 2000; Heil et al., 2006; Semenza, 2007). This process is mediated by increases in shear stresses in collaterals following the narrowing of a main artery, which results in recruitment of circulating cells that secrete factors to promote the enlargement of the vascular wall structures (Heil et al., 2006).
Another mechanism by which the vessel network can be expanded is the splitting of blood vessels by the insertion of tissue pillars resulting in daughter vessels, a process termed intussusception (Fig 1.9 B) (Djonov and Makanya, 2005). However little is known about the physiological role and molecular mechanisms regulating intussusception (Adams and Alitalo, 2007; Carmeliet and Jain, 2011). Finally albeit debatable, bone marrow-derived circulating endothelial progenitor cells (EPCs) are also thought to contribute regenerative or pathological vessel growth in the adult in a process known as post-natal vasculogenesis (Fig 1.9 C) (Adams and Alitalo, 2007; Rafii et al., 2002). EPCs are recruited to the sites of repair or pathology and incorporate into the endothelial lining of the vascular wall (Adams and Alitalo, 2007; Carmeliet and Jain, 2011). While the existence of EPCs remains controversial, there is enormous potential of harnessing EPCs in the treatment of diseases (Rafii et al., 2002).

1.3. THE VASCULAR SYSTEM IN PATHOLOGY

Angiogenesis requires tight coordinated changes in pro-angiogenic and stabilizing signals to establish functional vascularization. Dysregulation of this balance can lead to abnormal vessel growth and function, which contribute to many pathologies, including cancer, ischemia and inflammatory diseases (Potente et al., 2011). Hence angiogenesis has been suggested to be a potential therapeutic target in these diseases. To date however, clinical applications of angiogenesis targets are of mixed results.

1.3.1. IN CANCER

1.3.1.1. Angiogenesis as a clinical target in cancer

Cancer is one of the leading causes of death worldwide with people having approximately a 1 in 2 risk of developing cancer in their lifetime and 1 in 4 chance of dying from this disease (World cancer report 2014, World Health Organisation statistics, www.iarc.fr).
The process of tumourigenesis is a multistep, diverse and complex process, but several critical hallmarks have been defined of which the vascular system plays a major role in three of these (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Firstly, in order to progress to a larger size, tumours require active angiogenesis to drive tumour vessel expansion and infiltration into the growing tumour to supply oxygen and nutrients (Hanahan and Weinberg, 2000; Weis and Cheresh, 2011). Secondly, the vascular system is important in regulating tumour metastasis by providing a systemic conduit for primary tumour dissemination (Weis and Cheresh, 2011). Thirdly, immune cells, which can both promote and inhibit tumour growth, infiltrate the tumour through crossing the vascular barrier (Gajewski et al., 2013).

The idea of targeting tumour angiogenesis dates back to 1971, where Folkman proposed that tumour growth is angiogenesis-dependent and that stopping the blood supply would cause the tumour to die (Folkman, 1971). Since the initial proposition decades ago, discoveries including the identification of the main angiogenic growth factor, vascular endothelial growth factor (VEGF) (Keck et al., 1989; Leung et al., 1989; Senger et al., 1983) and its receptors (Millauer et al., 1993) have established angiogenesis as a viable therapeutic target.

Clinically, VEGF blockers have been approved for clinical use in cancer treatment; the most notable being the VEGF neutralizing antibody bevacizumab (Avastin) in the treatment of a variety of metastatic cancers (Carmeliet and Jain, 2011; Jain et al., 2006). Further, several multi-targeted tyrosine kinase inhibitors (e.g. sunitinib (Sutent) which block signaling pathways such as those downstream of VEGF have also been used (Carmeliet and Jain, 2011). However, the clinical use of VEGF and signaling blockers as anti-angiogenic therapy is proving to be more difficult than anticipated; treatment with inhibitors only prolongs survival in the order of months (Carmeliet and Jain, 2011; Jain et
al., 2006). This has been attributed to the activation of an invasive and metastatic tumour switch following vessel regression by anti-VEGF treatment (Keunen et al., 2011; Weis and Cheresh, 2011). Furthermore, patients inevitably develop resistance to anti-VEGFA inhibitors (Lu et al., 2013). An emerging concept of “vascular normalization” where restoring endothelial function and co-targeting the tumour is more promising (Goel et al., 2011; Weis and Cheresh, 2011). Such treatments include using convention cytotoxics (e.g. doxorubicin) in combination with normalization therapies including pericyte-targeted (PDGF-D overexpression) or anti-angiogenic therapies (bevacizumab, TNP-470) (Goel et al., 2011).

1.3.1.2. The tumour vasculature is abnormal

The first stage of tumourogenesis is the acquisition of DNA damage to induce oncogene activation or tumour suppressor inactivation resulting in uncontrolled cell proliferation and hyperplasia (Hanahan and Weinberg, 2000). Tumour growth however is confined to a few millimeters due to nutrient deprivation and hypoxia (Weis and Cheresh, 2011). Tumours overcome this by triggering an ‘angiogenic switch’; secreting pro-angiogenic factors, such as VEGF, into the microenvironment to stimulate the sprouting of ECs into the tumour (Weis and Cheresh, 2011). Other factors in the tumour microenvironment, including the cancer associated fibroblasts, myeloid cells and the ECM also secrete factors to drive tumour angiogenesis (Weis and Cheresh, 2011). The tumour vasculature, however, is highly abnormal at all levels of structure and function: the vessels lack the normal hierarchical organization, are heterogenous, tortuous, highly sprouting and branching and have uneven lumen (Fig 1.10 A) (Carmeliet and Jain, 2011; Jain, 2003; Potente et al., 2011; Weis and Cheresh, 2011). The tumour ECs lack cobblestone appearance, are poorly interconnected and form an imperfect and disorganized lining within the vessels (Jain, 2003; Potente et al., 2011). The basement membrane is irregular
and fewer loosely attached abnormal tumour-associated pericytes cover the tumour vessels (Jain, 2003; Potente et al., 2011). As a result, vessels are leaky and blood flow is heterogenous leading to the irregular distribution of nutrients and oxygen within the tumour (Potente et al., 2011; Weis and Cheresh, 2011). This deprivation further drives proangiogenic stimulation and continuous but non-productive and disorganised remodeling of the vasculature (Potente et al., 2011). This creates a hostile acidic and hypoxic microenvironment that selects for more malignant clones and promotes tumour cell intravasation and dissemination through the already loosely structured vessel wall (Potente et al., 2011; Weis and Cheresh, 2011).

1.3.1.3. Vascular normalisation for treatment of cancers

The vasculature is central in triggering the cascade from a hyperplastic lesion to a highly malignant and invasive lesion and this understanding has led to the development of anti-angiogenic therapies, such as bevacizumab (Willett et al., 2004). The abnormal tumour vasculature poses many challenges in the delivery of drugs to the tumour. The high interstitial pressure, leaky vessels and irregular distribution of vessels impedes drug penetrance throughout the tumour (Carmeliet and Jain, 2011; Potente et al., 2011). Further tumour hypoxia also reduces the efficacy of many chemotherapeutic agents, due to their reliance on the formation of oxygen radicals (Carmeliet and Jain, 2011). Excessive pruning of the vasculature by anti-angiogenic therapy however aggravates tumour invasiveness and metastasis by increasing hypoxia (Fig 1.10 B) (Ebos et al., 2009; Paez-Ribes et al., 2009). The pruning of immature vessels while retaining the more mature efficient vessels, the so called ‘vascular normalization’ concept, is believed to be the key to drug delivery into tumours (Fig 1.10 B) (Goel et al., 2011; Jain, 2005). VEGF inhibitors block tumour vessel branching and induce the regression of pericyte-devoid leaky vessels while maintaining the mature functional tight vessels to promote tumour
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oxygenation (Benjamin et al., 1999; Goel et al., 2011; Jain, 2005). Therapies to promote pericyte coverage on tumour vessels also endorse functional vessels (Goel et al., 2011; Jain, 2005). This increases the efficacy of combined cytotoxic therapies and may also be combined with hormone withdrawal therapies to reduce VEGF production (Izumi et al., 2002; Jain, 2005), or pericyte directed therapies to destabilize tumour vessels (Bergers et al., 2003; Goel et al., 2011). However recent clinical studies have demonstrated that vascular normalization following anti-angiogenic therapy is only transient, and thus future strategies will be aimed at optimizing cytotoxic therapies specifically while the window is open (Goel et al., 2011).

1.3.1.4. In Ischemic disease

Another leading cause of death is cardiovascular disease (CVD), which accounts for approximately one-third of deaths worldwide (Deaton et al., 2011; Khurana et al., 2005). While tumourigenesis is driven by an over-abundance of angiogenic signals, insufficient angiogenesis leading to tissue ischemia occurs in CVD, such as myocardial infarction, stroke and coronary artery disease (Khurana et al., 2005; Marti and Risau, 1999; Ware and Simons, 1997) and also in non-cardiovascular conditions, such as preeclampsia (Maynard et al., 2008) and limb ischemia (Tongers et al., 2008). Clinical studies of angiogenic cytokine therapy in patients with myocardial ischemia have been shown to promote neovascularization and improve myocardial perfusion (Losordo and Dimmeler, 2004). However, some patients do not show clinical improvement and this is postulated to be due to a lack of blood delivery by arteriogenesis rather than by angiogenesis (Potente et al., 2011). Further, various studies have suggested that neovascularization contributes to the growth of atherosclerotic lesions and is key in plaque destabilization and rupture (Khurana et al., 2005; Moussa and Moses, 1999). While an attractive approach, further clinical studies would be needed to identify suitable targets for the treatment of CVD.
1.3.1.5. **Inflammatory diseases**

The acute inflammatory response to infections and wounds involves the recruitment of the immune cells to local sites to facilitate repair or clearance of the pathogen. The changes in the vasculature are under strict regulation, opening to permit immune cell extravasation and rapidly returning the vessel to homeostasis (Dejana, 2004; Vestweber et al., 2009). However, in a chronic inflammatory setting, the balance between the positive and negative regulators is disturbed with accumulation of inflammatory cells and inflammatory mediators that ultimately leads to damage and destruction. Such factors include pro-angiogenic cytokines (e.g., VEGF, FGF) that promote angiogenesis, which in turn contributes to the inflammatory pathology by transporting additional inflammatory cells and providing nutrients and oxygen to the site of inflammation (Jackson et al., 1997). As such, angiogenesis has been shown to be important in many chronic inflammatory diseases such as rheumatoid arthritis (Bainbridge et al., 2006), retinopathy (Crawford et al., 2009) and psoriasis (Heidenreich et al., 2009). Clinical studies with bevacizumab have shown improvement in patients suffering from diabetic retinopathy with marked reduction in neovascularization and hemorrhage (Avery et al., 2006; Spaide and Fisher, 2006). Anti-VEGF therapies injected locally into the eye is also a treatment for age-related macular degeneration, inhibiting vascular leak and limiting neovascularisation (Kovach et al., 2012).

1.3.1.6. **Vascular anomalies**

In addition to contributing to other diseases, the vascular system also has direct pathological conditions. Vascular anomalies are congenital conditions of abnormal vascular development and include vascular tumours and malformations (Richter and Friedman, 2012). The genetic defects underlying these conditions, however, are poorly understood.
Hemangiomas are a common tumour in infants, occurring in approximately 10% of the population (Richter and Friedman, 2012). This usually involves hyperplasia of endothelial cells and results in rapidly growing tumours during the first 6 months, although most regress with time and are rarely infiltrative. Hemangiomas are often managed by close observation, with medical or surgical interventions occurring in a small proportion of tumours.

Vascular malformations are rare vascular anomalies composed of irregular vascular networks and are classified by the vessel type, for example venous malformations (Richter and Friedman, 2012). In contrast to hemangiomas, vascular malformations are slow growing, infiltrative and often destructive vascular lesions, and often require intervention. Vascular malformations do not regress and continue to expand with cycles of rapid growth, infiltration and soft tissue destruction. Therapeutic interventions depend on the type of malformation and can consist of laser therapy, surgical resection and sclerotherapy.

1.4. **MOLECULAR REGULATION OF SPROUTING ANGIOGENESIS**

One of the key steps in angiogenesis is sprouting angiogenesis, the formation of the initial vascular sprout. These vascular sprouts organize into the leading TCs that guide the direction of sprouting and the trailing SCs that support the elongation of the sprout. Many recent studies have begun extrapolating the molecular mechanisms that regulate sprouting angiogenesis. Here we describe two of the most important mechanisms: pro-angiogenic VEGF signaling and the stabilizing EC junctions.

1.4.1. **VEGF-NOTCH SIGNALING**

1.4.1.1. **VEGF receptors and ligands**
VEGFA is the principal master regulator of blood vessel sprouting during development and in disease. VEGFA was initially identified as vascular permeability factor (VPF), as it induces vascular leak (Keck et al., 1989; Senger et al., 1983) and is part of a large family of non-redundant angiogenic regulators including placental growth factor (PIGF), VEGF-B, VEGF-C and VEGF-D (Ferrara et al., 2003; Hoeben et al., 2004; Olsson et al., 2006). All members have a VEGF homology domain with a cysteine knot-core region that is important in dimerisation (Hoeben et al., 2004; Olsson et al., 2006). The ligands are organised as homodimers in an anti-parallel orientation with each pole of the dimer interacting with the receptor binding sites (Muller et al., 1997; Olsson et al., 2006). The VEGF ligands bind to three receptor tyrosine kinases (RTKs), known as VEGF receptor-1 (VEGFR1/Flt1), -2 (VEGFR2/Flk1) and -3 (VEGFR3/Flk4) as well as to co-receptors such as heparan sulphate proteoglycans (HSPGs) and neuropilins (Olsson et al., 2006). These VEGFRs follow the typical mechanism of RTK activation with ligand binding triggering receptor dimerization, tyrosine kinase activation, autophosphorylation of the receptor and opening docking sites for the recruitment of signal transducers (Ferrara et al., 2003). The receptors differ in ligand binding specificity and in function (Fig 1.1 A). VEGFA binds to VEGFR1 and VEGFR2 to regulate blood vessel morphogenesis, while VEGFC and VEGFD bind to VEGFR3 to primarily regulate lymphangiogenesis (Joukov et al., 1996; Olsson et al., 2006).

While VEGFA binds to both VEGFR1 and VEGFR2, the functional effects of the two receptors are very distinct. Binding to the cognate receptor Tyr kinase, VEGFR2, activates multiple downstream pathways including Ras/RAF/MEF/ERK (MAK), PI3K/Akt, phospholipase Cγ and small GTPases (Hoeben et al., 2004; Olsson et al., 2006) (Fig 1.1 B). As a result, VEGFA signaling regulates EC proliferation, survival, filopodial extension, degradation of the ECM and chemotaxis (Neufeld et al., 1999;
Olsson et al., 2006). VEGFA is primarily regulated by hypoxia and drives the motile and invasive behavior of TCs to activate the angiogenic response to tissue oxygen deficiency during development and disease (Gerhardt et al., 2003; Pugh and Ratcliffe, 2003). Consequently, \textit{flk1}(\textit{VEGFR2}) knockout and \textit{VeGa}^{+/−} mice are embryonically lethal and display severe vascular deficiencies (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995).

VEGFR1, on the other hand, has higher affinity for VEGFA than does VEGFR2, but possesses weak Tyr kinase activity and is considered to be a decoy receptor that counteracts angiogenic signaling (Seetharam et al., 1995) (Hiratsuka et al., 2005). Furthermore, alternative splicing of \textit{VEGFR1} generates a secreted isoform of VEGFR1 (soluble VEGFR1 (sVEGFR1)) that acts as a sink for VEGFA in the microenvironment (Hiratsuka et al., 2005). As a result, \textit{flt1}(\textit{VEGFR1}) knockout is associated with EC hypersprouting, abnormal angiogenesis and embryonic lethality in mice and zebrafish (Fong et al., 1995; Hiratsuka et al., 2005; Krueger et al., 2011).

Other co-receptors that lack distinct kinase signaling mechanisms are also important in modulating VEGFR signaling. Alternative splicing of the VEGFA transcript gives rise to variants that have divergent functions and bioavailabilities, with the most predominant being VEGFA165 (VEGFA164 in mice) and VEGFA121 (VEGFA120 in mice) that vary in the presence or absence of the heparan sulphate-binding and neuropilin-binding domains (Hoeben et al., 2004). The heparin sulphate-binding VEGFA165 is matrix associated and forms gradients to promote the directional migration and filopodial extension of ECs (Ruhrberg et al., 2002). In contrast, VEGF121 is unable to bind heparin sulphate, is highly diffusible and controls EC proliferation but not migration (Ruhrberg et al., 2002). Another variant is VEGF188, which is completely matrix bound (Hoeben et al., 2004). Furthermore, the NRP co-receptors modulate angiogenic sprouting by complexing
with VEGFR and enhancing VEGF signaling when bound to VEGA, but only the VEGFA165 isoform and not the VEGFA121 isoform (Gerhardt et al., 2004; Kawasaki et al., 1999).

Finally, while VEGFC-VEGFR3 signaling is crucial in lymphangiogenesis (Joukov et al., 1996; Olsson et al., 2006), recent studies have shown additional roles of VEGFC in regulating sprouting angiogenesis (Siekmann and Lawson, 2007; Tammela et al., 2011; Tammela et al., 2008). VEGFR3 is highly expressed in TCs and forms VEGFR2-VEGFR3 heterodimers that binds VEGFC to positively influence angiogenic sprouting (Tammela et al., 2011). The function of VEGFR3 signaling is dependent on VEGFR2 expression during angiogenesis but not during lymphangiogenesis (Zarkada et al., 2015). Hence, the correct spatiotemporal expression of VEGFR3 is considered to be an important determinant of TC function (Herbert and Stainier, 2011).

1.4.1.2. **VEGF-Notch signaling controls Tip and Stalk cell selection**

Sprouting angiogenesis requires the organization of angiogenic sprouts into the leading TCs and trailing SCs and is coordinated by VEGF-Notch signaling (Fig 1.12). ECs express multiple Notch receptors (Notch1 being the most important) and transmembrane Notch ligands (including Delta-like ligand 4 (DLL4), and Jagged 1) (Phng and Gerhardt, 2009). Cells expressing the Notch ligands transactivate Notch signals in the adjacent neighbouring cells. Ligand binding induces cleavage of the Notch receptor and release of the Notch intracellular domain (NICD), which functions in association with other transcription factors as a transcriptional regulator that controls cell fate specification (Phng and Gerhardt, 2009).

The default endothelial response to VEGFA is the TC phenotype. The ECs expressing the highest level of VEGFA signaling become selected as the TCs and transactivate Notch signals to laterally inhibit TC and promote SC fates (Herbert and Stainier, 2011; Potente
et al., 2011). VEGFA-VEGFR2 signaling promotes transcriptional DLL4 expression in TCs (Hellstrom et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007). DLL4 is expressed on the surface of the TCs and binds to the Notch1 receptor on the adjacent cells. DLL4-Notch signaling suppresses TC fate and VEGFA responsiveness by downregulating VEGFR2, VEGFR3 and NRP1 in addition to upregulating VEGFR1 (Hellstrom et al., 2007; Jakobsson et al., 2010; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Consequently, reduced DLL4-Notch signaling is accompanied by excessive TC formation, uncontrolled hypersprouting and disordered vessel branching (Hellstrom et al., 2007; Lobov et al., 2007; Suchting et al., 2007).

Whereas DLL4 negatively regulates sprouting angiogenesis, another Notch ligand, Jagged 1, promotes TC formation and angiogenesis (Benedito et al., 2009). Jagged 1 is expressed primarily by SC, and binds to Notch1 but poorly transactivates Notch signals. Accordingly, SC-restricted Jagged 1 competes with DLL4 for binding to Notch1 on TCs to suppress Notch signaling in TCs.

**1.4.1.3. ECs dynamically rearrange positions in vascular sprouts**

To expand the vascular network, ECs undergo cycles of sprouting, branching and tubulogenesis, which require the dynamic balance and transitions between the tip and stalk cell phenotypes. VEGFR2 inhibition causes sprouting defects with blunt ended channels, while DLL4 or Notch1 inactivation, although resulting in more vessels, leads to poorly perfused dysfunctional vessels (Phng and Gerhardt, 2009; Potente et al., 2011). Recent studies have demonstrated that the TC and SC fates are transient and that the ECs are continuously shuffling within the sprout and are in constant competition for the leading TC position (Fig 1.13 A) (Bentley et al., 2014; Bentley et al., 2009; Jakobsson et al., 2010). This is mediated by the relative expression of VEGFR2 and VEGFR1 in the neighbouring cells, such that cells with high VEGFR2 and low VEGFR1 stand a better
chance to occupy the leading position (Jakobsson et al., 2010). However, every cell within the sprout has a distinct level of Notch signaling compared to its neighbouring cells and continuously re-regulates its expression of VEGFR2 and VEGFR1 and position within the sprout (Bentley et al., 2014). Therefore rather than a single TC directing uniform migration, this dynamism allows the sprout to constantly assess the microenvironment and direct sprouting according to the availability of the pro-angiogenic growth factors (Bentley et al., 2014; Jakobsson et al., 2010). This tight sensory control of TC and SC fates is regulated by the dynamic changes in DLL4 expression. The TEL/CtBP repressor complex at the DLL4 promoter is transiently disassembled upon VEGFR2 signaling to allow a temporary pulse of DLL4 transcription (Fig 1.12) (Roukens et al., 2010). Hence, the cells that have highest VEGFA signaling express DLL4 more quickly or at higher levels transiently become the TCs. However, without the continuous VEGF signaling and DLL4 expression, these cells then lose the position to other cells that are more responsive to VEGF.

In line with a tightly controlled system, activation of Notch and the release of NICD not only regulates the expression of VEGFR but also promotes its own turnover to prevent sustained Notch activation (Fig 1.12) (Phng et al., 2009; Potente et al., 2011). The NICD complexes with the RBPj/CFB1 transcription factors and regulates the expression of Notch-regulated ankyrin repeat protein (NRARP), which negatively regulates Notch by disassembling the co-activator complex and promoting NICD degradation. Additionally, NRARP also promotes stalk cell proliferation and maintains the stability of the nascent vessel connections via signaling through the Wnt/β-catenin pathway (Corada et al., 2010; Phng et al., 2009).
1.4.2. **Endothelial Junctions**

The endothelial cell-cell junctions are the key regulators of vascular integrity in maintaining intercellular adhesion and regulating the flux of blood fluids, proteins and cells through the blood vessels. During responses to angiogenic or inflammatory stimuli, the EC junctions are dynamically remodeled to facilitate vascular permeability and leukocyte extravasation. However, the cell-cell junctions are not only sites of attachment between ECs; they also function as intercellular signals to modulate the limitation of cell growth, cell polarity, lumen formation and interactions with mural cells (Dejana, 2004; Dejana et al., 2009). One major implication of these signals is in the regulation of angiogenic sprouting.

**1.4.2.1. Organisation and function of the EC junctions**

The cell-cell junctions are organized into two specialized adhesive junctional regions: the tight junctions (TJs) and the adherens junctions (AJs) (Fig 1.1). The Gap junctions are also present in ECs, but have limited roles in regulating EC permeability and sprouting, so are therefore not further described here. The AJs are involved in the initiation and maintenance of cell-cell contacts while TJs regulate paracellular passage of ions and solutes and limit the free movement of lipids and proteins through the vessel (Bazzoni and Dejana, 2004; Dejana et al., 2009). Both the TJs and the AJs consist of homophillic interactions of transmembrane proteins that line and form zipper-like structures along the cell borders (Dejana et al., 2009). These transmembrane proteins interact through their cytoplasmic tails with cytoskeletal and signaling proteins to anchor the junctions to actin microfilaments and transfer of intracellular signals (Dejana et al., 2009). Cytoskeletal association is important for stabilization of the junctions, but also for regulation of the dynamic changes in junction opening and closure, and in controlling cell shape and polarity (Dejana, 2004; Dejana et al., 2009; Hartsock and Nelson, 2008). The AJs are first
formed at the initiation of cell contract and followed by TJ organization (Dejana, 2004). Moreover, the AJs are required for TJ assembly, although interestingly are not essential for TJ maintenance (Capaldo and Macara, 2007; Dejana, 2004).

The major functions of the TJs are in the regulation of vascular permeability and leukocyte extravasation (Matter and Balda, 2003). The TJs show considerable variability amongst different vascular beds. For example, the ECs that comprise the tight brain microvasculature are rich in TJs, while the post-capillary venules, which are the primary site of leukocyte extravasation, display less TJ complexity (Wallez and Huber, 2008). The core components of the TJs that promote cell-cell adhesion are the Claudin proteins. There are over 20 Claudin proteins, of which claudin-5 is endothelial specific (Wallez and Huber, 2008). While Claudin-5 deficiency has limited vascular defects in embryos, the mice suffer from post-natal defects in the blood-brain barrier leading to death after a few hours of birth (Nitta et al., 2003). The TJs are also comprised of other transmembrane proteins, including occludin and JAMs, which also contribute to intercellular adhesion (Dejana et al., 2009). These adhesive proteins all contribute to intracellular signaling by interacting with binding partners, such as the ZO proteins, which in turn can bind to TFs, such as ZONAB (Balda et al., 2003) and β-catenin (Rajasekaran et al., 1996). The stable TJs in confluent cells restrain the TFs away from the nucleus and as a result inhibit cell proliferation. However, to date there have been no studies examining the roles of TJs in sprouting angiogenesis.

In contrast to the TJs, the AJs are ubiquitous in all types of vessels (Dejana, 2004). The transmembrane proteins of the AJs complex are the cadherin proteins, which are calcium-dependent adhesion molecules (Giannotta et al., 2013). In ECs, these include VE-cadherin and N-cadherin. N-cadherin is also expressed in neural and mural cells and regulates EC-pericyte interactions, and loss of N-cadherin results in early embryonic death due to
defects in vascular development owing to an inability to stabilize the vasculature (Luo and Radice, 2005; Tillet et al., 2005). VE-cadherin is an EC-specific cadherin and is the major regulator of EC-EC adhesion. The cytoskeletal tails of the cadherins bind catenins, in particular p120, β-catenin and plakoglobin, which in turn interact with α-catenin that regulates the cell cytoskeleton to reinforce cell adhesion (Abraham et al., 2009; Giannotta et al., 2013; Vestweber et al., 2009).

Despite the essential function of VE-cadherin for EC adhesion, Cdh5−/− mice initially form the primitive vascular network, but then fail to develop into a mature vessel system (Carmeliet et al., 1999; Gory-Faure et al., 1999). Vascular sprouts still recognize each other and form connections but fail to stabilize and anastomose correctly (Crosby et al., 2005). Importantly, when sprouting tip cells from WT mice connect with each other to anastomose, they stop forming filopodia to regulate uncontrolled sprouting and vascular branching. However, in cells lacking VE-cadherin, they continue to form filopodia, but do not sense the cell to cell contact, and instead continue searching for other connections (Giannotta et al., 2013; Lenard et al., 2013). This is further supported in inducible Cdh5−/− mice, whereby loss of VE-cadherin leads to uncontrolled sprouting and branching in the retina (Gaengel et al., 2012). This indicates that while VE-cadherin is not essential for initial EC interaction, it is required for stabilising EC-EC adhesion to allow the fusion of vascular sprouts and in the transfer of negative signals to switch the cells to a quiescent state.

1.4.2.2. **VE-cadherin regulates VEGF signaling**

In addition to promoting EC-EC adhesion, VE-cadherin and the AJs also act as intracellular signaling mediators, underlining the concept that stable EC junctions act to inhibit growth signals and reinforce the quiescent state. The natural state of mature vessels is that of confluent, resting quiescent cells with VE-cadherin clustered at the cell-
cell contacts and structured organization of the AJs. Under these conditions, VE-cadherin signaling promotes vascular stability, such as contact inhibition, limiting cell migration, protection from apoptosis and control of endothelial permeability (Giannotta et al., 2013). In particular this is mediated through the interaction and clustering of VE-cadherin with GFRs, most notably VEGFR2 (Dejana and Orsenigo, 2013). VE-cadherin interacts with VEGFR2 and inhibits its downstream MAPK-ERK mediated proliferation signal while maintaining anti-apoptotic PKB/Akt signaling (Dejana and Orsenigo, 2013; Lampugnani et al., 2002). This is mediated via interactions with β-catenin and the DEP-1 phosphatase to retain VEGFR2 at the cell membrane where it is quickly dephosphorylated (Fig 1.15) (Lampugnani et al., 2006). VEGFR2 signaling is strongly influenced by internalization, including maintenance of signaling from the endosomal compartments to degradation and recycling of the receptors (Giannotta et al., 2013; Nakayama et al., 2013). VEGFA binding to VEGFR2 triggers Src activation, which in turn activates Rac signaling leading to the phosphorylation of Ser665 and other tyr residues on VE-cadherin (Gavard and Gutkind, 2006). These serve to recruit β-arrestin and activate the internalization machinery, thereby promoting VE-cadherin endocytosis into clathrin-coated vesicles (Gavard and Gutkind, 2006; Gavard et al., 2008) (Fig 1.15). This disassembly of the AJs increases vascular permeability and destabilizes cell-cell contacts, which are crucial steps in the initiation of angiogenic sprouting. Additionally, this also allows β-catenin and other VE-cadherin binding partners to translocate to the nucleus to regulate transcription, where they can act to control cell proliferation (Dejana et al., 2009).

Another mechanism of regulation is the association of VE-cadherin with the vascular endothelial receptor-type protein tyrosine phosphatase (VE-PTP), an endothelial specific phosphatase (Hayashi et al., 2013). VE-PTP dephosphorylates substrates at the EC junctions, including VE-cadherin, plakoglobin, and VEGFR2 (Hayashi et al., 2013).
During sprouting angiogenesis, VE-PTP regulates VEGFR2 activation in SCs to dephosphorylate VEGFR2 and VE-cadherin to stabilize EC junctions, establish cell polarity and lumen formation (Hayashi et al., 2013). Furthermore, it has been recently established that the EC junctions are differentially regulated during sprouting angiogenesis (Bentley et al., 2014). As described above, vascular sprouts exhibit a mosaic of TC and SC phenotypes that are constantly shuffling and transitioning and this is regulated by DLL4-Notch signaling (Fig 1.13). It has been further demonstrated that these Notch/VEGFR interactions regulates differential VE-cadherin dynamics such that Notch signaling stabilizes EC junctions and limits EC mobility within the stalk (Fig 1.13 B) (Bentley et al., 2014). On the contrary, the TCs that are high in VEGFR signaling and DLL4 expression exhibit active junctions with serrated localization of VE-cadherin at the junctions and increased mobility. Thus, sprouting angiogenesis is a dynamic process with constant regulation of VEGFR-Dll4-Notch signaling and differential VE-cadherin during cell rearrangement and the establishment of effective vascular patterning.

1.4.3. Other regulators

In addition to the aforementioned regulators, there are an abundance of other important regulators that are involved in angiogenic sprouting. These include other GF/GFR signaling pathways such as FGF2, PDGF, Wnt/β-catenin, the stabilising Angiopoietin/Tie2, VEGFA and the VEGF co-receptor Nrp1/2, Ephrin, and guidance signals such as Netrins, ROBO, Sema/Plexin D1. The roles of these pathways in sprouting angiogenesis are covered in other reviews (Adams and Alitalo, 2007; Herbert and Stainier, 2011; Potente et al., 2011). However, these are beyond the scope of this thesis, where the regulation by VEGFA and the EC junctions are of most concern.
1.5. **Rho GTPases in Angiogenesis**

The Rho GTPases are the major signaling molecules that regulate the actomyosin cytoskeleton. Through this, the Rho proteins control many cellular functions including cell migration and adhesion, two critical processes in sprouting angiogenesis. However, the exact roles that Rho GTPases play in angiogenesis are not completely understood. Here we discuss some of the mechanisms of the Rho GTPases in regulating the cytoskeleton during cell migration and adhesion.

1.5.1. **Rho Family and General Functions**

The Rho GTPases are small GTPases, members of the Ras superfamily and comprise over 20 intracellular signaling molecules that regulate the actinomyosin cytoskeleton (Heasman and Ridley, 2008). The best characterized of these Rho GTPases are RhoA, Rac1 and Cdc42, which are important in regulating actin stress fibres, lamellipodia and filopodia, respectively. These Rho proteins are further classified into eight subfamilies that share substantial homology within each (Fig 1.16) (Heasman and Ridley, 2008). This homology could account for functional redundancy between some of the closely related Rho proteins – upregulation of other Rho GTPases are seen in some Rho knockout models (Heasman and Ridley, 2008). Most Rho proteins act as molecular switches that cycle between active GTP bound and inactive GDP bound forms (Fig 1.17 A). This regulation of Rho activity is mediated by interaction with three sets of proteins, guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). The Rho GTPases interact with downstream effector proteins only when GTP bound and regulate various aspects of the actin cytoskeleton (Fig 1.17 B).

The Rho proteins are direct downstream targets of GF signaling such as VEGF. VEGFA stimulation leads to a dramatic and rapid activation of RhoA, Rac1 and Cdc42 and
localisation to the membrane (Bryan and D'Amore, 2007). This rapid activation then returns to baseline suggesting that these have critical functions in the early stages of angiogenesis, namely in regulating cell migration, adhesion and cell shape changes, but that there are also important negative regulators that restrict this activation.

1.5.2. In cell migration
During directed cell migration, there is a coordinated interplay between the three major Rho GTPases generating a protrusive force at the leading edge and simultaneous retractive force at the rear of the cell (Fig 1.18) (Bryan and D'Amore, 2007). This involves the activation of Cdc42 at the leading edge to promote the formation of fine, actin-rich filopodia, which function to sense the microenvironment and guide the direction of cell migration. This is followed by Rac-1 dependent actin polymersation at the cell periphery to form the lamellipodia to promote forward movement of the cell. Both Cdc42 signaling through WASP and Rac signaling via the WAVE complex converge on ARP2/3, which regulates actin polymersation to form new actin filaments (Ridley, 2001). This is then followed by Rho and Rac-dependent formation of new focal complex structures localized in the lamellipodia that mediates attachment of the extending lamellipodia to the ECM. Cell body contraction then occurs, and is dependent on Rho mediated actomyosin contractility (Ridley, 2001). This is mediated by Rho signaling via ROCK (also known as Rho-kinase) to phosphorylate MLC, thus enabling the myosin crossbridge to bind the actin filament and initiate contraction (Ridley, 2001).

1.5.3. In adhesion and permeability
Another important role of the Rho GTPases is in the regulation of cell-cell adhesion and vascular permeability. Under resting conditions, VE-cadherin regulates AJ stability and Rho GTPase activation through interactions with the catenins (Lampugnani et al., 2002) (Fig 1.19 A). VE-cadherin clustering increases the concentration of Tiam1 and Vav2, Rac
GEFs, and p21 activated kinase (PAK), a Rac effector, thus promoting lamellipodia formation that favours barrier maintenance (Lampugnani et al., 2002). Further, while it is generally assumed that RhoA mediated stress fibres and contraction impairs barrier integrity, it has been shown that in resting conditions a basal level of ROCK is essential for continuous EC junction remodeling and maintenance (Szulcek et al., 2013). In response to barrier destabilizing mediators such as thrombin and VEGF, the Rho GTPases act to regulate transient and sustained increases in vascular permeability. Upon thrombin stimulation, rapid RhoA signaling to ROCK and MLC phosphorylation leads to contractility, which in turn weakens the connected junctional complex (Giannotta et al., 2013; van Nieuw Amerongen et al., 2003). In contrast, VEGFA mediated Rho GTPase activation has a more direct effect on the disruption of the AJs (Fig 1.19 B). VEGFA stimulation activates Src, which stimulates Vav2 to activate Rac1 and PAK. This in turn phosphorylates VE-cadherin S665 leading to its endocytosis, and thus an increase in cell permeability (Gavard and Gutkind, 2006). While RhoA signaling can promote contractility and weaken cell-cell contacts through ROCK, it can also stabilize the AJs through downstream activation of mDia (Sahai and Marshall, 2002). mDia promotes actin polymerization, localization of the cadherins to the cell periphery, and microtubule organisation to effectively stabilise the AJs (Sahai and Marshall, 2002). Moreover, Angiopoietin-1 acts to stabilise the AJs through its activation of RhoA and mDia, which counteracts the effects of VEGFA by sequestering Src (Gavard et al., 2008). This has been attributed to the Rho GEF, Syx, which is recruited following Ang1 to activate Rho and mDia, and dissociated from the junctions following VEGFA stimulation (Ngok et al., 2012).

Also of interest is RhoC, which is part of the RhoA subfamily and shares 92% identity with RhoA. RhoC is not required for embryogenesis, which is likely due to compensation
by the other Rho subfamily members (Hakem et al., 2005). However, RhoC is functionally distinct from RhoA. RhoC displays higher affinity for ROCK than RhoA, and when overexpressed drives activation of ROCK and actomyosin contraction to disrupt the AJs (Sahai and Marshall, 2002). On the other hand, RhoA-mDia signaling drives AJ stabilization (Sahai and Marshall, 2002). This RhoC-ROCK signaling also regulates VE-cadherin mediated suppression of cell sprouting, whereby overexpression of RhoC reverses the increase in cord formation following VE-cadherin blockade (Abraham et al., 2009). RhoC is also critical in tumour cell biology, where it is overexpressed in many cancers and mediates disruption of the AJs to promote invasion and metastasis (Hakem et al., 2005; Iiizumi et al., 2008; Islam et al., 2014; Ruth et al., 2006; Simpson et al., 2004; van Golen et al., 2000a; van Golen et al., 2000b). However, little is known about the role of RhoC in endothelial cells and angiogenesis.

1.5.4. **RhoJ, an endothelial specific Rho GTPase**

RhoJ, an EC specific Rho GTPase is of recent interest. A few studies have reported RhoJ as a regulator of EC motility and tube morphogenesis via its effects on controlling actomyosin contractility and focal adhesions (Kaur et al., 2011). Further, RhoJ deficient mice display delayed radial growth of the retinal vasculature with increased vascular regression (Fukushima et al., 2011). Due to its endothelial specificity, RhoJ is seen as an effective and selective target for tumour vasculature disruption (Kim et al., 2014). Dual blockade of RhoJ and VEGFA signaling effectively suppresses tumour progression and metastasis (Kim et al., 2014).

1.5.5. **Regulation of Rho GTPase activity**

As mentioned briefly above, the activity of the RhoGTPases is regulated by interaction with the GEF, GAP and GDI proteins (Fig 1.17 A). GEFs promote the release of bound GDP and subsequent binding of the more abundant GTP while GAPs on the other hand
catalyse the intrinsic hydrolysis of GTP and therefore act to inhibit the GTPase activity (Vega and Ridley, 2008). GDI proteins bind to the C-terminal end of RhoGTPases and prevent membrane association and sequester them in the cytoplasm, thereby inhibiting access to downstream targets. There are over 70 GEFs, 80 GAPs, and 3 GDI proteins of which there is substantial redundancy and also some proteins that regulate multiple GTPases (Heasman and Ridley, 2008). Aberrant Rho GTPase signaling is one of the major causes of disease including cancer and vascular diseases (Vega and Ridley, 2008). However, unlike the Ras superfamily counterparts, the RhoGTPases are rarely mutated in cancers. Instead, aberrant activation of Rho signaling involves constitutively active splice variants of RhoGTPases, altered localization mediated by GDIs, and altered expression and function of the regulatory GEFs and GAPs (Vega and Ridley, 2008). Many GAPs and GEFs have been identified as oncogenes, including the Rac GEFs Vav2 and Tiam1 mentioned above (Cook et al., 2013), and DLC-1, a RhoA GAP and tumour suppressor that is frequently mutated and lost in liver, breast and many other cancers (Ng et al., 2000; Xue et al., 2008; Yuan et al., 2003).

Taken together, this highlights the importance of Rho signaling in regulating critical processes of angiogenesis, namely cell migration and junctional integrity. Aberrant Rho signaling through perturbations in the regulatory Rho GEF and Rho GAP proteins is associated with diseases, hence Rho GTPase signaling is recognized as an attractive therapeutic target (van der Meel et al., 2011; Vega and Ridley, 2008).

1.6. ARHGAP18

The vascular biology laboratory previously identified a role of ARHGAP18 in the regulation of EC biology. ARHGAP18 was first isolated from a macrophage library and termed MacGAP (NM_33515). Subsequent studies from our laboratory identified
ARHGAP18 in a screen for angiogenic genes, and showed a novel role in senescence (hence the alias SENEX). ARHGAP18 has also been implicated in epithelial migration and morphogenesis.

1.6.1. Bioinformatics analyses

1.6.1.1. Protein domains and isoforms

The human ARHGAP18 gene is located on chromosome 6q22.33 and encodes for a 3.5 kb transcript (Accession ID NM_033515.2). The translated protein is 663 amino acids with a predicted molecular weight of ~75 kDa and an isoelectric point of 6.4 (Accession ID NP_277050.2). The actual ARHGAP18 protein exists as two isoforms, an ~78 kDa and a smaller ~75 kDa, which is proposed to be due to translation from the downstream start codon at amino acid 55 (Coleman et al., 2010; Maeda et al., 2011). It is unclear if there are any functional differences between these two isoforms.

ARHGAP18 belongs in the family of RhoGAP proteins due to the presence of the RhoGAP domain. As described above, the RhoGAP proteins catalyse the GTPase activity of the RhoGTPases thus causing their inactivation. The RhoGAP domain in ARHGAP18 is located between amino acids 324-523 (Fig 1.20 A). Apart from the RhoGAP domain, there are no other known classic domains.

By phylogenetic analysis of ARHGAP18 sequences, ARHGAP18 is highly conserved in many species (Fig 1.20 B, Table 1.1). Amongst the mammals, including mice, ARHGAP18 shows over 84% identity and 90% similarity. ARHGAP18 is less conserved in the non-mammals including chicken (Gallus gallus), zebrafish (Danio rerio) and frogs (Xenopus laevis). However, the GAP domain of ARHGAP18 in these species is considerably more conserved compared to the rest of the protein. This indicates that the function of ARHGAP18 through its RhoGAP domain is likely to be similar in the different species.
Comparison of ARHGAP18 to the sequences of 17 of the closest other RhoGAP proteins reveals that it shows highest similarity to ARHGAP28 and ARHGAP40 (Fig 1.21) approximately 30% identity and 40% similarity between these two proteins. Further, the GAP domain of ARHGAP18 shares close to 50% identity and over 70% similarity respectively. This may indicate that ARHGAP18 is more functionally similar and shows redundancy to ARHGAP28 and ARHGAP40. There is currently one publication that has identified ARHGAP28 to be a predominantly bone-restricted RhoA GAP protein (Yeung et al., 2014). When overexpressed in epithelial cells, ARHGAP28 was found to inhibit stress fibre formation and induce membrane ruffles. However, ARHGAP28−/− mice have limited phenotypes, which is thought to be due to the compensatory upregulation of ARHGAP6, but not ARHGAP18, in these mice. There are currently no studies on the function of ARHGAP40.

1.6.1.2. Expression analysis

Using BioGPS (Wu et al., 2009) (biogps.org), murine ARHGAP18 is found to be ubiquitously expressed in different cells and tissues, albeit at different levels (Fig 1.22). Most notably, there is high-level expression of ARHGAP18 in mast cells and macrophages, which may suggest potential roles of ARHGAP18 in degranulation. In the mouse tissues, there are various levels of expression with the most notable in the hormonal tissues (e.g. mammary gland, ovaries) and the gastrointestinal tissues except for the liver. Intriguingly, there is very little expression of ARHGAP18 in the tissues of the nervous system. We and others (Maeda et al., 2011) have previously shown that ARHGAP18 is also expressed abundantly in many cell lines of different organ origins. However, ARHGAP18 is most highly expressed in ECs (Coleman et al., 2010; Maeda et al., 2011), which has been found to be 22-fold higher relative to other tissues (van Buul et al., 2014).
Analysis of cancer databases (COSMIC (cancer.sanger.ac.uk/cosmic), cBioPortal (cbioportal.org)) reveals that ARHGAP18 is aberrantly regulated in many types of cancer. ARHGAP18 is mutated in up to 2% of cancer cases with various mutations throughout the protein, including the GAP domain. ARHGAP18 shows little changes in copy number variation, however it has been found to be overexpressed in over 5% of cancers including those of the cervix, liver, central nervous system, stomach, large intestine and esophageal tissues.

1.6.2. Previous publications

1.6.2.1. In tube formation in vitro

The Vascular Biology laboratory originally identified ARHGAP18 (alias SENEX) through a PCR array screen of genes regulated during in vitro capillary tube formation (Coleman et al., 2010; Hahn et al., 2005). Using this model, the ECs undergo a synchronized process of angiogenesis with coordinated changes in EC proliferation, migration, stabilization, lumen formation and maturation (Hahn et al., 2005). It was found that ARHGAP18 is dynamically regulated with an initial downregulation during the early migratory phase then upregulated at the later stabilization phase (Coleman et al., 2010; Hahn et al., 2005). Furthermore, knockdown of ARHGAP18 did not affect cell migration and alignment but prevented the formation of stable tubes resulting in EC death. On the other hand, ARHGAP18 overexpression did not affect tube formation. These observations suggested that ARHGAP18 might have potential roles in regulating EC migration or stabilization. ARHGAP18 was chosen for further analysis based on its high level of expression in EC and its biphasic regulation of expression during capillary tube formation. In addition, at the time of its characterisation in angiogenesis, the function of ARHGAP18 in any cell had not been identified.

1.6.2.2. In anti-inflammatory EC senescence
While ARHGAP18 overexpression does not affect tube formation, it induces EC senescence (Coleman et al., 2010). The induction of senescence is a response to oncogene activation or DNA damage and is a critical tumour suppressive mechanism (Hanahan and Weinberg, 2000; Perez-Mancera et al., 2014). Senescent cells cease to proliferate and are characterized by a large flattened morphology, polyploidy and accumulation of senescence-associated β-galactosidase (SA-β-gal) (Perez-Mancera et al., 2014). ARHGAP18 overexpression induces premature senescence that is associated with activation of the p16INK4a, but not the p53/p21 pathway (Coleman et al., 2010). Furthermore, senescent cells induced by oxidative stress, but not replicative stress had an accumulation of ARHGAP18.

While senescence undoubtedly is a tumour suppressive response, it also has deleterious effects through the senescence-associated secretory phenotype (SASP) (Perez-Mancera et al., 2014). Senescent cells accumulate with age and disease and contribute to pathology through the secretion of the proinflammatory SASP mediators, such as TNF-α, and IL-6 (Coppe et al., 2010; Perez-Mancera et al., 2014). Intriguingly, ARHGAP18 overexpression mediates a profoundly anti-inflammatory senescence phenotype, with reduced expression of inflammatory adhesion molecules (VCAM1, E-selectin) and perturbed neutrophil attachment and transmigration (Coleman et al., 2010). This has been demonstrated to be dependent on caveolae, small specialized lipid rafts on the cell membrane, that act as critical signaling molecules (Powter et al., 2015). During endothelial senescence induced by oxidative stress, hypoxia, or shear stress, there is an equal mixture of pro- and anti-inflammatory senescent cells (Coleman et al., 2013). This ARHGAP18-associated anti-inflammatory population is speculated to have unique protective roles in limiting uncontrolled proliferation and the local inflammatory response (Coleman et al., 2013). Thus, the work by (Coleman et al., 2010) showed a novel cellular...
phenotype (anti-inflammatory senescent EC) induced by a newly described gene (*ARHGAP18*).

### 1.6.2.3. **As a RhoA GAP in epithelial cell rounding and migration**

Following our initial publication (Coleman et al., 2010), Maeda et al. (2011) then demonstrated that ARHGAP18 is a regulator of cell shape, spreading and motility through its RhoA GAP function. Knockdown of ARHGAP18 in HeLa and MDA-MB-231 epithelial cells induces a contractile phenotype with prominent stress fibres and focal adhesions. On the contrary, overexpression of full-length ARHGAP18, but not GAP deficient or a GAP mutant, could reverse the effects on stress fibres and focal adhesions. The contractile phenotype was further demonstrated to be dependent on RhoA and downstream ROCK activation. This has critical functions in regulating cell spreading and migration, with the knockdown contractile cells displaying reduced cell spreading and migration.

### 1.6.2.4. **Regulation of tissue tension**

A recent study by Porazinski et al. (2015) demonstrated a role of ARHGAP18 in regulating tissue tension. During vertebrate development, correct tissue and organ shape and alignment are essential for function. This process of tissue tension is primarily regulated by actomyosin contraction. It was identified that medaka fish embryos with a mutation in the YAP protein results in pronounced body and tissue flattening and collapse (Porazinski et al., 2015). ARHGAP18 was revealed as a downregulated gene in the screening of YAP mutants and knockdown of ARHGAP18 phenocopied the YAP mutants. Further, expression of a membrane-targeted ARHGAP18 was able to rescue the body flattening phenotype indicating that ARHGAP18 acts downstream of YAP in the regulation of the cortical actomyosin network.
1.6.2.5. In Drosophila as Conundrum

The ARHGAP18 orthologue, Conundrum (Conu), has been identified as a regulator of Rho1 during epithelial morphogenesis in Drosophila (Neisch et al., 2013). Rho1 (the RhoA homologue in Drosophila) regulates epithelial integrity and morphogenesis by controlling F-actin assembly and actomyosin contractility. Conu regulates Rho1 and Rac1 activity through its localization at the cell cortex and interaction with Moesin to control epithelial morphology and proliferation. However, phylogenetic analyses of ARHGAP18 related amino acid sequences revealed that Conu actually displays higher similarity to ARHGAP28 and ARHGAP40 than to ARHGAP18 (Porazinski et al., 2015).

1.6.2.6. In breast cancer

This PhD was initially aimed at investigating ARHGAP18 in breast cancer and breast epithelial cell senescence. However this was indefinitely suspended due to technical feasibilities after 1.5 years into the project. The detailed results of this project are not discussed in this thesis.

We identified that ARHGAP18 is overexpressed in breast cancer, with increasing expression correlating with cancer progression (unpublished data). ARHGAP18 is highly expressed in the mammary epithelium, and is regulated during lactation (unpublished data, BioGPS). Furthermore, analysis of cancer databases reveals that ARHGAP18, while rarely mutated (<1% of cases), is amplified in breast cancer patients, with one particular study identifying gene amplification in over 30% of cases (Eirew et al., 2015). In murine mammary tumourigenesis, the ARHGAP18 locus has been demonstrated to be a novel integration site for mouse mammary tumour virus (Kim et al., 2011). ARHGAP18 is overexpressed in murine mammary tumours, and its overexpression increases mammary epithelial cell proliferation, cell cycle and 3D acinar morphogenesis (Kim et al., 2011).
1.7. **PROJECT AIMS**

Angiogenesis requires a highly coordinated set of molecular and cellular changes in EC behavior to drive the process including sprouting, elongation, stabilization and remodeling. Sprouting requires a highly regulated balance of sprout inducing signals, such as VEGF, and stabilizing signals, such as maturation of EC junctions, to effectively pattern the vascular network. Both of these factors converge on the RhoGTPases, core signaling molecules that regulate the actin cytoskeleton and control fundamental cell processes.

We had previously identified ARHGAP18 as a key regulator of EC biology in its regulation of anti-inflammatory senescence. At the commencement of this study, there had been little understanding into the function of ARHGAP18 in cell biology. Taking into consideration the current literature on the function and regulation of expression of ARHGAP18 during tube formation and 3D morphogenesis, we hypothesise that ARHGAP18 acts via its RhoGAP function as a key regulator of angiogenesis (Fig 1.23).

To address this possibility, we have investigated ARHGAP18 in relation to:

1. Its role in Ras induced EC sprouting and EndMT. The initial project was aimed at investigating Ras-induced oncogene induced EC senescence and the role of ARHGAP18. However, the results refocused the overall aim to investigate the role of ARHGAP18 in regulating EC sprouting angiogenesis.
2. EC sprouting angiogenesis and TC/SC regulation.
3. Its mechanism of ARHGAP18 in regulating EC sprouting angiogenesis.
4. Its post-translation modifications that regulate its function.
CHAPTER 1.
INTRODUCTION

FIGURES
Angiogenesis plays a major role in health and disease. Angiogenesis is crucial in embryonic development and in organ development. Balanced angiogenesis is required to maintain vascular homeostasis. Under or over regulated angiogenesis are major contributors to pathology including heart disease, cancer, chronic inflammation and vascular malformations.
During early embryo development, endothelial progenitors (angioblasts) differentiate from mesodermal cells. When formed, angioblasts may acquire arterial (red) or venous (blue) fates and form the first blood vessels, the dorsal aorta and the cardinal vein. Angioblasts also aggregate to form blood islands, which fuse and remodel to generate the primary capillary plexus.

Following vasculogenic assembly, angiogenic remodeling assembles the hierarchical network of arteries, arterioles, capillaries, venules and veins. Oxygenated nutrient rich blood travels from the heart through the arteries and arterioles to the capillaries which facilitate the exchange of gases and nutrients. Deoxygenated blood returns through the veins to the heart and then to the lungs to be replenished with oxygen.

The lymphatic system runs parallel to the vascular system and functions in the clearance of fluids, and immune cells. The formation of the lymphatic system is initiated from the lymphangiogenic sprouting of lymphatic ECs originating from the early embryonic veins. Blind ended lymphatic capillaries feed into the larger collecting vessels and ducts and finally into the venous circulation.

Adapted from Herbert and Stainier (2011).
Fig 1. Cellular mechanisms of vessel formation.
A. Endothelial cells (ECs) line the luminal surface of quiescent blood vessels. The ECs are tightly interconnected by junctional molecules and are ensheathed by pericytes which help maintain the inactive state of the endothelium.
B. During sprouting, high levels of pro-angiogenic growth factors select tip cells (TCs) for sprouting. Sprouting requires the modulation of junctional contacts, pericyte detachment and basement membrane degradation. The TCs migrate towards gradients of pro-angiogenic factors along a provisional extracellular matrix (ECM) scaffold.
C. TCs continue migrating until reaching other vessels, whereby TC behaviour is repressed and the cells undergo anastomosis, which is assisted by myeloid cells. Stalk cells (SCs) trail the leading TC and support the extension of the sprout. SCs are responsible for depositing basement membrane, dividing to elongate the vessel and initiating lumen formation.
D. Nascent vessels are perfused and stabilised by pericyte recruitment. The pericyte-endothelium interactions, strengthening of the EC-EC contacts and ECM deposition re-establish the quiescent vessel.

Adapted from Herbert and Stainier (2011).
Fig 1.4. Vessel fusion and perfusion.
A. Tip cell fusion. Tip cells sprout until sensing other tip cells and initiate VE-cadherin mediated cell adhesion. Branch anastomosis is also facilitated by macrophages.
B-C. Models of lumen formation. The formation of the lumen in sprouting vessels can occur by cell hollowing (B) or cord hollowing (C). In cell hollowing, ECs form a lumen by fusing intracellular and intercellular pinocytic vacuoles. In cord hollowing, ECs rearrange the EC junctions, and recruit negatively charged glycoproteins that repel to open up a lumen that is reinforced by cytoskeletal retraction.

Adapted from Potente et al. (2011).
Fig 1.5. Vessel stabilisation and remodelling

A. Vessel stabilisation relies on the recruitment of pericytes and deposition of basement membrane. TCs and SCs secrete a variety of factors to recruit pericytes including PDGFB and mediate EC-pericycle interactions via N-cadherin. Pericytes ensheath and stabilise vessels by suppressing EC sprouting and proliferation and promote cell survival.

B. Blood flow remodels the EC shape and contacts in the nascent vessel. The delivery of oxygen to the perfused vessel inactivates oxygen sensors to suppress hypoxia driven VEGF responses to promote endothelial quiescence.

C. Vascular regression. Remodelling of the vessels occurs in vessels that fail to perfuse. Occlusions trigger EC regression via apoptosis or via a reverse migration mechanism and form empty basement membrane sleeves.

Adapted from Potente et al. (2011).
Fig 1.6. Arteriovenous differentiation

A. ECs have heterogeneity in structure and function by differentiating into arterial or venous phenotypes. Arterial ECs have a aligned spindle-like morphology in the direction of blood flow, and are surrounded by layers of elastin fibres. Venous ECs are thinner, are enveloped by fewer layers of vSMCs and possess flap-like structures to prevent backflow. This differentiation is regulated by hemodynamic factors. Arterial and venous ECs have distinct gene expression profiles such as the expression of Ephrin-B2 and EphB4 respectively.

Adapted from Adams and Alitalo (2007).
Fig 1.7. Endothelial heterogeneity and the regulation of organ regeneration

A. ECs from different organs display extensive heterogeneity in structure and function. ECs in the brain form the tight blood brain barrier that restrict flow while ECs in the endocrine glands are fenestrated allowing for secretion. The mechanisms that regulate tissue-specific EC structure and function are poorly understood but are postulated to be due to specific molecular signatures of microvascular ECs in response to the tissue microenvironment. From Hoffman and Calabrese (2014).

B. Tissue-specific ECs regulate organ-specific regeneration via specific angiocrine factors. Following lung or hepatic injury, ECs respond to the microenvironmental cues and secrete angiocrine factors to facilitate organ regeneration. However, ECs from the liver are unable to regenerate injured livers. Adapted from Ramasamy et al. 2015.
**Fig 1.8. Endothelial-to-mesenchymal transition**

A. Endothelial-to-mesenchymal transition (EndMT) can be initiated by the activation of multiple signaling pathways. During EndMT, ECs lose endothelial characteristics in polarity, adhesion and migration and the expression of EC makers, and gain mesenchymal characteristics and markers. EndMT is important in regulating embryonic heart development and has stem cell-like properties. Endothelial derived fibroblasts also have important roles in regulating fibrosis of the lung, kidney and heart, and are a significant proportion of the cancer-associated fibroblast population.

*Modified from LeBleu and Kalluri (2011).*
**Fig 1.9. Other forms of vessel formation**

A. Arteriogenesis. Following vessel blockage, there is an increase in shear stress in the existing collateral arteries. This results in enlargement of the collateral arteries to compensate for the function of the original vessel. *Adapted from Simons and Ware (2003).*

B. Vessel intussusception. The splitting of vessels through the insertion of tissue pillars. Little is known about the function or regulation of intussusception. *From Adams and Alitalo (2007).*

C. Post-natal vasculogenesis. Circulating endothelial progenitors can be recruited and incorporated into the endothelial wall and can contribute to angiogenic sprouting. *From Adams and Alitalo (2007).*
**Fig 1.10. The tumour vasculature and vascular normalisation**

A. The tumour vessels have a highly disorganised vasculature structure. The ECs lining the tumour vessels are poorly interconnected and are surrounded by few pericytes. The vessels are leaky and promote a hostile hypoxic and acidic environment that facilitates further angiogenesis, tumour cell dissemination, and inhibits drug delivery and the efficacy of chemotherapeutic agents.

B. Vascular normalisation for treatment of cancers. Anti-angiogenic therapy alone drives excessive vessel regression, leading to increased hypoxia and inflammation that drives an invasive tumour switch. Vascular normalisation, where vessel function is restored in the tumour, and the co-targeting of the tumour is an emerging concept. Restoring barrier function and tumour oxygenation would allow increased efficacy of chemotherapeutics and limit the invasive switch of tumours.

*Modified from Carmeliet and Jain (2011).*
Fig 1.11. VEGF receptors and signaling

A. The VEGF family includes VEGFA, VEGFB, VEGFC and VEGFD that bind to different VEGFRs to regulate different aspects of angiogenesis. VEGFA is the major ligand that binds to VEGFR2 and coreceptors such as NRP1/2, to regulate angiogenesis. VEGFA also binds to the decoy receptor VEGFR1 and its soluble isoform sVEGFR1. VEGFC and VEGFD are major regulators of lymphangiogenesis by signaling via VEGFR3. VEGFC also regulates angiogenesis via VEGFR2/VEGFR3 heterodimer signaling. Modified from Herbert and Stainier (2011).

B. VEGFA-VEGFR2 signaling pathways. Binding of VEGFA to VEGFR2 triggers typical receptor tyrosine kinase activation and recruits signal transducers to activate downstream pathways such as PI3K/Akt, p38MAPK, FAK, MAPK/ERK and PKC. These act to regulate processes such as cell survival, migration, proliferation, and cytoskeletal rearrangements which are all important in angiogenesis.
Tip cells (TCs):
- Highest expression of VEGFR2
- Highly motile
- Numerous filopodia sense microenvironment and guide angiogenesis
- Induces DLL4 expression to laterally inhibit TC phenotype in neighbouring cells

Stalk cells (SCs):
- Activated by Notch signaling from TC-induced DLL4
- Suppress VEGFR signaling
- Less motile
- Support cells to stabilise the sprouting vessel
- Initiate lumen formation

Fig 1.12. VEGF-Notch signaling regulates TC-SC differentiation

A. The vascular sprouts are organised into the leading TCs and the trailing SCs. The TCs guide angiogenesis by sensing the microenvironmental cues while the stalk cells support the vessel structure and initiate lumen formation. TCs have high levels of VEGFR signaling that induces DLL4 expression to laterally suppress the TC phenotype in the SCs.

B. VEGF signaling triggers the TC phenotype and transcriptionally activates DLL4 expression. DLL4 is expressed on the cell surface to activate NOTCH1 on the SCs. This liberates NICD, which acts as a transcription factor to upregulate Flt1/VEGFR1 and downregulate VEGFR3 to suppress the TC phenotype. SCs also express Jagged1 which competes with DLL4 to prevent Notch signaling in the TCs. NICD also regulates its own degradation via NRARP to prevent excessive Notch signaling.

Adapted from Herbert and Stainer (2011).
Fig 1.13. Sprouting angiogenesis involves dynamic rearrangements of TCs and SCs.
A. Static and dynamic models of angiogenic sprouting. Under static models, there is a single tip cell with the supporting stalk. Computer modeling has indicated that angiogenic sprouts contain a mosaic of TC and SCs that vary in the expression of VEGFRs, DLL4 and Notch and in shuffling capacity. This regulates the dynamic rearrangements of the cells within the sprout. The TC and SC fates are also transient through continuously regulation of the VEGFRs, DLL4 and Notch levels. This permits constant sensing of the microenvironment to allow for the development of new TCs and long range movement and vessel sprouting. Adapted from Bentley et al (2014).
B. Dynamic rearrangement of TCs and SCs involves differential regulation of cell junctions. Cells expressing high VEGF activity and high DLL4 have weaker active serrated EC junctions. As a result these cells have high shuffling capacity and a higher preference for the TC position.
Fig 1.14. Organisation of the EC junctions
A. The endothelial junctions are organised into the tight junctions (TJs) and the adherens junctions (AJs). Both junctions consist of transmembrane proteins, the claudins, JAMs, occludins (TJs) and the cadherins (AJs) that function to regulate cell-cell adhesion. The TJs and the AJs are anchored to the actin cytoskeleton through interaction with intermediate signaling molecules. Functionally, the TJs regulate the paracellular passage of fluids and cells, while the AJs are the main regulators of cell-cell contact.

From Lemichez et al (2010).
Fig 1.15. Regulation of VE-cadherin-VEGFR2 signaling

A. In resting cells, the stable AJs interact with DEP-1 phosphatase to retain VEGFR2 at the cell membrane and inhibit its phosphorylation. Following VEGF stimulation, VEGFR2 signals via Src and Rac to phosphorylate VE-cadherin. VEGFR2 activation promotes angiogenic signaling is also internalised. Phosphorylation of VE-cadherin weakens the cell junctions and recruits β-arrestin which triggers VE-cadherin endocytosis into clathrin-coated vesicles and thus causing junction disruption and vascular leak.

Adapted from Gavard et al. (2006).
**Fig 1.16. The Rho GTPase family**

A. Phylogenetic tree of Rho GTPases. The tree demonstrates the relationship between the different subfamilies and the individual Rho GTPases. The percentage indicates the amino-acid sequence identity between the subfamily members. The classical Rho GTPases cycle between the GTP and GDP bound states, whereas the atypical Rho GTPases are all GTP-bound.

From Heasman and Ridley (2008).
Fig 1.17. Regulation of Rho GTPase activation and function
A. The Rho GTPase cycle. RhoGTPases cycle through active GTP-bound and inactive GDP-bound forms. This is catalysed by interaction with GEF and GAP proteins, respectively. The active GTP-bound form is recruited to the cell membrane and interacts with downstream effectors. The GDIs inhibit Rho movement to the membrane by sequestering them in the cytosol. From Akitores (2011).

B. Downstream effector pathways of Rho, Rac and Cdc42. The RhoGTPases interact with a variety of different effectors to regulate the actomyosin cytoskeleton. In general, RhoA regulates cell contraction, Rac1 actin polymerisation at the lamellipodia, and Cdc42 regulates actin polymerisation in the filopodia.
1. Extension of filopodia

2. Lamellipodia protrusion & attachment to focal complexes

3. Retraction of trailing end

4. Contraction of cell body

**Fig 1.18. Rho GTPase activation in cell migration.**

A. Cell migration requires coordination activation of the Rho GTPases. Firstly, Cdc42 guides the cell and extends actin-rich filopodia at the leading edge towards the direction of migration. Rac1 then promotes lamellipodia protrusion and in combination with RhoA form focal complexes that attach the cell to the ECM. The rear of the cell retracts and the cell body contracts via RhoA activation.

*Modified from Mattila and Lappalainen (2008).*
Fig 1.19. Rho GTPases in the regulation of EC junctions.
A. In resting cells, Rac1 promotes lamellipodia and stable junctions through Tiam1, PAK and Vav2 RacGEFs. On the other hand, RhoA-ROCK regulation of stress fibres acts to impair barrier integrity.
B. Following VEGF stimulation, Rac1 and RhoA have opposite effects. VEGF stimulation of Rac1 phosphorylates VE-cadherin causing its endocytosis leading to junctional disruption. In the combined presence of the Ang-1 and VEGF, RhoA acts to inhibit the junctional disruption mediated by Rac1. RhoA activation activates mDia which sequesters Src and prevents phosphorylation and endocytosis of VE-cadherin.

Modified from Gavard et al. (2006) and Gavard et al. (2008).
Fig 1.20. Domains and phylogenetic analysis of ARHGAP18.

A. Human ARHGAP18 contains a single RhoGAP domain between amino-acids 320 and 523. No other domains are known.

B. Phylogenetic alignment of ARHGAP18 from other species. ARHGAP18 sequences from different species were aligned by ClustalW. ARHGAP18 is well conserved amongst different species with high conservation in the RhoGAP domain (red).
Table 1.1. Amino acid identity and similarity of ARHGAP18, and the ARHGAP18 RhoGAP domain, in different species compared to the human sequence.

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Fig 1.21. ARHGAP18 related proteins.
A-B. Phylogenetic tree of closely related proteins to ARHGAP18. The full length sequences (A) and RhoGAP domains (B) of similar human proteins were aligned using ClustalW. ARHGAP18 shows highest similarity to ARHGAP28 and ARHGAP40 when aligning the full-length sequences or RhoGAP domains.
Fig 1.22. Tissue expression of ARHGAP18.
BioGPS (biogps.org) data of ARHGAP18 expression in murine cells and tissues, ARHGAP18 shows highest expression in mast cells and macrophages. In tissues, ARHGAP18 is highly expressed in hormonal tissues and very lowly expressed in the central nervous system.
Fig 1.23. Functions of ARHGAP18
ARHGAP18 has roles in the regulation of EC tube formation and anti-inflammatory senescence. Recent studies have also demonstrated that ARHGAP18 is a regulator of epithelial cell shape and tissue morphogenesis. The project aimed to investigate the functions of ARHGAP18 in the regulation of angiogenesis.
CHAPTER 2

MATERIALS AND METHODS
2.1. MOLECULAR CLONING

2.1.1. MATERIALS FOR MOLECULAR CLONING

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>LB Broth</td>
<td>10 g/L Bacto tryptone (Sigma Aldrich), 5 g/L yeast extract (Sigma Aldrich), 10 g/L NaCl.</td>
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<td>LB-Amp</td>
<td>LB Broth containing 100 µg/ml ampicillin sodium salt (Sigma Aldrich)</td>
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<td>LB Agar</td>
<td>LB broth containing 15 g/L agar (Sigma Aldrich).</td>
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<td>TAE buffer</td>
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<td>10 mM Tris-HCl (Sigma Aldrich), 1mM EDTA, pH 7.5.</td>
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2.1.2. PLASMIDS AND RETROVIRAL VECTORS

pcDNA3.1-GFP and pcDNA3.1-GFP-ARHGAP18, consisting of the human ARHGAP18, were previously constructed by Paul Coleman (Centenary Institute). pcDNA3-mCherry was a gift from Dr. Carol Wadham (Centenary Institute). pMIG and pMIG-Ras retroviral vectors were a gift from Dr. Alex Swarbrick (Garvan Institute of Medical Research). pQCXIN was a gift from Dr. Joshua Moses (Centenary Institute). The retroviral envelope plasmid, pVSV-G, was a gift from Dr. George Sharbeen (Centenary Institute). The use of retroviral vectors was in compliance with OGTR guidelines of the Royal Prince Alfred Hospital IBC.

2.1.3. PLASMID AND DNA FRAGMENT MANIPULATIONS

Restriction enzymes for cloning were sourced from New England Biolabs (NEB) (MA, USA). Restriction enzyme digests were performed at 37°C using at least 500 ng plasmid or insert DNA according to manufacturers’ instructions. Fragments generated from restriction digests or PCR amplification were ligated into Antarctic phosphatase (NEB) treated vector backbones using T4 DNA ligase (NEB) at RT for 10 min or 4°C overnight.
2.1.4. **HIGH-fidelity POLYMERASE CHAIN REACTION (PCR)**

Specific DNA fragments were amplified using Phusion high-fidelity DNA polymerase (Finnzyme). Reactions were performed in 0.2 mL PCR tubes (Interpath, VIC, Australia) in a total volume of 25 µL reactions consisting of 1X Phusion master mix buffer, 0.4 U Phusion DNA polymerase, 1.5 mM MgCl₂, 200 µM dNTP, 0.5 µM forward and reverse primers and DNA template. PCR amplification was performed in a BioRad thermocycler (BioRad, NSW, Australia) using the conditions outlined in Table 2.1.

<table>
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<th>Step</th>
<th>Temperature</th>
<th>Time</th>
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</thead>
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<td>Denaturation</td>
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<td>Hold</td>
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</table>

2.1.5. **AGAROSE GEL ELECTROPHORESIS**

Electrophoresis of DNA fragments was performed using 1% (w/v) agarose gels dissolved in TAE buffer and containing 0.5X GelRed DNA stain (Jomar Bioscience, SA, Australia). DNA samples were mixed with DNA loading dye (Fermentas, Thermo Scientific) prior to loading. DNA samples were co-electrophoresed with the 1kb DNA plus ladder (Life Technologies) to determine the sizes of the DNA fragments. Electrophoresis was performed in a Bio-Rad sub cell (Bio-Rad) electrophoresis tank containing TAE buffer at 100 V for approximately 60 min. DNA bands were visualized using the ChemiDoc MP Gel imaging system (Bio-Rad).
2.1.6. Gel purification and PCR purification

DNA fragments from agarose gels were recovered by excision of the desired DNA band with a scalpel. The recovery of purified DNA was performed using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer’s instructions. All other DNA purifications were performed using the QIAquick PCR Purification Kit (Qiagen).

2.1.7. Assessment of DNA quality and concentration

Purified DNA was quantified using the NanoDrop 2000 spectrophotometer (NanoDrop Technologies, DE, USA). The absorption at 260 nm was used to determine the concentration of DNA, and the ratio of the absorbance at 260 nm to 280 nm ($A_{260/280}$) and 260 nm to 230 nm ($A_{260/230}$) was used to determine the quality of the DNA. A sample was considered clean with an $A_{260/280}$ ratio of ~1.8 and an $A_{260/230}$ ratio ~2.0. DNA fragments were purified using the PCR purification kit when the quality was poor.

2.1.8. DNA sequencing

DNA sequencing reactions were prepared using 1 µg of DNA and 10 pmol of sequencing primer and performed through the service offered by the Australian Genome Research Facility (AGRF) (Sydney, Australia). The DNA sequencing results were viewed using FinchTV (Geospiza, PerkinElmer, Seattle, USA).

2.1.9. Bacterial transformation

The chemically competent Escherichia coli DH5α (Life Technologies) were used for the propagation of plasmids and ligation products. Briefly, 5 µL of plasmid or ligation products was added to 50 µL of DH5α cells for 30 min on ice. The cells were then heat-shocked at 42°C for 45 s and recovered with 500 µL SOC media at 37°C for 1 h. The bacteria were then spread onto LB agar plates containing 100 µg/ml ampicillin. Single colonies were screened, cultured and used for plasmid preparations.
2.1.10. **Screening of positive transformants**

Single colonies were screened for the presence of DNA inserts by PCR and restriction digest of plasmids. Colony PCR was performed using crude DNA prepared by lysing a single bacterial colony in 100 µL of 1% (v/v) Triton-X100 (Sigma Aldrich) in TE and heating at 95°C for 5 min. Colony PCR reactions were performed in 10 µL reactions consisting of 1X Go Taq Green master mix (Promega, NSW, Australia), 0.5 µM forward and reverse primer and 4 µL crude colony DNA. Thermocycling conditions used are listed in Table 2.2.

**Table 2.2. Thermocycling conditions for colony PCR**

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<td>Initial Denaturation</td>
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<tr>
<td>Denaturation</td>
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<td>5 min</td>
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<tr>
<td>Hold</td>
<td>4°C</td>
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</tbody>
</table>

Positive transformants were subcultured in 2 mL LB-Amp for small-scale plasmid cultures to be used for screening by restriction digest. The bacteria were grown at 37°C with shaking at 250 rpm overnight. Plasmid DNA was isolated using the Qiagen Plasmid Mini kit (Qiagen), and screened by restriction enzyme digest and confirmed by DNA sequencing.

2.1.11. **Bacterial and plasmid propagation**

Large-scale plasmid preparations were performed on confirmed colonies. Starter cultures of positive clones were first established in 2 mL LB-Amp for 6-8 h at 37°C with shaking. Large scale 100 mL LB-Amp cultures were prepared in conical flasks using 100 µL of the starter culture and cultured for 12-16 h at 37°C with shaking. Endotoxin-free plasmid was
isolated using the EndoFree Plasmid Maxi Kit (Qiagen) and the plasmid resuspended in 0.1X TE in H$_2$O.

**2.1.12. Specific cloning methods**

**2.1.12.1. pMIG-ARHGAP18 & pQCXIN-ARHGAP18**

The human ARHGAP18 fragment was excised by EcoRI digest of pcDNA3.1-GFP-ARHGAP18 and cloned into the EcoRI site of pMIG or pQCXIN. Correct orientation of the ARHGAP18 fragment was determined by restriction digest and sequencing of the plasmid.

**2.1.12.2. pQCXIN-mCherry and pQCXIN-mCherry-ARHGAP18**

For pQCXIN-mCherry, the mCherry fragment was amplified by high-fidelity PCR using the forward primer 5’-GCGGATCCGCCAATGGTGAGCAAGGGCGAGGAGCC-3’ and reverse primer 5’-CGGAATTCCTTTACTTTGTACAGCTCGTCCATGCC-3’ to insert a 5’ BamHI site and 3’ EcoRI site. The fragment was digested using BamHI and EcoRI and inserted into BamHI and EcoRI-digested pQCXIN backbone. For pQCXIN-mCherry-ARHGAP18, the mCherry fragment was amplified by high-fidelity PCR using the forward primer 5’-GCGGATCCGCCAATGGTGAGCAAGGGCGAGGAGCC-3’ and reverse primer 5’-CGGAATTCCTTTACTTTGTACAGCTCGTCCATGCC-3’ to insert a 5’ BamHI site and 3’ EcoRI site preceeded by a linker codon. The fragment was digested using BamHI and EcoRI and inserted into BamHI and EcoRI-digested pQCXIN backbone. The human ARHGAP18 fragment was excised from pcDNA3-GFP-ARHGAP18 by EcoRI digestion and inserted into the EcoRI-digested pQCXIN-mCherry-linker backbone.
2.2. CELL BIOLOGY

2.2.1. CELL CULTURE

2.2.1.1. Tissue culture media and solutions

PBS
1X Dulbecco’s phosphate-buffered saline (PBS), pH 7.1, was prepared from a 10X PBS solution (Gibco, Life Technologies, CA, USA) and contained of 20 mg/L KCl, 20 mg/L KH$_2$PO$_4$, 800 mg/L NaCl, and 216 mg/L Na$_2$HPO$_4$.

PBS-EDTA
1X PBS containing 10 mM EDTA (Sigma Aldrich, MI, USA).

Trypsin-EDTA
1X Trypsin-EDTA, pH 7.1 was prepared from a 10X Trypsin-EDTA solution (Gibco, Life Technologies) in PBS and contained of 500 mg/L trypsin and 200 mg/L EDTA.

DMEM
Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Life Technologies) contained 4.5 g/L glucose, 4 mM L-glutamine, 25 mM HEPES and was supplemented with 10% fetal calf serum (FCS) (AusGeneX, Brisbane, Australia).

HUVEC base media
Medium M199 (Sigma Aldrich) supplemented with 20 mM HEPES (Gibco, Life Technologies), 1 mM sodium pyruvate (Gibco, Life Technologies), 1X MEM non-essential amino acids (Gibco, Life Technologies), 1.125 g/L NaHCO$_3$ (Gibco, Life Technologies) 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco, Life Technologies).

HUVEC SF media
HUVEC base media.

HUVEC wash media
HUVEC base media supplemented with 2% (v/v) FCS (HyClone, ThermoFisher, Scoresby, Australia).
HUVEC culture media  HUVEC base media supplemented with 15% (v/v) FCS (Hyclone), 15 µg/mL endothelial cell growth supplement (BD Biosciences, MA, USA) and 15 µg/mL heparin (Sigma Aldrich).

Gelatin  Gelatin coating solution consisted of 17.5 mg/mL gelatin (Sigma Aldrich), 3.75% FCS (Hyclone), 1.56 g/L NaHCO₃, in Hank’s buffered saline solution (Gibco, Life Technologies).

EGM-2  Endothelial Growth Medium 2 (EGM-2) (Lonza, Sydney, Australia) consisted of EBM-2 Basal Medium supplemented with EGM-2 growth factors.

2.2.1.2.  Cell line culture

HEK293T and GP2-293 cells were gifts from Dr. Charles Bailey (Centenary Institute). NIH-3T3 mouse fibroblasts were a gift from Dr. Chris Jolly (Centenary Institute). Cell lines were maintained at 37°C in a 5% CO₂ humidified tissue culture incubator. The cell lines were cultured in DMEM in vented tissue-culture treated flasks (Corning, VIC, Australia) and were routinely passaged by trypsin digest and re-seeded at 1:5 split ratios, three times per week.

2.2.1.3.  HUVEC isolation and culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords of donors at the Royal Prince Alfred Hospital, Sydney according to approved human ethics guidelines. HUVECs were isolated by collagenase digestion as previously described (Coleman et al., 2010). Isolation of HUVECs was performed by Lijun Wang, Elena Zaporoshenko and Lutfun Khan.

HUVECs were routinely split every 4 d or when confluent. Briefly, confluent HUVECs were washed once with PBS-EDTA to remove trypsin-inhibiting factors, and detached by
trypsin-EDTA digestion. The trypsin was inactivated by addition of HUVEC wash media, and cells were collected by centrifugation at 500 x g for 5 min. The cell pellet was resuspended in HUVEC culture media and re-seeded in gelatin-coated vented tissue culture flasks at 1:3 split ratios. HUVECs were routinely used between passages 2-4 for experiments.

2.2.2. **CELL TRANSFECTION FOR GENE OVEREXPRESSION AND KNOCKDOWN**

2.2.2.1. **Transient transfection of cell lines with plasmids**

HEK293T and GP2-293 cell lines were transiently transfected for retroviral production and protein overexpression experiments by lipid transfection. Transfections were performed using Lipofectamine 2000 (Life Technologies) according to manufacturer’s instructions. Briefly, cells were seeded at ~80% confluence overnight in poly-L-lysine (Sigma Aldrich)-coated tissue culture dishes or plates. The tissue culture media was replaced 30 min prior to transfection with fresh DMEM. For 10 cm dishes, 60 µg of Lipofectamine 2000 was first activated in 1.5 mL OptiMEM (Life Technologies) at RT for 5 min. In a separate tube, 24 µg of total DNA was diluted in 1.5 mL OptiMEM. The DNA and Lipofectamine solutions were then mixed and incubated for 20 min for complex formation. The complexes were then added drop-wise to cells. Penicillin and streptomycin was added 4-6 h following transfection to prevent contamination. The media was changed 24 h post-transfection. Transfected cells were routinely used 48-72 h following transfection for protein experiments and retrovirus production. Experiments were upcaled or downscaled using the equivalent ratios of DNA and Lipofectamine. Successful transfection was monitored by the examining EGFP or mCherry expression on a Nikon Eclipse Ti inverted fluorescence microscope (Nikon, NY, USA).

2.2.2.2. **siRNA transfection of HUVECs for gene knockdown**
HUVECs at P2, unless stated otherwise, were seeded at $2.0 \times 10^5$ cells in gelatin-coated 6-well plate wells overnight. Prior to transfection, the cell media was removed, washed once with serum-free HUVEC media and replaced with 1.5 mL EGM-2 media. Lipofectamine:siRNA complexes were formed by mixing 2.5 µL of Lipofectamine RNAiMax (Life Technologies), diluted in 250 µL OptiMEM, and 5 nM of siRNA, diluted in 250 µL OptiMEM, for 10 min. The complexes were added drop-wise to cells and incubated for 4-6 h after which the media was replaced with HUVEC media. Cells were fixed or harvested at 3 d following knockdown, which represented the time point of greatest knockdown effectiveness. For double knockdown experiments ECs were first transfected with ctrl or ARHGAP18 siRNAs for 1 d then transfected with RhoA/C or ctrl siRNAs on the next day and cells used 3 d following RhoA/C siRNA transfection. siRNA sequences used are listed in Table 2.3.

### Table 2.3. siRNA manufacturers and sequences

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### 2.2.3. Adenovirus Transduction for Gene Overexpression

Infectious adenovirus particles were prepared using the Adeasy system, as previously described (Coleman et al., 2010). The adenoviral particles transiently overexpress GFP and ARHGAP18 or empty vector (EV) off separate CMV promoters. For high level transduction (section 3.2.11) HUVECs were seeded overnight at $1.2 \times 10^5$ cells in gelatin-coated 6-well plate wells overnight. The cell media was replaced with HUVEC media containing 2% FCS and adenovirus particles were added at a predetermined MOI for ~70% transduction. After 2 h, the media was supplemented with HUVEC media containing 38% FCS and cultured overnight. The media was replaced completely at 24 h with HUVEC culture media and cells routinely used at 3 d following transduction. For low-level overexpression (section 5.2.5), HUVECs were seeded at $1.8 \times 10^6$ cells in gelatin-coated 10 cm dishes overnight and transduced with adenoviral particles in HUVEC media containing 2% FCS and 8 µg/mL polybrene (hexadimethrine bromide) (Sigma Aldrich) for 2 h. The media was completely changed and cells used 1 d following transduction.

### 2.2.4. Retrovirus Production and Transduction

#### 2.2.4.1. Retrovirus Production

Pantropic retroviruses were used for transduction of HUVECs for ectopic gene expression. Pantropic retroviruses were produced using the GP2-293 system (Clontech). In this system, GP2-293 cells, which stably express the Moloney Murine Leukemia Virus (MMLV) *gag* and *pol* genes, were transfected with the envelope vector, pVSV-G, which
confers pantropism, and a retrovirus expression vector (pMIG or pQCXIN backbones) to encode for genes of interest. GP2-293 cells were seeded at $1 \times 10^7$ cells in 10 cm dishes pre-coated with 0.1% (w/v) poly-L-lysine and allowed to attach overnight. Transfection of GP2-293 cells were performed as mentioned in section 2.2.2.1, using 9.6 µg pVSV-G and 14.4 µg retrovirus expression vectors, for a combined 24 µg. Following 24 h transfection, the cell media was replaced with 7 mL of DMEM media and incubated at 32°C overnight to promote virus stability. At 48 h post-transfection, the live retrovirus was collected by removing the culture supernatant and passing through a 0.45 µm polyvinylidene difluoride (PVDF) filter (MillexHV Millipore, VIC, Australia). The virus was distributed into 1.5 mL aliquots, snap-frozen in dry ice and stored at -80°C until use. The virus was also collected at 72 h post transfection after replacing the media with another 7 ml of media following virus harvest at 48 h. Retrovirus production was up and down-scaled as required.

2.2.4.2. Retroviral titre

To determine the retroviral titre for subsequent transduction experiments in HUVECs, NIH3T3 mouse fibroblasts were transduced with pantropic retroviruses. NIH3T3 cells are easily transduced, and are commonly used as a standard for determining viral titre by EGFP expression (Dolnikov et al., 2003). NIH3T3 cells were seeded at $2.0 \times 10^5$ cells in 6 well plate wells overnight. Retroviruses were diluted in 3.16-fold serial dilutions (half log$_{10}$) in DMEM and polybrene (Sigma Aldrich) added to a final concentration of 4 µg/mL. The cell media was replaced with 1.5 mL of diluted virus and the virus was spinoculated onto the cells by centrifugation at 1,500 rpm (520 x g) for 90 min at RT. Following centrifugation, the cells were returned to 37°C for 30 min, and subsequently media changed. The cells were collected by trypsin detachment at 48 h post-transduction,
fixed and analysed for GFP expression by flow cytometry (see section 2.2.5). The viral titre, green fluorescence units per mL, is represented by the following formula:

\[
\text{Viral titre (GFU/ml)} = \frac{\% \text{ of transduced cells x cell no. at time of transduction}}{\text{volume of virus (ml)}}
\]

This only holds true when the percentage of transduced cells is less than 15%, where it represents a single virus particle per cell (Dolnikov et al., 2003). At higher transduction efficiencies, there is a higher propensity for multiple viruses transducing the same cell, resulting in higher copy numbers. The multiplicity of infection (MOI) for HUVECs was defined as the number of virus particles per cell and is represented by:

\[
\text{MOI} = \frac{\text{GFU}}{\text{no of cells at time of transduction}}
\]

2.2.4.3. Retrovirus transduction of HUVECs

HUVECs were seeded at 1.5 x 10^5 cells in gelatin-coated 6 well plate wells overnight. The following day, the cells were washed once in serum-free HUVEC media to remove residual heparin (a polyanion) and replaced with virus-containing DMEM media with the addition of 8 µg/mL polybrene. Transduction of HUVECs was routinely performed at MOIs between 1-10 and represented at least 80% transduction efficiency. The cells were spinoculated at 1,500 rpm (520 x g) for 90 min at RT, and returned to 37°C for 30 min following centrifugation, after which the media was replaced with HUVEC media. EGFP and mCherry was readily expressed 2 d following transduction, but maximally from 4 d transduction. Cells were either harvested from 2 d transduction or split and kept in culture for other experiments up to 8 d transduction.

2.2.5. Flow cytometry for GFP expression

The measurement of GFP transfected and transduced cells was determined by flow cytometry. GFP-expressing cell lines and ECs were harvested by trypsin detachment and
fixed in 2% formaldehyde (w/v), 0.02% sodium azide (Sigma) in PBS for 30 min. The
cells were washed in PBS and resuspended in 1% (w/v) FCS in PBS in 5 mL round
bottom tubes (BD Biosciences). The samples were analysed on a FACS Canto II flow
cytometer (BD Biosciences) and the data analysed using FlowJo software (version 9.4,
Treestar).

2.2.6. CELL STIMULATION AND TREATMENT

2.2.6.1. Stimulation with growth factors and thrombin

For stimulation with growth factors and thrombin for molecular assays and for cell
imaging, the HUVECs were first starved in HUVEC SF media containing 2% FCS for 2
h. VEGF-A (Sigma) was used at 10 ng/mL. Thrombin (Sigma) was used at 1 U/mL.

2.2.6.2. Treatment with drug inhibitors

Treatments of HUVECs with signaling inhibitors following Ras activation were
performed in normal culture media. U0126 (30 µM), LY294002 (30 µM) and PD98059
(100 µM) were from Sigma and were added to culture media every 2 d.

ROCK inhibitor experiments were performed using the starvation conditions for thrombin
treatment. HUVECs were pretreated with 2.5 µM Y-27632 (Sigma) for 30 min prior to
thrombin stimulation.

2.2.7. CELL PROLIFERATION

Transduced or transfected HUVECs were seeded at 4 x 10^5 cells in 25 cm^2 flasks in
HUVEC media. Cell proliferation was determined at different days by trypsin detachment
of cells, and counting the number of cells in a hemocytometer. The media was replaced
every 3 days during the assay until the end of the experiment.
2.2.8. Cellular senescence by SA-β-gal staining

Cytochemical detection of Senescence-associated β-galactosidase (SA-β-gal) at pH 6.0 was performed according to (Charalambous et al., 2007). Control or HRasV12-transduced ECs cultured in 25 cm² flasks for 5 d were washed twice in PBS then fixed in 2% (w/v) formaldehyde, 0.2% gluteraldehyde in PBS for 15 min at RT. The fixed cells were washed once with PBS, then twice with dH₂O before staining overnight at 37°C with SA-β-gal staining solution consisting of: 5 mM potassium ferrocyanide (Sigma Aldrich), 5 mM potassium ferricyanide (Sigma Aldrich), 2 mM MgCl₂, 0.02% (v/v) NP-40 (Sigma Aldrich), 0.01% (w/v) sodium deoxycholate (Sigma), 40 mM sodium citrate (Sigma), 150 mM NaCl, and 1 mg/ml X-gal (Merck), pH 6.0. Stained cells were washed with dH₂O and imaged on the Nikon Ti Eclipse inverted microscope (Nikon).

2.2.9. Cell migration

Transfected or transduced HUVECs were seeded in triplicate wells at 3 x 10⁵ cells in 6 well plates and cultured for 3 d. Confluent ECs were first washed once with HUVEC wash media then scratched with a cell scraper in a vertical and horizontal direction. The cells were washed twice with HUVEC wash media to remove scratched and semi-detached cells, after which the media was replaced with HUVEC media. The scratches were imaged at 0, 3 and 5 h post-scratch and the scratch area determined by manual outline using Fiji (version 1.48 e, fiji.sc).

2.2.10. Spheroid sprouting

2.2.10.1. Solutions for spheroid sprouting assay

Methylcellulose stock 1.2% (w/v) High viscosity methylcellulose (Sigma Aldrich) in serum-free HUVEC media.
Chapter 2. Materials and Methods

2.2.10.2. Spheroid sprouting assay

The spheroid sprouting assay was performed according to (Korff and Augustin, 1998). Transduced or 1 d siRNA-transfected HUVECs were resuspended in spheroid media and 600 cells seeded into U-bottom 96 well plates (Greiner Bio One, Stonehouse, UK) overnight. The spheroids were harvested using a Pasteur pipette and collected by centrifugation at 500 x g for 3 min. The supernatant was removed, resuspended gently by scratching on a rough surface and overlayed with 500 µL of spheroid resuspension media. The collagen solution was freshly prepared and 500 µL mixed with the spheroids. Of the 1 mL, 800 µL, containing roughly 75 spheroids, were added to a pre-warmed 24-well suspension plate and the gel allowed to set at 37°C for 30 min. The spheroids were then stimulated with 200 µL of HUVEC media containing 125 ng/mL VEGFA or 125 ng/mL FGF-2. FGF-2-stimulated spheroids were cultured for 48 h and re-stimulated at 24 h, while VEGFA spheroids were cultured up to 24 h. Spheroids were imaged at the end-point using the Nikon Ti Eclipse at 100X total magnification. The total number and length of sprouts was determined by manual measurement using Fiji. At least 20 spheroids were analysed per experiment.

2.2.10.3. Spheroid sprouting competition

Spheroid media 20% (v/v) methylcellulose stock, 80% (v/v) HUVEC media, 15 µg/mL endothelial cell growth supplement, 15 µg/mL heparin

Spheroid resuspension media 60% (v/v) methylcellulose stock, 40% (v/v) FCS.

Collagen solution 2 mg/mL rat tail collagen (type I) (BD Biosciences), 1X EBSS, 20 mM NaOH.
A spheroid tip cell competition assay was performed using control and ARHGAP18 siRNA transfected HUVECs to determine the relative potential of each cell type to occupy the sprouting front position. One day control and ARHGAP18 siRNA-transfected HUVECs were stained using 2.5 µm CellTracker Orange CMTMR (5,6-4-chloromethyl-benzoyl-amino-tetramethyl-rhodamine) (Life Technologies) or CellTracker Green CMFDA (5-chloromethyl-fluorescein diacetate) (Life Technologies), and nuclei stained using 3 µg/ml Hoechst 33342 (Life Technologies) for 30 min in HUVEC media containing 5% FCS. The cells were then washed once with wash media, and incubated in culture media for 30 min to allow for excess dye secretion and chloromethyl modification. The cells were harvested by trypsinisation, mixed at 50:50 ratios and used to form spheroid sprouts, which were stimulated with 50 ng/ml VEGFA and FGF-2 to facilitate more pronounced and stable sprouts. The actual distributions of sictrl:siARHGAP18 cells were determined following mixing by imaging on the Nikon Ti Eclipse and determining the relative proportions of green and orange cells. The collagen gels were fixed overnight with 4% formaldehyde at 4°C, transferred to a glass-bottomed MatTek dish (MatTek, MA, USA), washed with H2O and partially air-dried to prevent xy-drift and imaged by confocal. The distribution of each cell population in the spheroid sprouts and at the tip position were determined manually.

2.2.11. CELL PERMEABILITY

The measurement of endothelial permeability was performed by measuring fluorescein isothiocyanate (FITC)-dextran (40 kDa) leak through an endothelial monolayer. ECs transfected with siRNAs for 2 d were seeded at 1.0 x 10^5 cells in triplicate on 3 µm 24-well polycarbonate transwell inserts (Corning) pre-coated with 50 µg/ml fibronectin (BD Biosciences) and allowed to attach overnight. The cells were stimulated with 0.3 U/mL thrombin (Sigma) or left non-stimulated together with 1 mg/mL FITC dextran (40 kDa) in
the upper chamber. At indicated time points, 20 µL was transferred from the lower chamber to a black 96-well plate (BD Biosciences) with 180 µL of serum-free HUVEC media. The samples were excited at 490 nm and read at 520 nm using the POLARstar Omega (BMG LabTech, Mornington, Australia).

2.3. MOLECULAR BIOLOGY

2.3.1. GENE EXPRESSION ANALYSIS BY QUANTITATIVE RT-PCR

2.3.1.1. RNA isolation from cell monolayers

Isolation of RNA from HUVECs was performed using TRIzol (Life Technologies). Cells seeded in 6 well plates were lysed with 500 µL TRIzol reagent at RT for 5 min, and either isolated immediately or stored at -80°C until RNA isolation. Fresh or thawed TRIzol lysates were mixed and 100 µL of chloroform (Sigma Aldrich) was added to the samples and shaken vigorously for 15 s. The samples were let to settle at RT for 2 min prior to centrifuging at 12,000 x g for 15 min at 4°C. The upper aqueous phase of the trizol:chloroform separation was then transferred to a new tube and 250 µL of isopropanol (Sigma Aldrich) added and mixed. The samples were incubated at RT for 10 min prior to centrifuging at 12,000 x g for 10 min at 4°C. The resultant RNA pellet was washed once with 1 mL of 75% (v/v) ethanol (Sigma Aldrich) and centrifuged at 7,500 x g for 5 min at 4°C, air dried and resuspended in 30 µL of nuclease-free H₂O (Sigma Aldrich). The yield and quality of the RNA was determined using the Nanodrop and used immediately for cDNA synthesis. A pure RNA sample had an A₂₆₀/₂₈₀ ratio of 1.8-2.0 and an A₂₆₀/₂₃₀ ratio of approximately 2.0.

2.3.1.2. RNA isolation from spheroid sprouts

The RNA from 96 spheroids was isolated following overnight (18 h) VEGFA sprouting using TRIzol LS, a more concentrated TRIzol reagent suitable for liquid samples.
Following sprouting, the stimulation media was removed, and the remaining 0.8 mL of collagen I gel lysed with 2 mL of TRIzol LS. The samples were pipette mixed for 10 min, transferred to tubes, and centrifuged at 12,000 x g for 10 min at 4°C to clear insoluble collagen and extracellular matrix materials. The RNA-containing supernatant was transferred to a new tube and 266 µL of chloroform added, mixed, settled and centrifuged as per normal monolayer RNA isolation. The aqueous phase was transferred to a new tube and 10 µg of glycogen (Life Technologies) was added as a carrier to facilitate RNA isolation. The RNA and glycogen was precipitated by the addition of 666 µL of isopropanol, incubating at RT for 10 min, before centrifuging at 12,000 x g for 10 min at 4°C. The RNA pellet was washed once with 1 mL of 75% ethanol, centrifuged at 7,500 x g for 5 min at 4°C, air dried and resuspended in 20 µL of nuclease-free H₂O. The RNA was used immediately for cDNA synthesis.

2.3.1.3. **DNase I treatment and cDNA synthesis**

Trizol extracted RNA contains trace levels of genomic DNA (gDNA). In order to prevent the contribution of residual gDNA to mRNA expression results, the samples were first treated with deoxyribonuclease I (DNase I) prior to cDNA synthesis using the high capacity reverse transcription kit (Life Technologies). DNase I treatment of RNA samples was performed using amplification grade DNase I (Sigma Aldrich). Specifically, 1 µg of freshly isolated total RNA was treated with 1 U of DNase I in 1X DNase reaction buffer at RT for 15 min in a final volume of 10 µL in 0.2 ml PCR tubes. The reaction was stopped by the addition of 1 µL of 50 mM EDTA and heating at 70°C for 10 min, before being cooled on ice. A 9 µL mix of cDNA reverse transcription reagents (Life Technologies) consisting of 1X RT buffer, 1X RT random primers, 4 mM dNTP, 1 U RNase inhibitor, and 2.5 U MultiScribe reverse transcriptase was then added for a total volume of 20 µL. Controls of DNase-treated RNA, but no reverse transcriptase enzyme,
were also prepared as a negative control for the downstream PCR. The reactions were run in a BioRad thermocycler according to the conditions listed in Table 2.4.

**Table 2.4. Reverse transcription conditions**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>25°C</td>
<td>10 min</td>
</tr>
<tr>
<td>RT</td>
<td>37°C</td>
<td>120 min</td>
</tr>
<tr>
<td>Terminate</td>
<td>85°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

**2.3.1.4. Quantitative reverse transcriptase PCR (qRT-PCR)**

qRT-PCR was performed on reverse transcribed cDNA samples using SYBR Green JumpStart *Taq* ReadyMix (Sigma Aldrich) to analyse relative gene expression changes. qRT-PCR reactions were performed in 0.1 mL strip tubes (Qiagen) and consisted of 1X SYBR Green Jumpstart Mix, 0.2 µM of forward and reverse primer, and cDNA at the equivalent of 2.5 ng of pre-RT RNA in a final volume of 10 µL. Triplicate reactions were setup and run in a Rotor-Gene 3000 real-time PCR machine (Corbett Research, Qiagen) using a 4-step PCR cycling protocol as listed in Table 2.5.
### Table 2.5. Quantitative RT-PCR thermocycling conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
<th>Acquisition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>15 s</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>40 s</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>40 s</td>
<td></td>
</tr>
<tr>
<td>Non-specific Melt</td>
<td>78°C</td>
<td>15 s</td>
<td>FAM</td>
</tr>
<tr>
<td>Melt Curve Hold</td>
<td>60°C</td>
<td>60 s</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>72-95°C</td>
<td>1°C/5 s</td>
<td>FAM</td>
</tr>
</tbody>
</table>

The fourth PCR step (non-specific melt) is added to denature any non-specific products prior to signal acquisition. The specific amplification of a single PCR product was confirmed by the presence of a single peak in the melt curve and by electrophoresis of DNA amplicons on an agarose gel.

#### 2.3.1.5. qRT-PCR primer design

qRT-PCR primers were designed using Primer3 (version 0.4.0, [http://bioinfo.ut.ee/primer3-0.4.0/](http://bioinfo.ut.ee/primer3-0.4.0/)) (Rozen and Skaletsky, 2000). PCR primers were designed to consist of 20-28 bp oligonucleotides with a melting temperature of 60°C, 50% GC content, and amplicon size of 140-300 bp. The primers used are listed in Table 2.6.
### Table 2.6. Primer sequences for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ARHGAP18</em></td>
<td>F</td>
<td>CGAGCAAGCACTCAATCAGAAGAGAG</td>
<td>273</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GCTGTCAATGGAACGCAAAAAAGACCAG</td>
<td></td>
</tr>
<tr>
<td><em>PECAM1</em></td>
<td>F</td>
<td>CCCAGCCCCAGGATTCTTAGT</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>ACCGCAGGATCTTTGAGTT</td>
<td></td>
</tr>
<tr>
<td><em>CDH5</em></td>
<td>F</td>
<td>CAGCCCAAGGTGTGTGAGAA</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAGCCCAAGGTGTGTGAGAA</td>
<td></td>
</tr>
<tr>
<td><em>ACTA2</em></td>
<td>F</td>
<td>TGACAATGGCTCTGGGTCTGTGAA</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TTCGTCACCCACGACTGTCTCTTT</td>
<td></td>
</tr>
<tr>
<td><em>SNAI1</em></td>
<td>F</td>
<td>ACCCACCATCTCTCTCCTCTG</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TACAAAAAACCACGCAAGA</td>
<td></td>
</tr>
<tr>
<td><em>FLT1</em></td>
<td>F</td>
<td>GGCTCTGTGGAAGTTCAGC</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GCTCACAAGTGTCATCCAAA</td>
<td></td>
</tr>
<tr>
<td><em>FLK1</em></td>
<td>F</td>
<td>GTGACCAACATGGAGTCG</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TGCTTCACAGAAGACCATGC</td>
<td></td>
</tr>
<tr>
<td><em>FLT4</em></td>
<td>F</td>
<td>GAGACAGGAGCAGCGAGAC</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TCACGAACACGTAGAGACTG</td>
<td></td>
</tr>
<tr>
<td><em>DLL4</em></td>
<td>F</td>
<td>AGGCCTGTAAAAAGAACAGAG</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTCCAGCTCACAGCTCCAC</td>
<td></td>
</tr>
<tr>
<td><em>GAPDH</em></td>
<td>F</td>
<td>GAGTCACAGGATTTGATGACCAG</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TTGATTTGGAGGGATCTG</td>
<td></td>
</tr>
<tr>
<td><em>ACTB</em></td>
<td>F</td>
<td>CCCTCCATCGGCCACGCAATGCTTC</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CGACTGCTGTCACCTCCACGCGTCAG</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.3.1.6. Mathematical model for relative gene expression quantification

The cycle threshold (CT), the cycle number at which the sample reached the threshold fluorescence value, was determined following qRT-PCR using the Rotor-Gene software (Corbett, Qiagen). This threshold fluorescence value represented the fluorescence
significantly above the background and that lied in the linear phase of the amplification. The relative gene expression changes were calculated using the $2^{\Delta\Delta CT}$ method (Pfaffl, 2001), where:

$$\text{Relative Gene Expression} = 2^{\Delta\Delta CT}$$

and

$$\Delta\Delta CT = (CT_{GOI} - CT_{HK})_{\text{Sample}} - (CT_{GOI} - CT_{HK})_{\text{Control}}$$

GOI represents the gene of interest and HK represents the housekeeping gene.

β-actin was used as the housekeeping gene. β-actin expression was verified to not change in the treatment conditions used. This expression model assumes the perfect amplification of the genes of interest and the housekeeping gene, and is valid only when the efficiencies of the genes of interest and housekeeping gene are similar. The efficiencies of the amplicons were determined by serial dilution of template and were, on average, around 1.8. While the genes did not have perfect amplification the net effect on actual fold changes are insignificant, and more importantly, the trend of up- or down-regulation are maintained, hence this method was valid to use for calculating expression changes.

### 2.3.2. Protein expression analysis by immunoblotting

#### 2.3.2.1. Immunoblotting solutions

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>50 mM Tris HCl, 150 mM NaCl, 2 mM EGTA, 1% NP40, pH 7.6.</td>
</tr>
<tr>
<td>IP lysis buffer</td>
<td>10 mM NaH$_2$PO$_4$, 150 mM NaCl, 1 mM EDTA, 0.5% (w/v) CHAPs, pH 7.2.</td>
</tr>
<tr>
<td>MOPS running buffer</td>
<td>50 mM MOPS, 50mM Tris Base, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.7.</td>
</tr>
<tr>
<td>MES running buffer</td>
<td>50 mM MES, 50 mM Tris Base, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.3.</td>
</tr>
</tbody>
</table>
Transfer buffer  
25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, 20% (v/v) Methanol, 1 mL NuPAGE anti-oxidant pH 7.2.

Blocking buffer  
5% (w/v) BSA or skim milk, 0.1% (v/v) Tween-20 in PBS.

Wash buffer  
0.1% (v/v) Tween-20 in PBS.

2.3.2.2. Cell lysis

Monolayer cells for protein harvesting were first washed twice with cold PBS. The PBS was aspirated and 80 µL (6 well plate well) or 500 µL (10 cm dish) of lysis buffer or IP lysis buffer containing 1X protease inhibitor cocktail (Sigma Aldrich) and 1X PhosSTOP phosphatase inhibitor cocktail (Roche) added to the cells. The cells were incubated on ice for 10 min and then scraped with a cell scraper. The cell lysate was transferred to a 1.5 mL tube and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to a new 1.5 ml tube and stored at -80°C until gel electrophoresis.

2.3.2.3. Protein preparation and SDS-PAGE

The protein concentration of whole cell lysates was determined using the Detergent Compatible Protein Assay (Bio-Rad) according to manufacturer’s instructions. The sample was read at 690 nm against a serially diluted BSA standard curve in the microplate reader (BioRad). Whole cell lysates were prepared at 1.0 mg/mL in 1X NuPAGE LDS sample buffer (Life Technologies) and 1X NuPAGE sample reducing agent (Life Technologies) and heated at 70°C for 10 min. Ten µg of protein samples were electrophoresed in NuPAGE Novex 4-12% Bis-Tris gels in MOPS or MES (for low molecular weight proteins) buffers containing 0.5 mL of NuPAGE antioxidant (Life Technologies) at 150 V until the dye front reached the end of the gel. The Precision Plus Protein Kaleidoscope standard was electrophoresed in conjunction with protein samples as a protein size marker.
2.3.2.4. **Protein transfer**

Electrophoresed proteins were transferred onto PVDF membranes (Millipore) by wet transfer using the XCell II blot module (Life Technologies). The PVDF membrane was activated in 100% methanol for 30 s, and equilibrated in transfer buffer for 2-3 min. Filter paper (Whatman, Dassel, Germany) and sponges (Life Technologies) were pre-soaked in transfer buffer. The gels were removed from the cassettes following electrophoresis, washed in transfer buffer and assembled into a stack and transferred at 30 V for 75 min.

2.3.2.5. **Blocking, antibody incubation and detection**

Following protein transfer, the membranes were removed from the assembly and blocked in blocking buffer for 1 h at RT with rocking. The membranes were washed once with washing buffer before incubation in primary antibody (prepared in wash buffer or BSA blocking buffer) overnight at 4°C with rotation. Primary antibodies and dilutions used are listed in Table 2.7. After overnight incubation, the membranes were washed 5 times for 5 min with washing buffer. The membranes were subsequently incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at RT, after which they were washed 5 times for 5 min. The HRP was detected using Enhanced Chemiluminescence (ECL) substrate (Thermo Fisher) or ECL Plus substrate (Thermo Fisher) with 1 min or 5 min pre-incubation, respectively, on the ChemiDoc MP gel imaging system (Bio-Rad).
### Table 2.7. Antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species and Clonality</th>
<th>Manufacturer</th>
<th>Concentration/Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARHGAP18</td>
<td>Mouse mAb (2A3)</td>
<td>In house</td>
<td>0.5 µg/mL</td>
</tr>
<tr>
<td>ARHGAP18</td>
<td>Rabbit pAb</td>
<td>In house</td>
<td>1:400</td>
</tr>
<tr>
<td>Pan-Ras</td>
<td>Rabbit pAb</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Rabbit pAb</td>
<td>Abcam</td>
<td>1:500</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Rabbit pAb</td>
<td>Abcam</td>
<td>1:500</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>Goat pAb</td>
<td>Santa Cruz</td>
<td>1:500</td>
</tr>
<tr>
<td>Snail</td>
<td>Rabbit mAb (C15D3)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-Erk1/2 (T202/Y204)</td>
<td>Mouse mAb (E10)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-Akt (S473)</td>
<td>Rabbit mAb (D9E)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>Akt</td>
<td>Mouse mAb (40D4)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>pMLC (S19)</td>
<td>Rabbit pAb</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>pAkt Substrate (RXXS<em>T</em>)</td>
<td>Rabbit mAb (110B73E)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>RhoA</td>
<td>Rabbit mAb (67B9)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>RhoC</td>
<td>Rabbit mAb (D40E4)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rac1</td>
<td>Mouse mAb</td>
<td>Cytoskeleton</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Mouse mAb</td>
<td>Cytoskeleton</td>
<td>1:1000</td>
</tr>
<tr>
<td>RhoJ</td>
<td>Mouse mAb (1E4)</td>
<td>Abnova</td>
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<tr>
<td>Ezrin/Radixin/Moesin</td>
<td>Rabbit pAb</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>Moesin</td>
<td>Rabbit pAb</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cortactin</td>
<td>Rabbit pAb</td>
<td>Cell Signaling</td>
<td>1:1000</td>
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<td>DsRed</td>
<td>Rabbit pAb</td>
<td>Clontech</td>
<td>1:1000</td>
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<tr>
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</tr>
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<td>Nucleolin</td>
<td>Rabbit pAb</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Mouse mAb (B-5-1-2)</td>
<td>Sigma Aldrich</td>
<td>1:1000</td>
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<tr>
<td>β-Actin</td>
<td>Rabbit pAb</td>
<td>Sigma Aldrich</td>
<td>1:2000</td>
</tr>
<tr>
<td>Rabbit IgG-HRP conjugated</td>
<td>Goat pAb</td>
<td>Cell Signaling</td>
<td>1:4000</td>
</tr>
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</table>
### Antigen

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species and Clonality</th>
<th>Manufacturer</th>
<th>Concentration/Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgG-HRP conjugated</td>
<td>Goat pAb</td>
<td>Cell Signaling</td>
<td>1:4000</td>
</tr>
<tr>
<td>Goat IgG-HRP conjugated</td>
<td>Donkey pAb</td>
<td>Santa Cruz</td>
<td>1:4000</td>
</tr>
</tbody>
</table>

#### 2.3.3. IMMUNOPRECIPITATION

**IP lysis buffer**  
0.5% (w/v) CHAPs, 10 mM NaH$_2$PO$_4$, 150 mM NaCl, 1 mM EDTA, pH 7.2.

**IP wash buffer**  
250 mM NaCl, 0.5% (v/v) Triton X-100, 5 mM NaF, 1 mM Na$_3$VO$_4$, 1mM Na$_4$P$_2$O$_7$.

**Elution buffer**  
1.5X NuPAGE LDS sample buffer, 1.5X NuPAGE sample reducing agent.

Confluent HUVECs cultured on 10 cm dishes were lysed with 600 µL of IP lysis buffer and cleared as per the immunoblotting procedure. The concentrations of the samples were determined and equalized to a concentration between 0.8-1.0 mg/mL. The proteins (400-500 µg) were precleared with 50 µL of 50% protein G sepharose beads (Life Technologies) on a MACSmix rotator (Miltenyi Biotec) at 4°C for 1 h. The beads were collected at 10,000 x g for 30 s and the supernatant transferred to another tube. Ten µg of ARHGAP18 (2A3) or MsIgG1 isotype control antibodies were then added and incubated overnight on the rotator at 4°C. The protein G sepharose beads were blocked with 2% (w/v) BSA/PBS for 2 h at 4°C, restored to a 50% slurry and 20 µL added to each sample for 2 h at 4°C. The samples were collected at 10,000 x g for 30 s, the unbound fractions were collected and the beads were washed with IP wash buffer 5 times. On the final wash, the samples were resuspended in 1 mL PBS, transferred to a new tube and collected by
centrifugation. The samples were eluted with 20 µL of elution buffer, heated at 70°C for 10 min, centrifuged and the supernatant collected for SDS-PAGE and immunoblotting.

2.3.4. IMMUNOFLUORESCENCE STAINING

2.3.4.1. Immunostaining of monolayer cells

Transfected, transduced or normal HUVECs were seeded overnight at 6 x 10⁴ cells on 8-well Lab-Tek chamber slides (Thermo Scientific) pre-coated with 50 µg/ml fibronectin (BD Biosciences). Basal or stimulated HUVECs were washed once with serum-free HUVEC media, prior to fixation with 4% methanol-free formaldehyde in PBS for 10 min at RT or with 50% (v/v) methanol, 50% (v/v) acetone at -20°C for 20 min, depending on the antigen. The cells were washed with PBS then permeabilised with 0.1% (v/v) Triton X-100 for 10 min at RT. The cells were then blocked with 2% (w/v) BSA (Sigma Aldrich) in PBS for 30 min at RT, then incubated in diluted primary antibody, prepared in 2% (w/v) BSA in PBS, for 1 h at RT. The unbound antibodies were washed 5 times for 2 min each with PBS, then incubated in Alexa Fluor-conjugated secondary antibodies (Molecular Probes, Life Technologies), prepared in 2% (w/v) BSA in PBS for 30 min at RT. A list of primary and secondary antibodies and the dilutions used are in Table 2.8. The secondary antibodies were washed off as per the primary antibodies. Where applicable, formaldehyde-fixed cells were stained with 0.2 U rhodamine phalloidin for 20 min at RT. The nuclei were counterstained with 200 ng/mL 4’,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich) for 10 min. The chamber slides were disassembled, and mounted with ProLong Gold antifade reagent (Life Technologies) using Nr 1 glass coverslips (Menzel-Glaser, Braunschweig, Germany) Immunostained slides were imaged by confocal microscopy.

Table 2.8. Antibodies used for immunofluorescence staining
2.3.4.2. Immunostaining of spheroid sprouts

Following sprouting induced by VEGFA or FGF-2, spheroid sprouts washed twice with PBS. The gels were fixed with 4% formaldehyde in PBS at RT for 2 h then overnight at 4°C. The gels were washed with PBS to remove the formaldehyde, then extracted with 100% methanol overnight at -20°C. The methanol was washed out with PBS and blocked.
with 1% (v/v) Triton X-100, 3% (w/v) BSA, 3% (v/v) NGS for 2 d at 4°C. The gels were then incubated in primary antibody diluted in the blocking buffer for 2 d at 4°C. The antibodies are listed in Table 2.9. The unbound antibodies were washed 8 times with 1% Triton-X in PBS for 1 h each. The gels were then incubated in Alexa Fluor-conjugated secondary antibodies, diluted in blocking buffer, for 2 d at 4°C, then washed as per the primary antibody. The nuclei were counterstained with 500 ng/mL DAPI for 30 min, then washed in PBS for 4 times 30 min each, dH2O for 4 times 30 min each and air-dried overnight on a StarFrost microscope slide (Waldemar Knittel, Germany) in a fume hood. The dried gels were mounted with glass coverslips with ProLong Gold Antifade reagent. Immunostained spheroid sprouts were imaged by confocal microscopy.

Table 2.9. Antibodies used for immunofluorescence staining of spheroid sprouts

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species and Clonality</th>
<th>Manufacturer</th>
<th>Concentration/Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARHGAP18</td>
<td>Mouse mAb (2A3)</td>
<td>In house</td>
<td>10 µg/mL</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Rabbit pAb</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>Rabbit mAb (D87F2)</td>
<td>Cell Signaling</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit IgG-AlexaFluor</td>
<td>Goat pAb</td>
<td>Life Technologies</td>
<td>1:500</td>
</tr>
<tr>
<td>488 conjugated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse IgG-AlexaFluor</td>
<td>Goat pAb</td>
<td>Life Technologies</td>
<td>1:500</td>
</tr>
<tr>
<td>594 conjugated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse IgG-AlexaFluor</td>
<td>Goat pAb</td>
<td>Life Technologies</td>
<td>1:500</td>
</tr>
<tr>
<td>647 conjugated</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3.5. **RhoGTPase activity assay**

The measurement of active RhoGTPase activity was performed using the GLISA kits (Cytoskeleton) according to manufacturers instructions. Transfected or transduced ECs were cultured to confluence in 10 cm dishes. The starved and stimulated or non-stimulated cells were quickly washed twice with cold PBS and lysed with 600 µL GLISA lysis buffer containing protease inhibitors for 1 min. The cells were scraped, collected and centrifuged at 10,000 x g at 4°C for 1 min. The cleared cell lysate was transferred and
distributed into aliquots, which were then snap-frozen in liquid nitrogen. Each aliquot was used to measure a single RhoGTPase and was not reused. The protein concentration was determined using the Dc Protein Assay kit and equalized between samples. The protein samples (25-50 µg) were added to the immobilised RhoGTPase protein binding wells and incubated on ice with shaking at 320 rpm for 30 min. The wells were washed to remove unbound samples, the antigens were retrieved and then incubated with primary antibodies against the RhoGTPases at RT with shaking at 320 rpm for 45 min. The primary antibodies for RhoA, Rac1 and Cdc42 were supplied with the GLISA kits. A RhoC antibody (Cell Signaling) was used with the RhoA GLISA kit to measure active RhoC and was used at a 1:100 dilution. A RhoJ antibody (Abnova) was used with the Cdc42 GLISA kit to measure active RhoJ and was used at a 1:100 dilution. The unbound primary antibodies were washed and the HRP-conjugated secondary antibodies were added and incubated at RT with shaking for 45 min. The secondary antibodies were washed and the HRP detection substrate was added for 10-15 min at 37°C after which the reaction was stopped. The absorbance at 490 nm was read in a POLARstar OMEGA plate reader. The signals were corrected against a blank sample and the readings were normalized against the total RhoGTPase levels as determined by immunoblotting.

2.4. MOUSE STUDIES

2.4.1. MOUSE LINES

C57BL/6J mice were purchased from the Animal resources centre (WA, Australia). The ARHGAP18<sup>−/−</sup> mouse was generated through the Knockout mouse project (KOMP) repository (CA, USA). The generation and features of the mouse is described in section 4.2.5. All mouse experiments were performed in accordance to animal ethics guidelines from the University of Sydney and Sydney local health district.
2.4.2. **Aortic ring sprouting assay**

The ex vivo aortic ring sprouting assay was performed on 6-week old mice as described (Baker et al., 2012). The aortas were isolated from the mice and washed with PBS. The aortas were cut into 1 mm rings, placed in 96-well plates containing 100 µL Matrigel (BD Biosciences) and covered with an additional 100 µL Matrigel. The aortas were cultured for up to 7 d and imaged by brightfield microscopy.

2.4.3. **Post-natal retina vascularisation model**

The analysis of post-natal retina vascularisation was performed according to (Pitulescu et al., 2010).

2.4.3.1. **Solutions for retina staining**

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA blocking buffer</td>
<td>1% (w/v) BSA, 0.3% (v/v) Triton-X in PBS</td>
</tr>
<tr>
<td>Serum blocking buffer</td>
<td>3% (v/v) NGS, 1% (w/v) BSA, 0.3% (v/v) Triton-X in PBS</td>
</tr>
<tr>
<td>Mouse on mouse blocking buffer</td>
<td>2X Mouse Ig blocking reagent (Vector Laboratories), 3% (v/v) NGS, 1% (w/v) BSA, 0.3% (v/v) Triton-X in PBS</td>
</tr>
<tr>
<td>PBLEC</td>
<td>1 mM MgCl2, 1 mM CaCl2, 0.1 mM MnCl2, 1% Triton-X in PBS</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>0.3% Triton-X in PBS</td>
</tr>
</tbody>
</table>

2.4.3.2. **Eye and retina dissection**

*WT* and *ARHGAP18*/*-/-* pups were euthanased on P6 by decapitation. The eyes were collected by surgical incision around the eye and severing the optic nerve. The eyes were transferred to cold PBS then fixed in 4% (w/v) formaldehyde for 2 h on ice. The fixative was washed out and replaced with PBS then dissected to remove the retina. Firstly, the
extra fat was removed from the exterior of the eye, and a small incision was made on the cornea-sclera junction of the eye. The cornea was subsequently removed by extending the incision across the junction. Next, the sclera and choroid layers were removed using fine forceps. The lens was extracted out and the iris and the hyaloid vessels were removed, leaving the retina. Each retina was cut into quadrants approximately two-thirds of the length of the retina and kept in PBS until staining.

2.4.3.3. Isolectin B4 staining of retinal vessels

Retinas for isolectin B4 (IB4) staining were first re-fixed with 4% (w/v) formaldehyde for 1 h at RT. The extra post-dissection fixation preserves the filipodia staining of retinal sprouts while preventing overfixation of hyaloid vessels onto the retina (Pitulescu et al., 2010). The retinas were washed with PBS then blocked overnight at 4°C BSA blocking buffer. The blocked retinas were equilibrated in PBLEC for 1 h then incubated with 10 µg/ml biotinylated Griffonia (Bandeiraea) Simplicifolia lectin I isolectin B4 (Vector Labs) in PBLEC overnight at 4°C. The retinas were washed 6 times for 10 min each with wash buffer then incubated in 10 µg/ml streptavidin-Alexa Fluor 594 or Alexa Fluor 405 (Molecular Probes, Invitrogen) for 2 h at RT. The stained retinas were washed 6 times for 10 min each with wash buffer, then 2 times for 5 min with PBS prior to mounting.

2.4.3.4. Antibody staining of retinas

Dissected retinas for antibody staining were blocked overnight with serum blocking buffer overnight at 4°C with rocking. The blocked retinas were incubated with primary antibodies prepared in serum blocking buffer overnight at 4°C. A list of antibodies and dilutions used is listed in Table 2.10. The retinas were washed as for IB4 staining of retinas, then incubated in Alexa Fluor dye-conjugated goat anti-mouse or -rabbit secondary antibodies (1:500) prepared in serum blocking buffer for 2 h at RT. The retinas were washed as per IB4 staining following secondary antibody incubation.
2.4.3.5. **ARHGAP18 staining of retinas**

ARHGAP18 staining of mouse retinas was performed with the mouse monoclonal antibodies against ARHGAP18. The dissected retinas were blocked with mouse on mouse blocking reagent overnight at 4°C with rocking. The retinas were washed twice with serum blocking buffer then incubated with anti-ARHGAP18 and co-staining antibodies prepared in serum blocking buffer overnight at 4°C (Table 2.10). The retinas were washed as for IB4 staining of retinas and incubated with the goat anti-mouse Alexa Fluor 647-conjugated F(ab’)2 antibodies (at 1:500 dilution) (Molecular Probes, Invitrogen) and goat anti-rabbit Alexa-Fluor 488-conjugated secondary antibodies prepared in serum blocking buffer for 2 h at RT. The retinas were washed as per IB4 staining following secondary antibody incubation.

**Table 2.10. Antibodies used for immunofluorescence staining of retinas**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species and Clonality</th>
<th>Manufacturer</th>
<th>Concentration/Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARHGAP18</td>
<td>Mouse mAb (2A3)</td>
<td>In house</td>
<td>20 µg/mL</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>Rat mAb (11D4.1)</td>
<td>BD Biosciences</td>
<td>1:400</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>Rabbit pAb</td>
<td>Abcam</td>
<td>1:200</td>
</tr>
<tr>
<td>NG2</td>
<td>Rabbit</td>
<td>Millipore</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Rabbit</td>
<td>Abd Serotec</td>
<td>1:200</td>
</tr>
</tbody>
</table>

2.4.3.6. **Mounting of stained retinas**

The stained retinas were transferred using 2 ml transfer pipettes to StarFrost glass slides and orientated with the inner retina facing the coverslip. The excess PBS was removed and the retinas were flat-mounted using Nr. 1 glass coverslips (Menzel-Glaser) and ProLong Gold anti-fade mounting media. The stained retinas were imaged by stereo and confocal microscopy.
2.4.4. Croton oil-induced ear inflammation and neutrophil infiltration

Ear inflammation was induced in WT and ARHGAP18−/− mice using the irritant, croton oil. Croton oil was dissolved in acetone at concentration of 2.5% (v/v) and 20 µL of croton oil applied topically on the left and right ear. The acetone vehicle control was applied to separate control animals. The ear thickness was measured using a micrometer. At 24 h, the cells were isolated for characterization by flow cytometry. The ears were dissected and split into the dorsal and ventral halves using forceps. The tissues were digested using 2 mg/mL collagenase type IV (Sigma Aldrich) in PBS for 1 h at 37°C. The digestion was stopped by the addition of FACS buffer (5% FCS, 2 mM EDTA, 0.02% sodium azide in PBS). Single cell suspensions were obtained by filtering through a 80 µm stainless steel mesh and the cell numbers determined by haemocytometer counting. For flow cytometric analysis of neutrophils, the cells were incubated with fluorochrome-conjugated antibodies (BD Biosciences) against CD45 (30-F11), Ly6G (1A8), and CD11b (M1/70) for 30 min on ice. The cells were washed three times with FACS buffer and resuspended in 300 µL of FACS buffer. Immediately prior to flow cytometric acquisition, DAPI was added as a viability marker. The samples were acquired on the LSR Fortessa (BD) and the data analysed using FlowJo.

2.5. Imaging and image analysis

2.5.1. Microscopes and objectives

Table 2.11. List of microscopes and objectives used.

<table>
<thead>
<tr>
<th>Imaging</th>
<th>Microscope</th>
<th>Objective</th>
<th>Immersion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightfield &amp; Widefield Fluorescence</td>
<td>Nikon Eclipse Ti</td>
<td>Nikon PL FL 4x/0.13 NA</td>
<td>Air</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nikon PL FL 10x/0.30 NA</td>
<td>Air</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nikon PL FL 20x/0.45 NA</td>
<td>Air</td>
</tr>
</tbody>
</table>
### Imaging Materials and Methods

#### 2.5.2. Image Analysis

**2.5.2.1. Cell circularity and cell size**

Analysis of cell circularity and size as parameters of cell morphology were performed on brightfield images using Fiji. Outlines of cells were traced manually and the area and perimeter determined. The size of the cell corresponds to the measured area and was converted to µm$^2$ units. The circularity of the cell, a measure of the elongation/roundness of the cell, is determined by the formula: $4 \times \pi \times \text{area} / \text{perimeter}^2$. A circularity value of 1.0 indicates a perfect circle while values approaching 0.0 indicates an increasingly elongated polygon. For measurement of these parameters, the results of a minimum of three independent experiments consisting of at least 20 individual cells were pooled.

**2.5.2.2. Cell density measurement by counting of cell nuclei**

Analysis of cell density was performed on DAPI-stained cells cultured on coverslips for immunostaining. Using Fiji, a macro was programmed to count the number of nuclei in a field of view. The macro was designed to convert the image to black and white, distinguished the nuclei from the background, blurred the image, set a threshold intensity and counted the particles above a certain size. The macro is detailed in Appendix A.1.1.

**2.5.2.3. Aortic ring sprouting**

The magnitude of aortic ring sprouting was defined as the sprouted area (SA). This was determined using Fiji by manually tracing the total area (TA) of the sprouted aortic ring and subtracting the ring area (RA). The SA was then corrected for the size of the aortic
ring by measuring the surface area (perimeter) of the ring (RP). This is also given by the formula: Corrected sprouted area = (TA - RA) / RP. Since each experiment had different rates of sprouting, the measurements were corrected to each experimental WT control.

2.5.2.4. Analysis of retina vascularization

The radial extension, the longitudinal distance to which the vessels had migrated to, was determined using whole retina images acquired on the stereo microscope by measuring the distance from the optic nerve to the angiogenic front.

For the other parameters of retinal vascularization, confocal images of whole retina quadrants were analysed by Wimasis GmbH (www.wimasis.com/, Germany) using a skeleton analysis program. As described in section 4.2.7, the analysis of the whole retina did not show noticeable differences in the vascular phenotype, which may be due to the complex architecture of the retina or the different sizes of the retina quadrants. In order to eliminate these irregularities, the analysis was redefined to a smaller area located in between the two large vessels (veins and arteries). A macro was programmed to execute the analysis in an automated and unbiased manner. This analysis is described in Fig 4.1 and the macro is listed in Appendix A.1.2. The macro utilised the original analysed image, and selected a 250 pixel (380 µm) x 250 pixel square, which was re-analysed for the individual parameters by colour thresholding. The area of the vessel outline (black) was determined as a percentage of the whole area and defined as the vessel density. The branch points were isolated into single (red dots), double (green dots) and triple (blue dots) branches which were individually counted and totaled.

2.5.2.5. Pericyte coverage

Analysis of pericyte coverage was determined post-acquisition on VE-cadherin and NG2 stained retinas and is described in Fig 4.17. A macro was programmed for the automated
analysis of pericyte coverage, as defined as the percentage of vessels that are covered by pericytes and is listed in Appendix A.1.3. VE-cadherin stains the junctions of ECs so the vessel outline was determined by filling the VE-cadherin outlines to define the vessels. A binary pericyte mask was created by thresholding the pericyte signal and an overlay of the pericyte mask (green) and the vessel mask (red) was created. The non-pericyte covered area was indicated by the red areas, while the pericyte covered vessels were the yellow areas, and the pericyte areas not associated with vessels as the green areas.

2.6. **Statistical analysis**

Statistical significance was analysed using Prism (v6.0d, Graphpad Software. For comparison of two groups, t-test were used. For multiple comparisons, analysis of variance (ANOVA) was used. A $P$-value less than 0.05 was considered significant.
CHAPTER 3

ARHGAP18 IN HRas-INDUCED ANGIOGENESIS
3.1. INTRODUCTION

One of the major regulators of cell signaling and function are the Ras GTPases. Aberrant signaling of the Ras pathway is one of the most common causes of cancer (Bos, 1989; Downward, 2003; Pylayeva-Gupta et al., 2011). Physiologically, the Ras proteins act in response to receptor tyrosine kinase (RTK) stimulation by GF to control fundamental cellular processes such as proliferation and migration (Pylayeva-Gupta et al., 2011; Rajalingam et al., 2007). Ras, like the Rho GTPases, cycle between active GTP-bound and inactive GDP-bound forms through interaction with GEFs and GAPs, respectively (Pylayeva-Gupta et al., 2011). Following GF binding, the RTK activate and recruit GEFs, which subsequently interact with Ras proteins and facilitate the exchange of GDP for GTP (Chardin et al., 1993; Karnoub and Weinberg, 2008). The active form of Ras then binds to effectors including Raf kinase and phosphatidylinositol 3-kinase (PI3K), which signal to downstream pathways such as the MAPK-ERK and Akt pathways, respectively (Rajalingam et al., 2007). Interaction with GAP proteins then facilitates the hydrolysis of GTP and a return to the inactive GDP-bound state.

The Ras family consists of 39 Ras proteins, of which the functions of three proteins, HRas, KRas and NRas have been the most well studied (Karnoub and Weinberg, 2008). Of the three major Ras proteins, only KRas is essential, as knockout of KRas, but not HRas or NRas, results in embryonic lethality (Esteban et al., 2001; Johnson et al., 1997; Koera et al., 1997; Umanoff et al., 1995). However $HRas^{+/} NRas^{+/-}$ double knockout animals have reduced Mendelian ratios while $KRas^{+/-} NRas^{+/-}$ animals are embryonic lethal, which suggests that there is partial overlap in function between proteins (Esteban et al., 2001; Johnson et al., 1997). Furthermore, mice that have had the KRas gene replaced with HRas display normal embryonic development indicating that the KRas promoter-
driven expression of the Ras protein, rather than the actual specific Ras isoform, is essential during development (Potenza et al., 2005).

Aberrant signaling of the Ras pathway is common in many disorders and involves several different aspects of the pathway. These include mutations of the Ras proteins, deletion of the GAP proteins, sustained GFR activation, and amplification or mutation of the downstream effectors (Downward, 2003). Mutations of the Ras proto-oncogenes are very common in cancers, comprising approximately 30% of all tumours (Bos, 1989). Notably, certain tumours are associated with mutations in specific Ras proteins, as for instance, KRas is mutated in 60% of pancreatic cancers compared to HRas and NRas which are mutated in 0% and 2% of cases respectively (Karnoub and Weinberg, 2008). This association has been largely attributed to the preferential expression of the specific Ras isoforms in the different organs (Leon et al., 1987). Most oncogenic mutations of Ras target its inactivation by GAPs (Pylayeva-Gupta et al., 2011). In particular, the substitutions of the Gly residues at positions 12 or 13 (most frequently with Val or Asp) prevent the formation of van der Waals bonds between Ras and the GAP through steric hindrance (Scheffzek et al., 1997), while mutations of Gln61 prevent GTP hydrolysis (Buhrman et al., 2010). These mutations result in persistence of the GTP-bound form and hyper-activation of the downstream effector pathways leading to increased cell proliferation, transformation, survival, migration and metastasis, metabolism and induction of angiogenesis, all of which are hallmarks of cancer (Hanahan and Weinberg, 2000; Pylayeva-Gupta et al., 2011). Therapeutically, this has resulted in the development of molecules to target aberrant Ras signaling in the treatment of cancers (Downward, 2003).

Aberrant Ras signaling is also common in vascular diseases. Mutations in Ras have been identified in angiosarcomas, an uncommon malignant neoplasm arising from endothelial
cells (Boivin-Angele et al., 2000; Przygodzki et al., 1997; Weihrauch et al., 2002; Wen and MacKenzie, 2013). Additionally, mice lacking the Ras GAP, RASA1, develop defects in the vascular system (Henkemeyer et al., 1995). In humans, mutations in RASA1 are associated with arteriovenous malformations with complications including bleeding and heart failure (Boon et al., 2005; Eerola et al., 2003; Revencu et al., 2008). Furthermore, deficiency of another Ras GAP, NF1, results in cardiovascular defects in mice and humans (Brannan et al., 1994; Friedman et al., 2002; Gitler et al., 2003).

In ECs, Ras functions as an essential signaling molecule downstream of growth factor pathways including VEGFA and FGF-2 (Meadows et al., 2001; Rennel et al., 2003). In particular, HRas, which is the highest expressing isoform in ECs (Bajaj et al., 2010; Haeussler et al., 2013), is required for proliferation, migration and tube formation following VEGFA activation, as introduction of a dominant negative HRas (HRasN17) inhibits such phenotypes (Meadows et al., 2001). During sprouting angiogenesis, ERK, a downstream molecule of Ras-MAPK signaling, is highly activated at the angiogenic front while absent from the mature quiescent vessels (Westenskow et al., 2013). Conversely, the negatively regulator of Ras signaling, RASA1 is absent at the angiogenic front while highly expressed in the mature vessels, suggesting that the regulation of RASA1 expression is the critical factor in controlling Ras signaling during angiogenesis (Westenskow et al., 2013). Overexpression of constitutively active HRas (HRasV12) in ECs has been shown to promote tube formation, migration and proliferation (Meadows et al., 2004) and induce angiosarcoma formation when introduced into mice (Arbiser et al., 1997; Rennel et al., 2003). However, other groups have conflicting studies that show that overexpression of active HRas results in an inhibition of invasion (Rennel et al., 2003), induction of cellular senescence (Spyridopoulos et al., 2002) and abnormal vascular morphogenesis (Bajaj et al., 2010).
Given this controversy, the project was initially aimed at investigating whether active HRas overexpression invoked an oncogene-induced senescence response in ECs. Further, given the established role of ARHGAP18 in oxidative-stress induced senescence, the project was focused at determining whether there was a role of ARHGAP18 in the regulation of HRas-induced senescence. However, the early results indicated that HRas induced a dualistic phenotype of OIS in addition to pro-angiogenic EC phenotype. Furthermore, this coincided with a dramatic downregulation of ARHGAP18. Hence the aim of this chapter was to characterize the phenotype induced by active HRas overexpression and to determine whether there was a role of ARHGAP18 in the regulation of this phenotype.
3.2. RESULTS AND DISCUSSION

3.2.1. OVEREXPRESSION OF ACTIVE HRas IN ECs INDUCES A DOSE-DEPENDENT TRANSFORMED-LIKE AND SENESCENT-LIKE CELL APPEARANCE

In order to determine the phenotype induced by Ras activation, a retroviral vector overexpressing constitutively active HRas (G12V mutation, subsequently denoted as HRasV12) was used to transduce HUVECs. HRas was the chosen isoform as it is the most abundant isoform in ECs (Bajaj et al., 2010; Haeussler et al., 2013). To produce the retroviruses GP2-293 cells, which stably express the Murine Moloney Leukemia Virus (MMLV) gag and pol genes, were transfected with the pMIG or pMIG-HRasV12 retrovirus expression vectors in combination with the VSV-G envelope plasmid. These retroviruses, which encode for HRasV12 and GFP from a bicistronic mRNA or GFP alone, were titred by transducing NIH3T3 cells, and determining the number of GFP positive cells by flow cytometry (See section. 2.2.4.2) (Dolnikov et al., 2003).

To determine the effect of HRasV12 overexpression on ECs, HUVECs were transduced at two different doses of HRasV12 retrovirus, which represented a multiplicity of infection (MOI) of 10 and 1, while control cells were transduced with the pMIG retrovirus at an MOI of 3. At these doses the ECs were efficiently transduced with over 80% of control and 90% of HRasV12 cells expressing GFP (Fig 3.1 A and B). The GFP intensity levels indicated that the cells were indeed expressing at different levels and this is likely to be due to additional copies of the transgene integrating into the genome (Fig 3.1 B and C).

This was confirmed by the expression of the HRas transgene, and was accompanied with hyper-activation of the Ras signaling pathway, as evident by the increased phosphorylation of ERK1/2 and Akt (Fig 3.1 D, see also Fig 3.11 A). Notably however, there was no further activation of Akt at the higher dose. Surprisingly, ARHGAP18 was
markedly downregulated following HRasV12 overexpression regardless of the dose of virus used (Fig 3.1 D, see also Fig 3.11 A). This is unexpected as it was hypothesized that since ARHGAP18 is upregulated and crucial during oxidative stress-induced senescence, there would be a similar function of ARHGAP18 in OIS. Therefore, in order to establish the function of ARHGAP18 in the context of chronic Ras activation, it was first imperative to first ascertain the HRas phenotype.

Transduction of the ECs with the HRasV12 retroviruses resulted in two very distinct and different phenotypes: a senescent-like (SL) phenotype at the higher dose, and a transformed-like (TL) cell appearance at the lower (Fig 3.1 A). These SL cells had a large flattened morphology, increased vacuolation and polyploidy, which are all characteristics of senescent cells (Campisi and d’Adda di Fagagna, 2007). On the other hand, at the lower dose, the TL cells did not have the typical cobblestone phenotype of confluent HUVECs but had increased cell density, irregular cell shapes, and extensive cell protrusions that resemble the phenotype of fibroblasts.

3.2.2. ECs overexpressing HRasV12 at high levels are senescent

To confirm that the SL phenotype was indeed cellular senescence and not another cell phenotype such as cell spreading, the cells were stained for the senescence marker, senescence-associated β-galactosidase (SA-β-gal). In this assay, the activity of β-galactosidase is measured at pH 6.0, a suboptimal pH for β-galactosidase staining, with positive SA-β-gal staining resulting from an increase in lysosomal-mass and β-galactosidase in senescent cells (Kurz et al., 2000; Lee et al., 2006). HRasV12SL cells exhibited strong SA-β-gal staining, while control and HRasV12TL cells had little to no staining. Quantification of the number of senescent cells based on SA-β-gal staining and cell morphology showed a significant difference between the HRasV12SL dose and the control and HRasV12TL dose, confirming that the level of HRasV12 overexpression
induces a dose-dependent senescence response (Fig 3.2 B). However, although senescent cells were present, most cells (90%) transduced at the SL dose were not senescent, but had the typical TL phenotype.

This is likely due to two reasons. Firstly, although the cells were transduced at a high dose, most cells were likely to integrate only a single copy of the transgene, rather than the multiple copies needed for senescence. Secondly, the senescence response induces cell-cycle arrest, while the non-senescent cells continue to proliferate and gradually overtake the population. Such a dose-dependent response to oncogene activation in mediating cell transformation and senescence has been described previously (Sarkisian et al., 2007). While oncogene activation promotes proliferation and tumourigenesis, further oncogene hyper-activation results in hyper-proliferation and DNA hyper-replication resulting in accumulation of DNA damage and a p53 driven senescence response (Di Micco et al., 2006; Sarkisian et al., 2007; Serrano et al., 1997). This dose-dependent response to HRas activation is likely to be the cause of the conflicting phenotypes seen by other groups (Meadows et al., 2004; Spyridopoulos et al., 2002). Notably, the (Spyridopoulos et al., 2002) study utilized an adenoviral vector, which routinely overexpress proteins to extremely high levels (100-1000 fold) and thus is likely to account for the HRas-mediated cellular senescence observed.

Although the senescence phenotype was interesting, the remainder of the chapter is devoted to TL phenotype and the role of ARHGAP18 in this phenotype as: 1. Transduction with the TL dose reproducibly resulted in a near pure population of HRasV12TL cells, whereas isolating the HRasV12SL cells from the HRasV12TL cells for experiments was difficult, and 2. There was no difference in the regulation of ARHGAP18 between the HRasV12TL and the HRasV12SL ECs (also see section 3.2.9).
3.2.3. **The HRasV12TL Cells Are Smaller, More Elongated, Exhibit Long Filopodial Extensions and Have Profound Changes in the Cytoskeleton**

The HRasV12\textsuperscript{TL} cells had increased elongation of cells coupled with long filopodial extensions (Fig 3.3 A top panels). Additionally, the cells were substantially smaller and had a higher cell density. In order to quantify the changes in cell morphology, the cell size and circularity, a measure of the elongation/roundness (0.0 being a straight line, 1.0 a perfect circle), was assessed (Fig 3.3 A, bottom panels and 3.3 B). The HRasV12\textsuperscript{TL} cells were ~35% smaller in size, and significantly more elongated.

Given the gross changes in the cell morphology, the organization of the cytoskeleton was next assessed. The cytoskeleton is composed of three main kinds of cytoskeletal filaments: actin filaments, intermediate filaments and microtubules, which collectively regulate cell shape, structure and function, such as in migration and division (Fletcher and Mullins, 2010; Rodriguez et al., 2003). To visualize the cytoskeleton, the cells were stained for F-actin (actin filaments), vimentin (intermediate filaments) and tubulin (microtubules) (Fig 3.4 A). The arrangements of all of the filaments in the HRasV12\textsuperscript{TL} cells were distinctly different from normal cells with the evident localization of microtubules and intermediate filaments in the long protrusive extensions of HRasV12\textsuperscript{TL} cells. Furthermore, the arrangement of the actin microfilaments were profoundly different; in control cells, the actin cytoskeleton was organized into predominantly cortical actin fibres surrounding cell junctions and the occasional actin stress fibres running across the cells. On the other hand, the HRasV12\textsuperscript{TL} had a disorganized actin structure with large actin clusters, a distinct lack of cortical actin and prominent membrane ruffles.
3.2.4. **The HRasV12\textsuperscript{TL} phenotype is pro-migratory and pro-sprouting**

Since the long filipodial extensions and membrane ruffles are a characteristic of motile cells (Le Clainche and Carlier, 2008), the migratory ability of the HRasV12\textsuperscript{TL} cells was next assessed (Fig 3.5 A and B). Using a 2D wound healing assay, the HRasV12\textsuperscript{TL} had a ~50% increase in cell migration. This was accompanied with increased spreading of the leading migratory cell, consistent with the prominent membrane ruffles.

To further confirm this phenotype, the ability of HRasV12\textsuperscript{TL} to form 3D endothelial spheroid sprouts was investigated. The 3D model represents a more physiological model of angiogenesis in which cells are maintained in a tight quiescent cell mass and only sprout in response to the presence of GF (Korff and Augustin, 1998). In this model, HUVECs were transduced with control or HRasV12 then the monolayer of cells harvested and resuspended in the viscous methylcellulose media to form a 600-cell spheroid (Fig 3.6 A). These spheroids were collected and embedded in a collagen I gel, where the cells remain quiescent until stimulated with GF to induce endothelial sprouting (Fig 3.6 C vs E). Even prior to collagen I embedding, overexpression of HRasV12 resulted in striking difference in the spheroid morphology with an overall increased looseness with the cells in the spheroid, and the formation of cell blebs (Fig 3.6 B). The HRasV12\textsuperscript{TL} cells had extensive sprouting in the absence of stimulation, while control cells did not sprout at all (Fig 3.6 C and D). The morphology of EC sprouts vary with the different stimuli, with VEGF\textsubscript{A} induced sprouts more numerous, but less stable sprouts (as indicated by the number of detached cells) with many filopodia, while FGF-2 stimulation results in less numerous sprouts that have long multi-cellular and stable EC sprouts (Fig 3.6 E). HRasV12 overexpression resulted in sprouting phenotype that was more reminiscent of VEGF-induced spheroids, rather than by FGF-2. The migratory and sprouting phenotype is consistent with one other study where HRasV12 overexpression
resulted in increased membrane ruffle formation and transwell migration (Meadows et al., 2004).

### 3.2.5. **HRasV12 does not alter cell proliferation but promotes bypass of contact-mediated growth inhibition**

One other major function of Ras and its downstream signaling is in cell proliferation (Bos, 1989; Rajalingam et al., 2007). To next determine whether HRasV12 overexpression was affecting EC proliferation, control and HRasV12 transduced ECs were cultured for up to 5 days and the cell numbers determined at days 1, 3 and 5 (Fig 3.7 A). Both cell populations exhibited a growth lag phase initially after 1 d of cell seeding and reached close to confluence by the third day, but the cell proliferation was indifferent. The rate of cell growth was also measured in other experiments at 2 d, during the exponential growth phase, but was found to be unchanged (data not shown). This is in contrast to other studies that have shown an increase in EC proliferation in response to active HRas (Bajaj et al., 2010; Meadows et al., 2001; Rennel et al., 2003). However, these studies were performed in suboptimal growth medium suggesting HRas activation permits the bypass of growth factor receptor activation. We did not examine the growth of HRas$^{TL}$ cells in these conditions. On the other hand, we observed that HRas activation permits the bypass of cell contact inhibition. While the control cells grew minimally after reaching confluence, the HRasV12 transduced ECs had an increased ability to further increase in cell numbers (Fig 3.7 A). To quantify the changes in cell density, the cells were grown for 2 d following confluence, DAPI stained for cell nuclei, and the number of cells determined by counting the nuclei (Fig 3.7 B). The HRasV12$^{TL}$ cells had a ~40% increase in density compared to control cells. This suggests that rather than undergoing quiescence with G0-G1 arrest following cell confluence, the HRasV12$^{TL}$ cells bypass this arrest and continue the cell cycle. Further analysis of the cell cycle and the cell cycle
proteins is subject to future studies, but has demonstrated by others to be attributed to the increase in cyclin D1 (Meadows et al., 2004).

3.2.6. **The HRasV12\textsuperscript{TL} Cells Have Vastly Altered Cell-Cell Junctions**

The EC junctions are essential in maintaining cell-cell contacts and regulating cell contact inhibition by controlling GF signaling (Grazia Lampugnani et al., 2003). In order to determine whether the result of the bypass of contact inhibition was due to altered EC junctions, control and HRasV12\textsuperscript{TL} cells were stained for the junctional proteins PECAM-1, β-catenin and VE-cadherin (Fig 3.8 A). Indeed, while control cells had typical junctional localization of these proteins, their localization in the HRasV12\textsuperscript{TL} cells were severely disorganized. PECAM-1 was predominantly absent in HRasV12\textsuperscript{TL} cells. The adherens junctions proteins, VE-cadherin and β-catenin appeared to be downregulated and were largely expressed in the cell protrusions and lacked the distinct junctional distribution.

3.2.7. **Overexpression of HRasV12 Induces a Partial EndMT**

We next hypothesized whether this may be the result of an endothelial-mesenchymal transition (EndMT). EndMT is a specialized form of epithelial-mesenchymal transition (EMT) that occurs in ECs, whereby EC identity is lost and the cells acquire a mesenchymal phenotype (Kovacic et al., 2012). EndMT is crucial in the development of the cardiac system in the formation of the heart valve and septum (Kovacic et al., 2012). EndMT, however is also a main contributor to many diseases, including cardiac fibrosis (Zeisberg et al., 2007b), CCM (Maddaluno et al., 2013), and cancer (Potenta et al., 2008), where EndMT accounts for 40% of cancer-associated fibroblasts (Zeisberg et al., 2007a). EndMT is characterized by loss of EC-EC junctions, acquisition of an invasive and migratory phenotype, the loss of endothelial markers (PECAM-1, VE-cadherin) and gain
in mesenchymal markers (α smooth muscle cell actin (α-SMA), fibroblast specific protein-1 (FSP1)), and is driven by the increased expression of the Snail family of transcription factors (Snail, Slug, Twist) (Potenta et al., 2008).

In agreement with the immunostaining, the HRasV12TL cells had a marked reduction in PECAM-1 at the protein (Fig 3.9 A) and the mRNA level (Fig 3.9 B), although not a complete loss of PECAM-1 as seen by others (Medici et al., 2010). However, the total expression of VE-cadherin protein and mRNA were unchanged. Furthermore, the expression of the mesenchymal marker, α-SMA was absent at the protein level, although interestingly upregulated at the mRNA level. HeLa lysates were run as the positive control for α-SMA. This lack of the mesenchymal phenotype was confirmed by the absence of FSP1 (data not shown). This partial EndMT was confirmed with the upregulation of Snail at both the protein and mRNA level. Snail is a key initial regulator of EndMT as it drives the loss of cell-cell junctions by mediating VE-cadherin downregulation (Potenta et al., 2008). Other family members of the Snail family, including Slug and Twist were unchanged (data not shown). Overall, this suggested that HRasV12TL cells were undergoing a partial EndMT with incomplete loss of endothelial and an absence of mesenchymal identities. EndMT, however, is a progressive process that involves the gradual loss and gain of the respective identities (Zeisberg et al., 2007a). One potential explanation was that the HRasV12TL cells were undergoing a transition phase at the time assessed. To address this, the HRasV12TL cells were cultured for up to 20 d, but there was no change in the phenotype compared to 5 d transduced cells (data not shown) suggesting that the timing was not the cause of the phenotype.

Another explanation could be that the intermediate transition phase required another signal to trigger complete EndMT. One of the most crucial signaling pathways controlling EMT and EndMT is TGF-β signaling (Chen et al., 2012; Cooley et al., 2014; Kalluri and
Weinberg, 2009; Kumarswamy et al., 2012). TGF-β is abundantly expressed in the microenvironment during injury and in tumours and is thought to be the main signaling mechanism to drive EndMT (Potenta et al., 2008). It has been previously reported that treatment of ECs including human coronary endothelial cells (Zeisberg et al., 2007b), mouse lung endothelial cells (Zeisberg et al., 2007a), and HUVECs (Cooley et al., 2014) (Kitao et al., 2009) induces EndMT, with the characteristic loss of endothelial and gain of mesenchymal identity. In contrast to the other studies, TGF-β1 or TGF-β2 treatment did not induce a morphological change in either control or the HRasV12TL cells nor did the stimulation result in any changes in the expression of the EndMT genes (data not shown). This was despite multiple time, dose and media optimization experiments. We concluded that this is likely to be due to the source of ECs used, where the freshly isolated primary HUVECs were responding differently to ECs used in other studies. Indeed in reviewing the literature, most if not all EC seemed to be either long term passaged or transformed EC lines. Interestingly, it has also been shown that FGF signaling attenuates TGF-β signaling to retain endothelial identity (Chen et al., 2012), and a difference in the inherit FGF signaling may be another explanation for the differences between the cells. The response of HRasV12 and TGF-β signaling in EndMT is subject to further study. Overall, however this establishes that HRasV12TL cells are undergoing a partial EndMT, and that the expression of the EndMT genes and proteins are suitable markers for examining the function of ARHGAP18 in the context of HRas activation.

3.2.8. **The HRasV12TL Phenotype is Dependent on the Raf/MAPK/ERK Pathway and Not the Akt Pathway**

We next sought to determine the influence of the downstream Ras signaling pathways on the HRasV12TL phenotype. Ras activation has been shown to regulate a multitude of downstream signaling pathways including the Raf/MEK/ERK, Akt, JNK and the
p38MAPK pathways to control cell phenotypes including proliferation, migration, cell survival (Meadows et al., 2004).

To determine which signaling pathway was important for the HRasV12TL phenotype, control and HRasV12 transduced HUVECs were treated with MEK (U0126 and PD98059) and PI3K (LY294002) inhibitors. Overexpression of HRasV12 resulted in the activation of ERK and Akt, and treatment with the inhibitors restored basal ERK (Fig 3.10 A) and Akt (Fig 3.10 B) activity. We observed that the inhibition of the MAPK/ERK pathway reverted the elongated phenotype to a more cobblestone-like phenotype (Fig 3.10 C) and is quantified by the restoration of cell circularity (Fig 3.10 D). On the other hand, treatment with the PI3K inhibitor did not alter the elongated phenotype and also resulted in increased cell death, which is in agreement with the function of the Akt pathway in mediating cell survival (Kennedy et al., 1999). Inhibition of the MAPK-ERK pathway also resulted in a decrease in spheroid sprouting (Fig 3.10 E and F).

These results are in agreement and in conflict with several similar studies. By also overexpressing HRasV12, (Meadows et al., 2004) demonstrated that Ras activation leads to an altered cell morphology, increased tube formation, and migration. The migratory ability was dependent on both ERK and Akt activation. Further, (Serban et al., 2008) utilized additional mutations of HRas that elicit PI3K (HRasV12C40) or ERK (HRasV12S35) only activation and demonstrated that both of these pathways promote angiogenesis. In contrast, (Bajaj et al., 2010) found that proliferation was ERK dependent, and HRas activation results in abnormal vascular morphogenesis that was dependent on Akt and not ERK. While we dismissed the functional effects of Akt based on its lack of effect on cell morphology, these other studies indicate that it would be of interest to determine its actual effects on EC sprouting. Furthermore, the influences of the other downstream pathways such as JNK and p38MAPK remain to be elucidated.
3.2.9. **The RhoGAP, ARHGAP18, is downregulated following HRasV12 overexpression**

The major focus of the project and thesis involves the functional characterization of the relatively novel protein ARHGAP18. The Vascular Biology laboratory at the Centenary Institute previously identified a role of ARHGAP18 in oxidative stress-induced senescence (Coleman et al., 2010). ARHGAP18 is upregulated following hydrogen peroxide-induced senescence and overexpression of ARHGAP18 induces cellular senescence in ECs. Therefore the initial hypothesis was that ARHGAP18 is also upregulated and is crucial in HRas-driven OIS. Unexpectedly, and as described above, both the HRasV12 SL and HRasV12 TL cells downregulated the levels of ARHGAP18, indicating that overexpression of HRasV12 results in a senescence-independent downregulation of ARHGAP18 (Fig 3.11 A). However, transduction at the SL dose of virus also results in a high amount of HRasV12 TL cells (Fig 3.2 B). To delineate these different responses, the HRasV12 SL cells were purified by partial trypsin digestion, recultured and assessed for ARHGAP18 expression. The expression of ARHGAP18 was found to be indifferent in this population compared to the HRasV12 SL and HRasV12 TL cells (data not shown). To further confirm this, the expression and localization of ARHGAP18 was assessed by IF staining. Optimisation and confirmation of this staining is described in section 5.2.1. While the localization of ARHGAP18 in the control and HRasV12 transduced HUVECs was predominantly in the cytosol, the expression of ARHGAP18 was markedly reduced in the HRasV12 transduced ECs, in agreement with the immunoblots (Fig 3.11 B). Notably, the expression of ARHGAP18 was further reduced in the HRasV12 SL cells (white arrows) in comparison to the HRasV12 TL cells. As for the reasons described earlier, the rest of this chapter focused on determining the role of ARHGAP18 in the HRasV12 TL phenotype.
To first characterize the downregulation of ARHGAP18 following HRasV12 overexpression, the protein and mRNA levels were assessed at early (2 d), mid (5 d) and late (8 d) timepoints, which respectively represent the time points prior to, during and following the transition to the TL phenotype (Fig 3.12 A). At the protein level ARHGAP18 was significantly downregulated at the mid and late time points (Fig 3.12 B and C). Notably at the mRNA level, the expression of ARHGAP18 in control cells was significantly upregulated from 2 to 5 and 8 d, which correlates with an increase in cell density to confluence (Fig 3.12 C). On the other hand, transduction with HRasV12 resulted in a time-dependent downregulation of ARHGAP18, and an extensive downregulation when compared to the control at the same time point.

3.2.10. Downregulation of ARHGAP18 by HRasV12 overexpression is dependent on MAPK/ERK activation

We next sought to determine which of the Ras activating pathways were regulating the downregulation of ARHGAP18. HUVECs were transduced with HRasV12 expressing retroviruses for 2 d then treated with U0126 or LY294002 and the expression of ARHGAP18 was determined by immunoblotting (Fig 3.13 A). While vehicle and LY294002 treated HRasV12 overexpressing HUVECs both resulted in the downregulation of ARHGAP18, treatment with U0126 resulted in substantially more ARHGAP18 suggesting that activation of the MAPK/ERK, but not the Akt pathway, controlled ARHGAP18 downregulation. However, inhibition of the MAPK/ERK pathway only partially prevented the downregulation of ARHGAP18. We next sought to determine whether prolonged MAPK/ERK inhibition could restore ARHGAP18 to baseline levels. To investigate this, HUVECs were transduced with HRasV12 retrovirus for 2 d then treated with U0126 every second day and harvested 3, 5 and 7 d post-transduction. While vehicle treated HRasV12-transduced HUVECs displayed a continuous downregulation of
ARHGAP18, HRasV12 transduced HUVECs treated with U0126 had similar levels of ARHGAP18 following 1, 3 and 5 d of U0126 treatment (Fig 3.13 B). This suggested that MAPK/ERK inhibition did not restore ARHGAP18 levels to basal control levels, but only inhibited the downregulation, hence resulting in a constant level of ARHGAP18. The reason for the partial downregulation of ARHGAP18 is postulated to be due to the time of U0126 treatment following HRasV12; the initial 2 d MAPK/ERK signaling triggers ARHGAP18 downregulation to the intermediate levels, which following U0126 treatment remains constant during the whole time course. This may have been circumvented by earlier addition of U0126, but it is important to note that successful retrovirus transduction and expression requires active proliferating cells, and that treatment with U0126 suppresses cell proliferation. Regardless, this suggested that HRasV12 overexpression triggers the downregulation of ARHGAP18. Furthermore, like the HRasV12TL phenotype, this downregulation was dependent on the activation of the MAPK/ERK and not the PI3K/Akt pathway. On the contrary however, while MAPK/ERK inhibition restored the control cell phenotype, ARHGAP18 expression could only be maintained and not restored to basal levels. However, the simultaneous regulation of ARHGAP18 and the TL phenotype by MAPK/ERK suggested that ARHGAP18 itself might have a role in regulating the HRasV12TL phenotype. In order to address this, we sought to determine the effects of rescuing the expression of ARHGAP18, by overexpression of ARHGAP18, in HRasV12 transduced ECs.

3.2.11. Retroviral overexpression of ARHGAP18 does not induce EC senescence

The overexpression of ARHGAP18 was previously reported to induce EC senescence (Coleman et al., 2010). This was mediated using an adenoviral vector overexpressing GFP and ARHGAP18 off two separate CMV promoters. HUVECs transduced with control or
ARHGAP18 adenoviral vectors indeed induced EC senescence as indicated by the large flattened morphology (Fig 3.14 A). The control cells, however, had a spread phenotype that is distinctly different to NT control cells. Immunoblots confirmed ARHGAP18 overexpression and was in the magnitude of 100-1000 fold levels (Fig 3.14 B). There are several disadvantages of the adenovirus expression system (Warnock et al., 2006). Firstly, cell transduction results in episomal adenoviral DNA viral particles, which dilute following cell division and hence results in a transient overexpression. Secondly, transduction with adenoviruses results in a varied expression between cells with populations of cells not transduced and other cells expressing varying levels of the transgenes. Finally, adenoviral transduction results in many copies of the episomal particles, which leads to the extremely high levels of overexpression seen (Fig 3.14 B). Given that the HRasV12 phenotype develops over several days, this transient and inconsistent system was deemed to be inappropriate for rescue studies. It is to be noted also that plasmid transfection into HUVECs is very toxic, inefficient and inconsistent. As the ECs described above were successfully transduced with HRasV12 and the control retroviruses, a retroviral system was designed to overexpress ARHGAP18.

The pMIG retroviral vector, as used for HRasV12 overexpression, was first utilized to overexpress ARHGAP18. Human full-length ARHGAP18 was subcloned from the pcDNA3 vector into the pMIG vector to express ARHGAP18 and GFP from a bicistronic transcript under the control of an MSCV promoter. Transduction of HUVECs with pMIG-ARHGAP18 resulted in poor transduction efficiency (~30-50% efficiency), despite repeated attempts to concentrate the retroviral particles (Fig 3.14 C). Immunoblotting for ARHGAP18 indicated that there was a ~2 fold overexpression of ARHGAP18 (Fig 3.14 D). Importantly, the transduced ECs had an indifferent phenotype to control cells, the normal cobblestone morphology as opposed to the senescence phenotype induced by
adenoviral overexpression. This lack of senescence may be due a few reasons: firstly, the low level of overexpression of ARHGAP18 in the retroviral system compared to the adenoviral system or secondly, a combination of the non-specific effects of the adenovirus itself with the high levels of ARHGAP18 overexpression. The additive effects of ARHGAP18 and the adenovirus on EC senescence are not disputed though.

Overexpression of ARHGAP18 with the pMIG backbone brought upon a few problems: 1. low level overexpression, 2. low transduction efficiency, and 3. GFP expression, which would not distinguish between cells overexpressing HRasV12, ARHGAP18 or both transgenes. Therefore, in order to address all of these problems, the pQCXIN backbone was utilized to overexpress ARHGAP18. The pQCXIN backbone encompasses a self-inactivating retroviral vector to express ARHGAP18 and the neomycin resistance gene in a bicistronic transcript off a CMV promoter. This theoretically allows for higher expression off the stronger CMV promoter, but also allows for the selection of transduced cells by G418 (Geneticin). Furthermore to address the GFP issue, a separate vector denoted pQCXIN-mCherry-ARHGAP18 was designed to express the red fluorophore, mCherry. To do this, mCherry was first cloned into pQCXIN and ARHGAP18 was subcloned immediately downstream of the mCherry to produce an N-terminal mCherry-ARHGAP18 fusion protein vector (details of this are listed in the methods section 2.1.12).

An N-terminal GFP-ARHGAP18 fusion protein was previously used in the Vascular Biology laboratory and shown to be functionally similar to non-tagged ARHGAP18. Transduction with pQCXIN-mCherry-ARHGAP18, unlike the control mCherry vector, resulted in a predominantly cytosolic expression of mCherry (Fig 3.14 E) that was typical of ARHGAP18 localisation in normal cells (see Fig 3.11 B and Fig 5.1), but there was no notable alteration in cell phenotype. Confirmation of overexpression by immunoblotting, however, revealed that while there was ectopic overexpression of fusion ARHGAP18,
these were in the form of two distinct protein products ~93 kDa and ~88 kDa in sizes (Fig 3.14 F). Immunoblotting for mCherry by a DsRed antibody suggested that the smaller of the two was in fact the mCherry-fusion protein. However, the presence of the larger protein could not be explained and therefore the construct was not used for rescue experiments, as any potential effects could not be attributed to one form or the other. This vector however was useful for preliminary experiments investigating the changes in ARHGAP18 localisation following stimulation with thrombin (section 5.2.4).

3.2.12. Restoration of ARHGAP18 does not alter the HRasV12TL phenotype

Overexpression of ARHGAP18 was eventually performed using the pQCXIN-ARHGAP18 retrovirus vector, to express non-tagged ARHGAP18 and the neomycin resistance gene. HUVECs were transduced with pQCXIN-ARHGAP18 or empty vector control and selected for 1 week with G418. Non-transduced (negative control) and pQCXIN-mCherry (positive control) transduced ECs were performed concurrently to monitor G418 efficacy and transduction efficiency following selection and was routinely >90%. Transduction with pQCXIN-ARHGAP18 resulted in overexpression of ARHGAP18 (Fig 3.15 C, lane 1 vs 3) that was more consistent than the pMIG vector. However, like the pMIG vector, there was no indication of cellular senescence (Fig 3.15 A, panel 1 vs 3). This further indicates that the AdvARHGAP18 senescence phenotype is likely due to a combined effect of ARHGAP18 overexpression with the non-specific adenoviral effects.

To finally determine whether ARHGAP18 restoration could rescue the effects of HRasV12, the control or pQCXIN-ARHGAP18 transduced cells were further transduced with pMIG-HRasV12 or a pMIG control vector and assessed for changes in the cell phenotype and analysed for the EndMT phenotype. Contrary to the hypothesis,
ARHGAP18 overexpression did not alter the phenotype induced by HRasV12 with the presence of the typical dense morphology with extensive cell protrusions (Fig 3.15 A). These changes were quantified by measurement of the cell circularity and were found to be indifferent (Fig 3.14 B). Overexpression of ARHGAP18 and rescue of ARHGAP18 in the presence of HRasV12 expression was confirmed by immunoblotting (3.14 C, top panel) and qRT-PCR (Fig 3.14 D, top panel). HRas overexpression reduced endogenous levels of ARHGAP18 in the pQCXIN-ARHGAP18 transduced ECs resulting in ARHGAP18 levels similar to that of the control ECs.

While there was no change in the gross phenotype, we next sought to assess whether ARHGAP18 was regulating the development of the partial EndMT phenotype by assessing the markers at the protein (Fig 3.14 C) and mRNA level (3.14 D). In the absence of HRasV12, ARHGAP18 overexpression did not alter the expression of any of the aforementioned EndMT markers. Furthermore, while HRasV12 overexpression induces the downregulation of PECAM-1 (protein and mRNA), alteration of VE-cadherin, and upregulation of α-SMA (mRNA) and Snail (protein and mRNA), restoration of ARHGAP18 did not alter the expression of these markers. This convincingly concluded that ARHGAP18 did not have a direct role in regulating the HRasV12 phenotype. In retrospect, given the role of ARHGAP18 in EC sprouting (chapter 4), an ideal functional assay to assess the contribution of ARHGAP18 in the HRas phenotype would be the spheroid sprouting assay. This was not performed due to the timing of the experiments (the ARHGAP18 rescue experiments were performed much prior to establishing the spheroid sprouting assay, which was predominantly used for assessing ARHGAP18 knockdown in chapter 4).
3.3. **Summary and Conclusions**

Ras is one of the major signaling pathways downstream of growth factor receptor signaling, including that of VEGF. Here, we describe that in agreement with some other studies (Meadows et al., 2001; Meadows et al., 2004; Serban et al., 2008), chronic HRas activation induces a pro-angiogenic phenotype in ECs. This is accompanied with a drastic alteration in the cell morphology, with profound changes in the cell cytoskeleton and EC junctions, and elicits a partial EndMT, with semi-loss of endothelial status and incomplete gain of the mesenchymal phenotype. The gross phenotype is dependent on chronic activation of the MAPK/ERK and not the PI3K/Akt pathway. Finally, we observe that the expression of the novel protein, ARHGAP18, is drastically downregulated also via a MAPK/ERK dependent pathway, however, restoration of ARHGAP18 does not influence the HRas phenotype directly (Fig 3.16).

As there was no clear indicator of ARHGAP18 as a crucial regulator of the drastic HRasV12<sub>TL</sub> phenotype, the project was left in this preliminary state. Since we have further established roles of ARHGAP18 in regulating EC sprouting (chapter 4) and junctional regulation (chapter 5), it would now be of future interest to determine whether ARHGAP18 restoration could affect these functional aspects of HRas. In addition, we established that ARHGAP18 acts as a RhoC GAP protein in its regulation of EC junctions (chapter 5). The RhoGTPases are the major regulators of cell morphology and the cytoskeleton and there is substantial cross-talk between the Ras and Rho pathways (Bar-Sagi and Hall, 2000; Sahai et al., 2001). A major future direction would be investigating the roles of the different Rho proteins in regulating the HRasV12<sub>TL</sub> phenotype. In the context of ARHGAP18, it would be very interesting to determine whether there are elevated levels of RhoC in the HRasV12<sub>TL</sub> cells and whether there is a direct role of ARHGAP18 in regulating this. We would predict that, regardless of the changes in
activity in the Rho proteins, there would be a minimal role of ARHGAP18 in regulating this due to the substantial redundancy within the RhoGAP family members. It would be interesting to analyse the expression of the other RhoGAPs and RhoGEFs to determine whether there is a collective response in the regulation of Rho activity or whether this downregulation of ARHGAP18 is a specific phenomenon.

It would also be of future interest, in the context of ARHGAP18 and Ras, to investigate the effects of chronic activation of the downstream pathways, such as using membrane tagged-Akt (myr-Akt) and mutated BRAF, or mutated Ras transgenes that only signal through one of the pathways (Serban et al., 2008). Further, the JNK and p38MAPK are also associated with Ras activation so investigating these would collectively assist in delineating the pathways that regulate the HRasV12TL phenotype and ARHGAP18 expression. Additionally, the results hinted that Ras overexpression directly affects the transcription of ARHGAP18, so further investigations into this regulation by ARHGAP18 promoter analysis and the identification of the associated transcription factors may give further insight into the function of ARHGAP18.

Aberrant signaling of the Ras signaling pathway is common in many vascular diseases, including angiosarcomas and vascular malformations. A detailed understanding of the regulators and downstream pathways can therefore potentially lead to the development of novel targeted therapeutics. Additionally, chronic activating mutations of Ras and its downstream signaling proteins, such as BRAF, occurs in a majority of many cancers (Brose et al., 2002; Davies et al., 2002). It would therefore be of major significance to determine whether there is similar regulation of ARHGAP18 in other epithelial cells and whether there is a crucial role of ARHGAP18 in regulating Ras-mediated cancer development.
Chapter 3. ARHGAP18 in HRas-induced Angiogenesis

CHAPTER 3.
ARHGAP18 in RAS-INDUCED ANGIOGENESIS

FIGURES
Chapter 3. ARHGAP18 in HRas-induced Angiogenesis

A

Brightfield

GFP

B

NT  pMIG  HRasV12\textsuperscript{TL}  HRasV12\textsuperscript{SL}

Cells (%) vs. GFP

C

Normalized Mean Fluorescence Intensity

D

Control  HRasV12\textsuperscript{TL}  HRasV12\textsuperscript{SL}

Pan-Ras  21 kDa

p-ERK1/2  44 & 42 kDa

p-Akt  60 kDa

ARHGAP18  78 kDa

Actin  42 kDa
Fig 3.1. Overexpression of constitutively active HRas in ECs induces a dose-dependent transformed-like and senescent-like cell appearance.

A. Brightfield and GFP widefield images of non-transduced (NT), pMIG retrovirus-transduced, and HRasV12 retrovirus-transduced HUVECs following 6 d transduction. Cells were transduced with two different MOI of HRasV12 viruses that induced a senescent-like morphology (HRasV12SL) and a transformed-like cell appearance (HRasV12TL). Bar 100 μm.

B. Flow cytometric analysis of GFP expression from NT (grey), pMIG (black), HRasV12TL (blue) and HRasV12SL transduced HUVECs. Representative of n=3 experiments.

C. Quantification of the mean fluorescence intensity (MFI) of the GFP expression of HUVECs transduced in B. Data was normalised to the pMIG control and represents the mean ± SEM from n=3 experiments. * P<0.05; one-way ANOVA, Tukey’s post-test.

D. Immunoblots of Ras, downstream phosphorylated kinases (p-ERK and p-Akt) and ARHGAP18 from HUVECs transduced with pMIG or RasV12 retroviruses for 5 d. Actin was used as a loading control. Representative blots of n=3 experiments.
Fig 3.2. HUVECs overexpressing HRasV12 at SL MOI are senescent.
A. SA-β-gal staining of NT, pMIG and HRasV12TL and HRasV12SL HUVECs. Senescent cells exhibit strong β-galactosidase activity as evidenced by intense blue staining. Bar 100 μm.
B. Quantification of senescent cells in NT, pMIG and HRasV12TL and HRasV12SL transduced HUVECs. Senescent cells were classified based on the large flattened morphology, polyploidy, increased vacuolation and positive staining for SA-β-gal and expressed as a percentage of the total population. Data represents the mean ± SEM from n=4 experiments. * P<0.05, ** P< 0.01; one-way ANOVA, Tukey’s post-test.
Fig 3.3. The transformed-like cells of HRasV12TL overexpression are smaller, more elongated and exhibit long filopodial extensions.

A. Top panels, High magnification of control and HRasV12TL ECs showing the altered cell morphology. Active HRas overexpression results in a reduced cell size, an increase in cell elongation and the appearance of long filopodial extensions (arrow). Bottom panels, manual outlines of the cells (yellow) were performed in Fiji to quantitate the changes in morphology. Bar 50 μm.

B. Quantification of the cell size and cell circularity from control and HRasV12TL ECs. Data represents the mean ± SEM from n=3 experiments. * P<0.05, ** P<0.01; t-test.
Fig 3.4. The HRasV12-induced elongated cell phenotype is associated with profound alterations in the cell cytoskeleton.

A. HUVECS transduced with control (left panels) or HRasV12 (right panels) retroviruses were stained for Ras and cytoskeletal proteins, including vimentin, tubulin and F-actin, counterstained with DAPI (blue) and imaged by confocal microscopy. HRasV12<sup>TL</sup> ECs accumulate vimentin and tubulin in the long filopodial filaments and actin forms large clusters as opposed to the organised structure in control ECs. Bar 25 μm.
Fig 3.5. HRasV12^{TL} ECs have increased cell migration.
A. HUVECS transduced with control or HRasV12 retrovirus were subjected to a scratch wound assay and imaged at 0 and 5 h post-scratch. The sizes of the wounds were determined using Fiji. Bar 100 μm.
B. Quantification of the wound recovery at 3 and 5 h post-scratch in A. Data represents the mean ± SEM from n=4 independent experiments. * P<0.05, *** P<0.001; t-test.
Chapter 3. ARHGAP18 in HRas-induced Angiogenesis

A

Monolayer ECs → Harvested → Single cell suspension → 24 h → Collected Embedded → Spheroids → Embedded spheroids → Stimulated → Spheroid sprouts

B

Control

HRasV12TL

C

Control

HRasV12TL

Non-stimulated

Sprouted Area (x 1000 µm²)

D

0 20 40 60 80 100

Sprouted Area (x 1000 µm²)

Control HRasV12TL

E

VEGF

FGF-2

****
Fig 3.6. HRasV12 overexpression promotes EC spheroid sprouting.

A. Schematic diagram of the spheroid sprouting assay. Monolayer ECs were resuspended in methylcellulose to form suspended spheroids. These spheroids were embedded into collagen I gels and stimulated to sprout.

B. Morphology of the spheroids formed from control and HRasV12 transduced HUVECs. Control cells formed a tight spheroid structure while the spheroids from HRasV12 transduced ECs had a relatively looser and irregular structure with apparent cell blebs. Bar 100 μm.

C. Non-stimulated spheroids comprised of control or HRasV12 transduced ECs were embedded in collagen I gels and imaged after 24 h. EC spheroids overexpressing HRasV12 resulted in prolific cell sprouting. Bar 200 μm.

D. Quantification of the sprouted area in C. Data represents the mean ± SEM of 16 control and 13 HRasV12 sprouted spheroids from n=3 independent experiments. **** P<0.0001; t-test.

E. HRasV12 spheroid sprouts have a VEGF-like sprouting appearance. Representative morphology of normal HUVEC spheroids stimulated with VEGF (25 ng/mL, 24 h) and FGF-2 (25 ng/mL, 48 h). Non-phase contrast in VEGF and FGF-2 stimulated sprout spheroid images. Bar 200 μm.
Fig 3.7. Overexpression of active HRas does not alter cell proliferation but promotes bypass of contact-mediated growth inhibition.

A. Control and HRasV12 transduced HUVECs were seeded in 6 well plates and cultured for up to 5 d. The cells were collected and counted after 1, 3 and 5 d. Data represents the mean ± SD from n=3 experiments. * P<0.05; two way ANOVA, Sidak’s post-test.

B. Control and HRasV12 transduced HUVECs were cultured on chamber slides for 1 d following cell confluence and stained for DAPI. Fluorescent images of DAPI alone (top panels) or DAPI and GFP (bottom panels) were taken and the number of cells per field determined by counting the cell nuclei. Bar 50 μm.

C. Quantification of cells per field in B. Data represents the mean ± SEM from n=4 independent experiments from at least 4 fields each. * P<0.05; t-test.
Fig 3.8. Overexpression of constitutively active HRas profoundly alters the expression and localisation of cell junction proteins.

A. Control (left panels) and HRasV12 (right panels) transduced HUVECs were cultured on chamber slides, fixed, stained for the junction proteins: PECAM-1, β-catenin and VE-cadherin; counterstained with DAPI and imaged by confocal microscopy. Bar 25 μm.
Fig 3.9. Overexpression of constitutively active HRas results in partial EndMT.

A. Immunoblots of endothelial (PECAM-1, VE-cadherin), mesenchymal (α-SMA), and EMT (Snail) markers from 5 d control and HRasV12 transduced HUVECs. HeLa lysate was used as a positive control for α-SMA. Actin was used as a loading control. Representative blots from n=3 independent experiments.

B. The mRNA levels of PECAM-1, VE-cadherin, α-SMA and Snail from 5 d control or HRasV12 transduced HUVECs were determined by qRT-PCR and normalised to β-actin. Data represents the mean ± SEM from n=4-7 independent experiments. ** P<0.01; t-test. Statistical tests were performed on log2(fold change) values.
Chapter 3. ARHGAP18 in HRas-induced Angiogenesis

A

<table>
<thead>
<tr>
<th></th>
<th>Control Vehicle</th>
<th>U</th>
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<tbody>
<tr>
<td>p-ERK1/2</td>
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<tr>
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B

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<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
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C

Control

HRasV12TL

+ Vehicle

+ U0126

+ PD98059

+ LY294002

D

Cell Circularity (Arbitrary Units)

E

HRasV12TL

+ Vehicle

+ U0126

F

Sprouted Area (x 1000 µm²)

141
Fig 3.10. The HRasV12TL phenotype is dependent on MAPK/ERK and not Akt activation.

A. Confirmation of MAPK inhibition by the MEK inhibitors, U0126 and PD98059. Immunoblots for p-ERK1/2 from control or HRasV12 transduced HUVECs treated with DMSO Vehicle, 30 μM U0126 (U) or 100 μM PD98059 (PD). Pan-Ras was used to show successful transduction; Actin was used as the loading control.

B. Confirmation of Akt inhibition by the PI3K inhibitor, LY294002. Immunoblots for p-Akt from control or HRasV12 transduced HUVECs treated with DMSO vehicle or 30 μM LY294002 (LY). Actin was used as the loading control.

C. Inhibition of MAPK/ERK but not PI3K/Akt partially reverts the HRasV12TL elongated phenotype. Brightfield images of HUVECs transduced with control or HRasV12 retroviruses for 2 d were treated with vehicle, 30 μM U0126, 100 μM PD98059 or 30 μM LY294002 for 2 d. Bar 50 μm.

D. Quantification of cell elongation in C. Cell circularity of control or HRasV12TL HUVECs treated with MEK or PI3K inhibitors were assessed using Fiji. Data represents the mean ± SEM of n=3 independent experiments. ** P<0.01; one way ANOVA, Tukey’s post-test.

E. MAPK inhibition suppresses the sprouting phenotype of HRasV12 overexpression. HRasV12 EC spheroids were embedded in collagen I gels and treated with vehicle or 30 μM U0126. Bar 200 μm.

F. Quantification of the sprouted area of HRasV12 EC spheroids treated with vehicle or U0126 in E. Data represents the mean ± SEM from 11 vehicle and 14 U0126 spheroids from n=2 independent experiments. **** P<0.0001; t-test.
Fig 3.11. ARHGAP18 is downregulated in ECs overexpressing HRasV12.

A. Immunoblots of ARHGAP18 from 5 d control and HRasV12 (TL and SL doses) transduced ECs. Overexpression of HRasV12 at different levels was confirmed by immunoblotting for pan-Ras. Actin was used as a loading control.

B. Confocal images of control and HRasV12 transduced ECs immunostained for ARHGAP18 (red). Cell nuclei were counterstained with DAPI (blue). White arrows represent the senescent HRasV12 cells. ARHGAP18 is localised predominantly in the cytosol of ECs and is expressed at a lower level in HRasV12 overexpressing cells relative to control ECs. Bar 50 μm.
ARHGAP18 is downregulated following HRasV12 overexpression in a time-dependent manner.

A. Brightfield images of HRasV12 overexpressing HUVECs following 2, 5 and 8 d transduction. Bar 100 μm.

B. Immunoblots of ARHGAP18 from 2, 5 and 8 d control and HRasV12 transduced ECs. Representative blots from n=3 independent experiments. p-ERK1/2 was used as an activation control for HRasV12 transduction. Actin was used as a loading control.

C. The mRNA level of ARHGAP18 from 2, 5 and 8 d control and HRasV12 transduced ECs was determined by qRT-PCR and normalised to β-actin. Data represents the mean ± SEM from n=3 independent experiments. **** P<0.0001, *** P<0.001, ** P<0.01; two-way ANOVA, Sidak’s post-test. Blue and red stars represent the significance of 5 and 8 d relative to 2 d from control and HRasV12, respectively. Black stars represent the significance of the differences between control and HRasV12 at the same time points. Statistical tests were performed on log₂(fold change) values.
ARHGAP18 downregulation is dependent on MAPK/ERK activation and is not restored to basal levels following MAPK inhibition

A. ARHGAP18 downregulation by HRasV12 overexpression is inhibited by MAPK/ERK inhibition but not PI3K/Akt inhibition. Three day control and HRasV12 transduced HUVECs were treated with vehicle, 30 μM U0126 or 30 μM LY294002 for 2 d, lysed and immunoblotted for ARHGAP18, p-ERK1/2 and p-Akt were used to confirm activation and inhibition of the MAPK/ERK and Akt pathways respectively. Actin was used as the loading control. Representative blots from n=3 independent experiments.

B. Long-term inhibition of MAPK/ERK signaling does not restore ARHGAP18 levels. Two day control and HRasV12 transduced HUVECs were treated with vehicle or 30 μM U0126 for 1, 3 and 5 d, lysed and immunoblotted for ARHGAP18. p-ERK1/2 was used to confirm activation and inhibition of the MAPK/ERK pathway. Actin was used as the loading control. Representative blots from n=2 independent experiments.
Chapter 3. ARHGAP18 in HRas-induced Angiogenesis

A

Adv-Control | Adv-ARHGAP18

Brightfield | Brightfield

GFP | GFP

B

<table>
<thead>
<tr>
<th>NT</th>
<th>Adv-Control</th>
<th>Adv-ARHGAP18</th>
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<tbody>
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<td>ARHGAP18</td>
<td>78 kDa</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>42 kDa</td>
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</table>

C

pMIG | pMIG-ARHGAP18

Brightfield | Brightfield

GFP | GFP

D

<table>
<thead>
<tr>
<th>NT</th>
<th>pMIG</th>
<th>pMIG-ARHGAP18</th>
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</thead>
<tbody>
<tr>
<td>ARHGAP18</td>
<td>78 kDa</td>
<td></td>
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<tr>
<td>Actin</td>
<td>42 kDa</td>
<td></td>
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</tbody>
</table>

E

pQCXIN-mCherry | pQCXIN-mCherry-ARHGAP18

Brightfield | Brightfield

mCherry | mCherry

F

| pQCXIN-mCherry | pQCXIN-mCherry-ARHGAP18 |
|-------------------------------------------------|
| ARHGAP18 | 78 kDa | |
| DsRed | 97 kDa | |
| Actin | 42 kDa | |
Fig 3.14. Retroviral overexpression of ARHGAP18 does not induce EC senescence.
A. Brightfield and GFP images of HUVECs transduced with control or ARHGAP18 adenoviruses. Adv-ARHGAP18 cells are senescent with the typical enlarged and flattened cell appearance. Bar 100 μm.
B. Immunoblots of ARHGAP18 from non-transduced, control and ARHGAP18-adenovirus transduced HUVECs. Actin was used as the loading control.
C. Brightfield and GFP images of HUVECs transduced with control or ARHGAP18 pMIG retroviruses. pMIG-ARHGAP18 cells are phenotypically indifferent from control cells. Bar 100 μm.
D. Immunoblots of ARHGAP18 from non-transduced, control and ARHGAP18-pMIG-retrovirus transduced HUVECs. Actin was used as the loading control.
E. Brightfield and mCherry images of HUVECs transduced with control or mCherry-ARHGAP18 pQCXIN retroviruses. pQCXIN-mCherry-ARHGAP18 cells are phenotypically indifferent from control cells. mCherry expression is throughout the cell in control-transduced cells, but predominantly cytosolic in cells expressing the mCherry-ARHGAP18 fusion protein. Bar 100 μm.
F. Immunoblots of ARHGAP18 and DsRed from control and mCherry-ARHGAP18 pQCXIN-retrovirus transduced HUVECs. Actin was used as the loading control. Expression of the fusion protein resulted in two distinct bands of which the lower stronger band was immunoreactive to the DsRed antibody.
Chapter 3. ARHGAP18 in HRas-induced Angiogenesis

A

Brightfield

Control  HRasV12TL  pQ-ARHGAP18  pQ-ARHGAP18 + HRasV12TL

B

Cell Circularity (Arbitrary Units)

HRasV12TL  -  +  -  +
pQ-ARHGAP18  -  -  +  +

C

ARHGAP18  78 kDa
PECAM-1  130 kDa
VE-cadherin  130 kDa
Snail  29 kDa
Actin  42 kDa

D

ARHGAP18 mRNA

PECAM-1 mRNA

α-SMA mRNA

SNAI1 mRNA

HRasV12TL  -  +  -  +
pQ-ARHGAP18  -  -  +  +
Fig 3.15. ARHGAP18 restoration does not revert the Ras phenotype

A. Brightfield images of HUVECs overexpressing ARHGAP18, HRasV12 or both ARHGAP18 and HRasV12. ARHGAP18 expression did not affect the typical TL phenotype of HRasV12. Bar 100 μm.

B. Quantification of cell circularity in A. Data represents the mean ± SEM of 40 cells from n=2 independent experiments.

C. Immunoblots of ARHGAP18 and the EndMT proteins (PECAM-1, VE-cadherin, Snail) from HUVECs overexpressing ARHGAP18, HRasV12 or both ARHGAP18 and HRasV12. Actin was used as the loading control. Representative blots from n=2 independent experiments.

D. Quantitative RT-PCR of ARHGAP18 and EndMT genes (PECAM-1, α-SMA and Snail) from HUVECs overexpressing ARHGAP18, HRasV12 or both ARHGAP18 and HRasV12. β-actin was used as the normalising gene. Data represents the mean ± SEM from n=2 independent experiments.
Fig 3.16. Proposed pathway for the HRas mediated TL phenotype and the role of ARHGAP18
A. Constitutive activation of HRas results in a partial EndMT phenotype with altered cell morphology, disrupted cell-cell junctions and increased cell sprouting and migration that is dependent on MAPK/ERK, but not Akt activation. Constitutive HRas activation also results in MAPK/ERK-dependent downregulation of ARHGAP18, however the HRas phenotype is not dependent the loss of ARHGAP18. The functional consequence of ARHGAP18 downregulation in the context of constitutive HRas activation remains to be elucidated.
CHAPTER 4

ARHGAP18 IN SPROUTING ANGIOGENESIS
4.1. **INTRODUCTION**

ARHGAP18 was identified from an array of genes regulated during *in vitro* tube formation (Coleman et al., 2013; Hahn et al., 2005). It was investigated further because it showed an unusual pattern of regulation, being downregulated during the migratory phase, followed by a later upregulation during tube stabilization (Coleman et al., 2010). This suggested that ARHGAP18 may have roles in regulating EC migration or vessel stabilization. Furthermore, in the previous chapter, we established that chronic HRas activation induces a pro-angiogenic phenotype with cytoskeletal and junctional defects and is associated with near complete loss of ARHGAP18 expression. However, since Ras regulates a multitude of signaling pathways and elicits an extreme gross change in phenotype, we were unable to identify a role of ARHGAP18 in this instance. On the other hand, this result indicated that there was an environment where there was a loss of ARHGAP18 expression, and thus makes the investigation of ARHGAP18 knockdown physiologically relevant. The aim of this chapter was to investigate whether there was a role of ARHGAP18 in the regulation of sprouting angiogenesis.

By *in vitro* and *in vivo* models of angiogenesis, we describe here that loss of ARHGAP18 results in EC hypersprouting. This is an EC-centric phenotype and is mediated by changes in VEGFA responsiveness, and the development of a tip cell (TC) profile and phenotype.

4.2. **RESULTS AND DISCUSSION**

4.2.1. **Optimisation of ARHGAP18 knockdown in ECs**

In the previous studies, ARHGAP18 knockdown was performed using the HiPerFect transfection reagent (Coleman et al., 2010). However, using the same conditions (50 nM siRNA), a consistent level of gene silencing was unable to be reproduced (data not shown). Therefore, the conditions for gene knockdown were re-established. The
Lipofectamine RNAiMax reagent was used, as this had been reported to be efficient and low in toxicity to ECs (Life Technologies manufacturer’s documentation). The concentrations of siRNA and lipofectamine reagent were first optimized using a pool of the three siRNAs used in the previous study (Fig 4.1 A and B). Even at low doses of siRNA/lipofectamine (5 nM siRNA, 2.5 µL lipofectamine), ARHGAP18 expression was significantly reduced, with over 90% decrease in protein expression (Fig 4.1 B). Transfection at the higher dose of siRNA/lipofectamine (10 nM siRNA, 5 µL lipofectamine) resulted in noticeable toxicity, as indicated by the prominent change in cell morphology in the control siRNA transduced cells (Fig 4.1 A). The lower dose of siRNA/lipofectamine was therefore chosen and the efficiency of transfection was confirmed to be over 90% by monitoring the uptake of a fluorescent oligo (Fig 4.1 C). The siRNA gene silencing ability is known to be transient and time-dependent. A time course of cells transfected with siRNAs was performed to establish the time conditions for future experiments. As expected, the siRNAs were marginally effective 1 d following transfection, but highest at 3 to 5 d. By day 7 the level of ARHGAP18 expression had returned to near endogenous levels (Fig 4.1 D). Therefore, all subsequent experiments were performed on cells following 3-4 d transfection.

Finally, the effects of the individual siRNAs targeting ARHGAP18 were examined (Fig 4.2 A-C). All the siRNAs targeted the coding sequence of ARHGAP18 and not the 5’ or 3’ untranslated regions (Fig 4.2 A) Individual transfection of the original siRNAs (siGAP18-1, siGAP18-2, siGAP18-3) all resulted in efficient gene knockdown. However, out of these three, one of the siRNAs (siGAP18-2) resulted in an elongated phenotype (Fig 4.2 C, white arrows). Therefore, to determine whether this was due to a non-specific effect, four other siRNAs (siGAP18-4, siGAP18-5, siGAP18-6, siGAP18-7) were tested for their ability to knockdown ARHGAP18 and induce an elongated phenotype.
Transfection with these siRNAs resulted in relatively poor gene knockdown in comparison to the original siRNAs, with only siGAP18-6 resulting in acceptable knockdown. This is likely due to the different targeting sequences or chemistries used by each manufacturer. However, importantly none of these siRNAs resulted in any alteration of the cell phenotype. Overall, it is likely that siGAP18-2 induced non-specific effects in ECs, and was therefore omitted from further experiments. From these optimization experiments, it was decided that a pool of siGAP18-1 and siGAP18-3 were to be used as the transfection conditions for subsequent knockdown experiments.

4.2.2. **ARHGAP18 Knockdown does not alter the gross EC phenotype or affect EC proliferation**

Using the aforementioned conditions from the optimization experiments, we first assessed whether knockdown of ARHGAP18 altered the gross EC phenotype (Fig 4.3 A-C). As previously described for the HRasV12 TL cells, the gross phenotype was assessed by analyzing cell size and circularity. Knockdown was confirmed at the protein (Fig 4.3 D) and mRNA (Fig 4.3 E) levels and was routinely over 90% and 80% respectively. Knockdown of ARHGAP18 resulted in a typical cobblestone EC morphology (Fig 4.3 A) and had no changes in cell size (Fig 4.3 B) or circularity (Fig 4.3 C).

The effect of ARHGAP18 on cell proliferation was next assessed. HUVECs transfected with siRNAs were cultured for 3 d and counted using a hemocytometer. Knockdown of ARHGAP18 did not have an effect on cell proliferation (Fig 4.4 A). While other proliferation assays (e.g MTT assays, CFSE) could have been performed to more accurately assess proliferation, there was no indication that ARHGAP18 was affecting cell proliferation and so these were not performed. Next, the effects of ARHGAP18 knockdown on the cell density was assessed by determining the number of cells per field after culturing the chamber slides. Intriguingly, knockdown of ARHGAP18 resulted in a
slight ~10% decrease in cell density (Fig 4.4 B and C), which given that there was no
effect on cell proliferation, may indicate a more spread out phenotype, and one possibly
involved in migration.

4.2.3. ARHGAP18 knockdown results in a pro-migratory and pro-sprouting phenotype

We next assessed the effects of ARHGAP18 knockdown on cell migration using a 2D
wound healing assay. Control knockdown ECs had migrated predominantly as a uniform
front within the first 5 h (Fig. 4.5 A). In contrast, ARHGAP18 knockdown resulted in an
irregular and protruded migratory front and an overall 16% increase in wound closure at
this early time point (Fig. 4.5 A and B). Notably, while the protrusive lamellipodium were
unchanged, the proximal edge of the leading cell at the migratory front had disrupted cell-
cell junctions (Fig. 4.5 C). The increased migratory phenotype was also observed in the
3D spheroid sprouting assay although with a more pronounced effect. Following VEGFA
stimulation for 20 h, knockdown of ARHGAP18 resulted in a significant increase in both
the number of sprouts and the cumulative sprout length of the spheroids (Fig. 4.6 A-C).
Unlike HRasV12TL cells, neither knockdown nor control spheroids sprouted in the
absence of stimulus (data not shown). We confirmed that ARHGAP18 was silenced in the
spheroid assay by assessing the ARHGAP18 mRNA levels (Fig 4.6 D). One of the
reviewers of the publication indicated that the ARHGAP18 knockdown
migratory/sprouting phenotype should be reproduced using individual siRNAs. Therefore,
using the spheroid sprouting assay (as the effects of ARHGAP18 depletion were more
pronounced and consistent), we confirmed the sprouting phenotype of spheroids
generated from ECs transfected with the individual siRNAs. Each of these siRNAs
resulted in a significant increase in spheroid sprouting, and was no different to the other
ARHGAP18 siRNAs nor the combined siRNA approach (Fig 4.6 E). Notably, these
results are in contrast to those seen by (Maeda et al., 2011) in epithelial cells. This may be due to intrinsic differences between epithelial and endothelial cells or a result of different targets in these cells. This is discussed further in section 5.2.5.

4.2.4. **Knockdown of ARHGAP18 promotes zebrafish ISV sprouting**

We next sought to confirm the sprouting phenotype using *in vivo* models of angiogenesis, firstly with the zebrafish model performed by Dr. Ka Ka Ting, from the same laboratory. The zebrafish embryo model has emerged as an excellent model to study *in vivo* angiogenesis for several reasons: the ability to be genetically manipulated, small size, rapid and external development, and optical transparency that allows it to be visualized microscopically (Gore et al., 2012). Initial blood vessel morphogenesis occurs by vasculogenesis to form the dorsal aorta (DA) and the posterior cardinal vein (PCV) (Ellertsdottir et al., 2010). Following the primary axial vessels, the elaborate vascular structure predominantly forms via sprouting angiogenesis, with the initial development of the intersegmental vessels occurring ~22 hours post fertilization (hpf) (Ellertsdottir et al., 2010).

A morpholino based approach was used to silence the expression of ARHGAP18 using two different morpholino approaches: a splice morpholino to inhibit splicing of ARHGAP18, and a translational morpholino to inhibit the initiation of translation of ARHGAP18 (Fig 4.7 A). The morpholinos were injected in the 2-4 cell stage of the zebrafish embryos and monitored for up to 48 h. Injection of the SpMO resulted in retention of ARHGAP18 intron 3 in the mRNA, and decreased expression of ARHGAP18 exon 5, thus confirming efficacy of the injection and the morpholino (Fig 4.7 B). Assessment of the gross morphology at 48 h indicated the presence of hindbrain, pericardial and yolk odema in both the TrMO and SpMO (Fig 4.7 C) and this was coupled
with reduced zebrafish viability (data not shown). This was not due to non-specific apoptotic effects, as reported by others (Gerety and Wilkinson, 2011), since there was an absence of TUNEL staining in the MO injected embryos (Fig 4.7 D).

To assess the effects of ARHGAP18 silencing on angiogenesis, the flil-GFP transgenic zebrafish embryos, which have the ECs expressing GFP, were injected with the morpholinos and imaged by confocal microscopy. At 24 hpf, both the TrMO and the SpMO resulted in a significant increase in ISV lengths compared to the control MO (Fig 4.8 A and B). This was coupled with an increase in filopodial extensions further confirming the hyper-sprouting phenotype. It was also observed that the lumen diameter of the dorsal aorta (DA) was notably reduced in the SpMO, and trending in the TrMO, whereas the lumen diameter of the posterior cardinal vein (PCV) in both ARHGAP18 morphants were similar (Fig 4.8 C and D). This may be an indicator of the increased migration of the cells from the DA into the ISV, which occurs first at ~22 hpf, whereas the cells migrate from the PCV at ~32 hpf (Ellertsdottir et al., 2010). Further, in collaboration with Dr. Neil Bower and Dr. Ben Hogan, at the Institute for Molecular Biosciences, University Queensalnd, they observed similar effects with the MOs on vascular hypersprouting, but also showed that the development of the lymphatics was unchanged (Chang et al., 2014), indicating that the effects of ARHGAP18 knockdown is vascular specific.

4.2.5. Genomic characterisation of the ARHGAP18 Knockout mouse

To next investigate the effects of ARHGAP18 loss in mammalian vascular development, a global ARHGAP18 KO mouse was generated through the KOMP repository. This mouse utilizes a splice cassette which is inserted downstream of the mouse ARHGAP18 exon 1 and just upstream of exon 2 (Fig 4.9 A). The splice cassette encodes for a splice
acceptor that continues to the expression of lacZ driven by an IRES element. In addition, a neomycin resistance gene is expressed from a separate constitutive promoter, which allows for neomycin/G418 selection of the murine ES cells for the generation of the KO mouse. Expression of the lacZ gene would have occurred in the KO cells under the control of the murine ARHGAP18 promoter and would have been be detected by β-galatosidase staining (Fig 4.9 B). However, following genotyping and sequencing of the mRNA (performed by Dr. Angelina Lay), it was concluded that, in the actual ARHGAP18 KO mouse, there was a cryptic splice donor site present in the cassette (Fig 4.9 B). This results in re-splicing of the cassette element and joining of the rest of the ARHGAP18 exons. As a result, this forbids the use of lacZ, (since it is spliced out) to be used as a marker of ARHGAP18 expression. Fortunately, despite the presence of the cryptic splice donor, there was still a stop codon present in the splice cassette, which prematurely terminates expression of the ARHGAP18 protein product. Analysis of mouse lung lysates confirmed the absence of ARHGAP18 in the ARHGAP18−/− mouse (Fig 4.9 C). The endothelial protein, VE-cadherin, was used as a loading control for the lung endothelium, thus confirming the absence of ARHGAP18 also in the endothelial population. The mice are phenotypically normal and display no changes in the rate of pregnancy or litter size.

4.2.6. **ARHGAP18 LOSS PROMOTES EX VIVO AORTIC RING SPROUTING ANGIOGENESIS**

To determine the effects of ARHGAP18 loss on angiogenesis, the aortic ring assay was used. The aortas from WT and ARHGAP18−/− mice were isolated, dissected into small sections and embedded and cultured in Matrigel. This matrix contains growth factors that allows the ECs to invade into the 3D environment and generate EC sprouts. The loss of ARHGAP18 resulted in significantly increased sprouting of the aortas, with earlier onset
and increased expansion of the sprouts (Fig 4.10 A and B). Strikingly, while the WT cells formed mainly linear sprouts, the EC from the ARHGAP18<sup>−/−</sup> mice had an increased propensity to form branches (Fig 4.10 B iii and iv).

### 4.2.7. Loss of ARHGAP18 results in Hypersprouting of the Developing Retina Vasculature

We next utilized the retina model to investigate the effects of ARHGAP18 on angiogenesis in vivo. This is a widely used and well-characterised model of developmental angiogenesis in vivo (Gariano and Gardner, 2005; Pitulescu et al., 2010; Stahl et al., 2010). Retinal vascularization occurs post-natally and begins with sprouting angiogenesis and radial expansion of the superficial layer (Fig 4.11 A). The vasculature undergoes constant remodeling and expands until it reaches the peripheral edges of the retina at P8. This is then followed by invasion of the vasculature into the deeper retinal layers, which is accompanied by pruning and further remodeling until the complete development of the adult retinal vessels at P21 (Stahl et al., 2010). Preliminary analysis of the adult retinal vasculature of the ARHGAP18<sup>−/−</sup> mice appeared to indicate more vascularization but did not reveal any major defects (Fig 4.11 B).

#### 4.2.7.1. ARHGAP18<sup>−/−</sup> mice have delayed expansion of the retinal vasculature

We investigated the effects of ARHGAP18 loss in P6 mice, as this represents a time point where angiogenesis is occurring at a linear rate (Stahl et al., 2010). The retinas from WT and ARHGAP18<sup>−/−</sup> mice were dissected and stained for the vessels using isolectin B4. We observed a significant (~10%) decrease in radial extension in the vessels of the ARHGAP18<sup>−/−</sup> mice (Fig 4.12 A-C). This was regardless of whether it was determined in the actual distance radiating from the optic nerve (Fig 4.12 B) or as a percentage of the total retina diameter (Fig 4.12 C).

#### 4.2.7.2. ARHGAP18<sup>−/−</sup> mice have increased vascularization of the central plexus
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The vascular network was next assessed using whole quadrants of the retinas. Images of the quadrants were analysed using Wimasis GmbH (www.wimasis.com, Germany) (Fig 4.13 A and B). Here using a skeleton algorithm, a number of parameters, including the vessel density, the number of branch points and the loops can be analysed. There was slight but not significant increase in each of these parameters (Fig 4.13 C-E). From discussion with Prof Ralf Adams, an expert with multiple publications in the field of the retina vasculature (Benedito et al., 2012; Ehling et al., 2013; Gaengel et al., 2012; Nakayama et al., 2013), he advised us that analysis of the whole retina can be inconsistent due to differences in vessel numbers in different areas, for example next to the larger arterial and venous vessels. Therefore, we reanalyzed the plexus to focus on a smaller area in the central vascular plexus that was between two large vessels (usually arteries and veins) and approximately ~5 branches away from the sprouting front. To do this, a macro was created in which the whole quadrant image was re-analysed to the smaller area while keeping the results from the original algorithm (Fig 4.14, Appendix A.1.2). Firstly, the image was isolated in the individual parameters, each of which was re-coloured and merged for downstream re-analysis. A suitable area of 380 x 380 µm region was selected and the parameters were re-analysed. Analysis of the central plexus revealed significant increases in vessel density (6%), the number of branch points (13%) and in the number of loops (13%) (Fig 4.15 A-D).

4.2.7.3. *ARHGAP18<sup>−/−</sup> mice have increased sprouting at the angiogenic front*

The vessels at the angiogenic front were next assessed. Loss of ARHGAP18 resulted in a 23% increase in sprout formation (Fig 4.16 A and B). Furthermore, examination of the sprouts revealed that the *ARHGAP18<sup>−/−</sup>* sprouts had extensive filopodia formation (Fig 4.16 C), which is consistent with the zebrafish ISV phenotype and the overall hypersprouting phenotype.
4.2.7.4. The hypersprouting phenotype is not associated with changes in pericyte coverage

Mural cells, such as pericytes, facilitate the stabilization of angiogenic vessels, and reduced pericyte coverage is associated with increased hypersprouting (Feng et al., 2007). To determine whether loss of ARHGAP18 results in changes with pericyte coverage, the retinas were stained with NG2 to label the pericytes and VE-cadherin to label the vessels (Fig 4.17A). In retrospect, the vessels would ideally be stained with isolectin B4, as VE-cadherin stains the junctions of ECs resulting in an outline of the ECs. However, in order to assess the pericyte coverage in these VE-cadherin stained vessels, the images were post-processed and analysed using a Fiji macro (Appendix A.1.3). This macro was designed to fill in the VE-cadherin junctions to fill in the cell outlines to create an EC mask. Next, the pericyte image was also converted into a mask and the overlaying of the two images resulted in three regions: the green pericyte only area, the red EC only area, and the yellow pericyte and EC area (Fig 4.17B). Therefore, the overall pericyte coverage is defined as the percentage of the yellow area of the total red and yellow area. We observed that there was a subtle but non-significant decrease in pericyte coverage in the ARHGAP18^−/− retinas (Fig 4.17C).

Overall, these results confirm the hypersprouting phenotype as observed in vitro, ex vivo and in vivo in the zebrafish embryos. While the decrease in radial extension appears contradictory, this highlights that there are major changes in vascular patterning with favouring of TCs over SCs that results in the excessive branching rather than extension of the vascular plexus. Furthermore, these in vivo results further indicate that the effects of ARHGAP18 loss are likely to be EC specific and not due on the effects of other cells such as lymphatic ECs or pericytes.
4.2.8. **ARHGAP18 loss promotes VEGF hyperresponsiveness and a tip cell phenotype**

4.2.8.1. **ARHGAP18 knockdown promotes Akt but not ERK hyperactivation following VEGF stimulation.**

The *in vitro* and *in vivo* observations indicated that the loss of ARHGAP18 promotes hypersprouting, with earlier and more prominent development of angiogenic vessels. One of the main regulators that controls sprouting angiogenesis is VEGFA and its downstream signaling pathways. We next sought to determine whether the hypersprouting phenotype was due to a hyper-responsiveness to VEGFA by examining the activation of the downstream MAPK/ERK and Akt pathways. Control or ARHGAP18 depleted HUVECs were starved and stimulated with VEGFA for different times, then the activation of ERK and Akt assessed by immunoblotting. Both the ERK and Akt are activated at early time points and restored to basal levels at the later time points. While ARHGAP18 knockdown EC displayed a similar level of ERK activation, there was a higher maximal, albeit subtle, and prolonged activation of Akt (Fig 4.18 A).

4.2.8.2. **ARHGAP18 loss have increased expression of TC genes**

VEGF-Notch signaling is the major regulator of TC-SC differentiation and a coordinated regulation of VEGF-Notch driven TCs and SCs is required for effective vascular patterning (Herbert and Stainier, 2011; Potente et al., 2011). Based on the *in vitro* and *in vivo* results, loss of ARHGAP18 resulted in a typical TC-like phenotype, with increased sprouting and migratory capacity and extensive filopodial extensions. These TCs initiate sprouting angiogenesis by extending filopodial extensions and migrating towards the guidance cues and are characterized by increased expression of genes including the VEGF receptors *Flk1* (VEGFR2), *Flt4* (VEGFR3), the Notch ligand *Dll4*, and guidance receptors including *Pdgfb* and *Unc5b* (Gerhardt et al., 2003; Phng and Gerhardt, 2009).
To assess whether ARHGAP18 promotes a TC phenotype, the expression of *Dll4*, *Flk1*, *Flt4* and *Flt1* (VEGFR1) were assessed by qRT-PCR (Fig 4.19). Knockdown of *ARHGAP18* was confirmed and was unchanged following 6 h VEGFA stimulation (Fig 4.19 A). We observe that the expression of *Dll4* was significantly upregulated in both basal and VEGFA stimulated ARHGAP18 knockdown ECs (Fig 4.19 B). VEGF signaling upregulates the expression of *Dll4*, which in a physiological environment acts on the neighbouring EC Notch receptor to suppress the TC phenotype to promote the stabilizing SC phenotype. Consistent with the VEGF hyper-responsiveness, we observe that ARHGAP18 loss further drives *Dll4* expression following VEGFA stimulation. This hyper-responsiveness is likely due to the increased signaling of the main VEGF receptor, *Flk1/VEGFR2*, which we found was upregulated ~1.7 fold (Fig 4.19 C). Furthermore, we observed that *Flt4/VEGFR3* is also significantly upregulated in ARHGAP18 knockdown EC (Fig 4.19 D). VEGFR3 is also crucial in angiogenesis and is highly expressed in TC, where it forms VEGFR2/VEGFR3 heterodimers that are enriched in TCs and promote angiogenic sprouting (Tammela et al., 2011). On the other hand, we observe that the expression of the inhibitory VEGF receptor, *Flt1/VEGFR1*, was unchanged in ARHGAP18 knockdown EC. Overall, these results indicate that the loss of ARHGAP18 promotes the expression of the TC genes. It would be interesting to determine the changes in the expression of the other TC genes, *Pdgfb* and *Unc5b*, that are involved in guidance and also to assess whether ARHGAP18 loss affects the SC phenotype and downstream Notch signaling, but this would be for future study. One of the major difficulties for assessing SC phenotypes is in the use of monolayer cells, as it is ineffective in replicating the intricacies of TC-SC signaling and interactions.

### 4.2.8.3. *ARHGAP18 knockdown EC have increased preference for the TC position*
One of the main characteristics of TC is in the preference for the leading position, hence known as the TC position. We next sought to determine whether the TC phenotype translated to a preference for the TC position. This was performed using the 3D spheroid assay to replicate sprouting \textit{in vitro} by creating chimeric spheroid sprouts comprised of control and ARHGAP18-knockdown cells. These cells were labeled with the different cell tracker dyes, mixed at 1:1 ratios and used to establish EC spheroid sprouts (Fig 4.20 A). The ARHGPA18 knockdown EC displayed a significantly increased number of cells in the sprouts, consistent with the increased sprouting ability of these cells (Fig 4.20 B). More importantly, these cells also had double the chance of occupying the TC position (Fig 4.20 C), thus confirming that ARHGAP18 loss promotes both the expression of TC genes and a TC phenotype.

\textbf{4.3. \textsc{Summary and Conclusions}}

In this chapter, by using a variety of \textit{in vitro} and \textit{in vivo} models of angiogenesis, we have established that the knockdown of ARHGAP18 promotes EC hypersprouting. This is further associated with changes in VEGF signaling and a TC phenotype, with increased preference of the TC position and expression of the TC genes. It remains to be determined as to whether the ARHGAP18 pro-sprouting effects are dependent on the slight activation of Akt, although interestingly, it has been demonstrated that VEGFR2 signaling via Akt and not MAPK/ERK drives DLL4 signaling (Liu et al., 2003). VEGF-Notch signaling is the major regulator of TC selection and vascular patterning. The increased expression of the VEGF receptors 2 and 3, and most importantly the Notch ligand, DLL4, are responsible for the TC favoured imbalance. This ultimately results in abnormal vascular patterning with increased hypersprouting and branching and delayed peripheral expansion of the vascular network.
CHAPTER 4.
ARHGAP18 IN ANGIOGENIC SPROUTING

FIGURES
Chapter 4. ARHGAP18 in Angiogenic Sprouting

**A**

siCtrl Transfection Dose

Lo | Hi
---|---

**B**

Transfection Dose: | Lo | Hi
---|---|---

siCtrl | siGAP18 | siCtrl | siGAP18

ARHGAP18

78 kDa

Actin

42 kDa

**C**

Brightfield

Alexa Fluor 555

**D**

Time following transfection (d): | 1 | 3 | 5 | 7
---|---|---|---|---

NT | siCtrl | siGAP18 | NT | siCtrl | siGAP18 | NT | siCtrl | siGAP18 | NT | siCtrl | siGAP18

ARHGAP18

78 kDa

Actin

42 kDa
Fig 4.1. Optimisation of ARHGAP18 siRNA transfection in HUVECs.
A. Brightfield images of HUVECs 3 d following transfection with Lo dose (5 nM siRNA, 2.5 µL Lipofectamine RNAiMax) or Hi dose (10 nM siRNA, 5 µL Lipofectamine RNAiMax) control siRNA. Transfection at the Hi dose results toxicity as evident by significant alteration in cell shape. Bar 50 µm.
B. Immunoblots for ARHGAP18 of lysates from HUVECs transfected for 3 d with Lo dose or Hi dose control or ARHGAP18 (pool of siRNA 1, 2 & 3) siRNAs. Actin was used as the loading control.
C. Brightfield and fluorescent images of HUVECs 2 d following transfection with BLOCK-iT Alexa Fluor Red Fluorescent Control. Uptake of the oligo is indicated by the fluorescent signals in the nucleus and as punctate dots in the cytosol. Bar 50 µm.
D. Time course of ARHGAP18 knockdown in HUVECs. HUVECs were transfected with control or ARHGAP18 siRNAs (pool of siRNA 1, 2 & 3) or non-transfected, lysed following 1, 3, 5 and 7 d following transfection and immunoblotted for ARHGAP18. Actin was used as the loading control.
Fig 4.2. Optimisation of ARHGAP18 siRNAs.

A. Location of ARHGAP18 siRNA targeting sequences. All seven siRNAs target the coding sequence (CDS) of ARHGAP18.

B. HUVECs were transfected for 3 d with the individual ARHGAP18 siRNAs (1-7) and two different siRNA controls, lysed and immunoblotted for ARHGAP18. Actin was used as the loading control.

C. Brightfield images of HUVECs transfected for 3 d with the individual ARHGAP18 siRNAs (1-7) and two different siRNA controls or non-transfected. Transfection with siARHGAP18-2 results in significant elongation of ECs (arrow). Bar 50 μm.
Fig 4.3. Knockdown of ARHGAP18 does not affect the gross phenotype in HUVECs.

A. Brightfield images of HUVECs transfected for 3 d with control or ARHGAP18 (pool of siRNA 1 & 3) siRNAs. Bar 50 μm.

B. Quantification of cell size of control and ARHGAP18 siRNA transfected ECs. Data represents mean ± SEM from n=2 independent experiments comprised of 15 cells each. ns, non-significant; t-test.

C. Quantification of cell circularity control and ARHGAP18 siRNA transfected ECs. Data represents mean ± SEM from n=2 independent experiments comprised of 15 cells each. ns, non-significant; t-test.

D. Immunoblots for ARHGAP18 of lysates from HUVECs transfected for 3 d with control or ARHGAP18 (pool of siRNA 1 & 3) siRNAs. Actin was used as the loading control.

E. The mRNA level of ARHGAP18 from HUVECs transfected for 3 d with control or ARHGAP18 (pool of siRNA 1 & 3) siRNAs were determined by qRT-PCR and normalised to ACTB. Data represents the mean ± SEM from n=4 independent experiments. ** P<0.01; t-test. Statistical tests were performed on log₂(fold change) values.
Fig 4.4. Knockdown of ARHGAP18 does not affect cell proliferation, but reduces cell density.

A. HUVECs transfected with control or ARHGAP18 siRNAs for 3 d were collected and counted as a percentage of the control cells. Data represents the mean ± SEM from n=3 independent experiments, P>0.05, non-significant; t-test.

B. HUVECs transfected with control or ARHGAP18 siRNAs were cultured in chamber slides at confluent densities (6 x 10^4 cells) overnight, fixed and stained for F-actin (red), nuclei counterstained with DAPI (blue), and imaged by confocal microscopy. Bar 50 μm.

C. Quantification of cell density in B. The number of cells per field were determined by counting the nuclei in B. Data represents the mean ± SEM from n=6 independent experiments. * P<0.05; t-test.
Fig 4.5. Knockdown of ARHGAP18 promotes 2D wound healing and disruption of the proximal junctions of the leading cells.

A. Brightfield images of HUVECs transfected for 3 d with control (i & iii) or ARHGAP18 (ii & iv) siRNAs at 0 (i & ii) and 5 (iii & iv) h following scratch wound. Bar 250 μm.

B. Quantification of wound recovery in A. Data represents the mean ± SEM from n=5 independent experiments. * P<0.05; t-test.

C. High magnification brightfield images of the migratory front of siCtrl and siARHGAP18 HUVECs following 5 h scratch wound. The leading migratory cells in ARHGAP18 knockdown ECs, but not control ECs, displayed disrupted proximal junctions (arrows). Bar 25 μm.
Fig 4.6. Knockdown of ARHGAP18 promotes 3D spheroid sprouting.

A. Brightfield images of siCtrl and siARHGAP18 HUVEC spheroid sprouts stimulated with 50 ng/ml VEGF for 20 h. Bar 100 μm.

B-C. Quantification of the number of sprouts (B) and the cumulative sprout length (C) per spheroid. Data represents the mean ± SEM from n=4 independent experiments, consisting of a minimum of 20 spheroids per group. ** P<0.01; t-test.

D. Confirmation of ARHGAP18 knockdown in the spheroid assay. Spheroid sprouts generated from siCtrl, siARHGAP18 and a 50:50 chimera of siCtrl:siARHGAP18 ECs or a collagen only negative control were lysed and the mRNA level of ARHGAP18 relative to ACTB determined by qR-TPCR. Data represents the mean ± SEM from 3 technical replicates from n=1 experiment.

E. Confirmation of ARHGAP18 knockdown effects using individual ARHGAP18 siRNAs. Spheroids sprouts were established using HUVECs transfected with the indicated siRNAs and the cumulative sprout length quantified. Data represents the mean ± SEM from 15 individual spheroids. Representative data of n=2 individual experiments. *** P<0.001; * P<0.05; ns non-significant; one-way ANOVA, Tukey’s post-test.
Fig 4.7. Morpholino-mediated knockdown of ARHGAP18 induces oedema in zebrafish embryos

A. Design of morpholinos. A translational morpholino (TrMO) (i) and a splice (SpMO) (ii) were used to silence ARHGAP18 expression in zebrafish embryos. The TrMO blocks translation from the start codon on the ARHGAP18 mRNA whereas the SpMO blocks splicing resulting in retention of intron 3.

B. Confirmation of ARHGAP18 morpholino efficiency. Zebrafish embryos were injected with control MO or ARHGAP18 SpMO or uninjected, total RNA collected and indicated amplicons amplified by RT-PCR. The absence of exon 5 and presence of intron 3 indicates successful injection and efficacy of the morpholino.

C. Representative images of zebrafish embryos injected with control MO, ARHGAP18 SpMO or TrMO or uninjected at 48 hpf. Arrows indicate the observable hindbrain, yolk and heart oedema. Bar 500 μm.

D. ARHGAP18 TrMO effects are not due to non-specific apoptotic effects. Zebrafish embryos injected with control or ARHGAP18 SpMO were TUNEL-stained (red). A DNase I treated zebrafish embryo was used as a positive control. The absence of the stain in the ARHGAP18 injected embryos suggests an absence of apoptosis. Bar 250 μm.

Experiments performed by Dr. Ka Ka Ting
Fig 4.8. Knockdown of ARHGAP18 promotes zebrafish ISV sprouting.
A. Representative GFP confocal images of intersegmental vessels (ISV) in flI1-GFP zebrafish embryos injected with control MO, ARHGAP18 SpMO or TrMO at 24 hpf. ISV from ARHGAP18 MO injected zebrafish have increased length and filopodial extensions. Red = dorsal aorta (DA), blue = posterior cardinal vein (PCV). Bar 100 µm.
B. Quantification of ISV lengths. Data represents the mean ± SEM from n=134 control MO, 306 SpMO, 105 TrMO ISV. * P<0.05, *** P<0.001; t-test.
C-D. Quantification of (C) dorsal aorta (DA) and (D) posterior cardinal vein (PCV) lumen diameter. Data represents mean ± SEM from n=21 control, 10 SpMO, 10 TrMO ISV. **, P<0.01; t-test.

Experiments performed by Dr. Ka Ka Ting
**Fig 4.9. Genomic map of the ARHGAP18⁻/⁻ mouse.**

A. Genomic map of the ARHGAP18⁻/⁻ mouse generated by the KOMP repository. E1-E15 denote the ARHGAP18 exons. The splicing cassette was inserted upstream of exon 2.

B. Schematic of the mRNAs expected from WT and ARHGAP18⁻/⁻ mice, and the actual ARHGAP18⁻/⁻ mRNA. The WT mRNA consists of all 15 exons. The ARHGAP18⁻/⁻ mouse was expected to encode for exon 1 and the lacZ gene from the IRES, however the presence of a cryptic splice site in the cassette results in the altered transcript with an absence of the lacZ gene, but retention of the stop codon that results in the knockout of the ARHGAP18 protein.

C. Confirmation of ARHGAP18 absence in ARHGAP18⁻/⁻ mice. Western blot of mouse lung lysates from WT and ARHGAP18⁻/⁻ mice blotted for ARHGAP18, and the loading controls VE-cadherin and actin.
Fig 4.10. ARHGAP18 loss promotes \textit{ex vivo} aortic ring sprouting.

A. Brightfield images of sprouted aortic explants from WT and ARHGAP18\textsuperscript{-/-} mice. The aortas of WT and ARHGAP18\textsuperscript{-/-} mice were harvested, embedded in Matrigel and cultured for up to 5 d. Low magnification of the sprouted area (i & ii). The sprouted area is outlined in red, the aorta is outlined in yellow. High magnification of sprouted area (iii & iv) shows extensive branching in explants from the ARHGAP18\textsuperscript{-/-} mice. Bar 500 \textmu m (top panels), 100 \textmu m (bottom panels).

B. Quantification of the sprouted area as depicted by the red-yellow area in A. Data represents mean \pm SEM from \textit{n}=7 experiments. * \textit{P}<0.05; t-test.

Experiments performed by Dr. Angelina Lay and Dr. Paul Coleman. GHKC performed the analysis.
Fig 4.11. Adult ARHGAP18−/− mice have no major retina vascularisation defects.

A. Schematic of the post-natal retinal angiogenesis model. Retinal vascularisation occurs post-natally with expansion to the vascular periphery at P8. The vessels constantly remodel and invade into the deeper layers until complete development at P21. Top images, flatmount; bottom images, cross sections of retinas. Adapted from Gerhardt et al. (2003).

B. Confocal images of the retinas of adult WT and ARHGAP18−/− mice stained using antibodies against PECAM for the vasculature. The image slices were stacked and merged post-acquisition to distinguish between the superficial (red) and the deeper layers (green). Bar 100 μm.
Fig 4.12. ARHGAP18<sup>−/−</sup> mice have delayed radial expansion of the retinal vasculature.

A. Retinas from P6 WT and ARHGAP18<sup>−/−</sup> mice were stained with isolectin B4 to visualise the developing vasculature and the whole retina was imaged by stereo microscopy. The yellow ring indicates the radial extension in the WT and is superimposed onto ARHGAP18<sup>−/−</sup> image. Bar 1 mm.

B-C. Quantification of the radial extension in B. The radial extension length was defined as the distance from the optic nerve to the angiogenic front and expressed in mm (C) or as a percentage of the total retinal length (D). Data represents mean ± SEM from n=32 WT and 24 ARHGAP18<sup>−/−</sup> mice. **** P<0.0001; t-test.
**Fig 4.13. Assessment of the quadrant plexus shows indifferent changes.**

A. Confocal images of the quadrant plexus of isolectin-B4 stained P6 retinas from WT and ARHGAP18⁻/⁻ mice. Bar 250 μm.

B. Analysis of the respective quadrant plexi in A. Green outline = analysed area; blue = vessel area; red = vessel skeleton; white dots = branch points; yellow crosses = loops.

C-E. Quantification of the vessel density (C), branch points (D), and loops (E) in whole quadrants of retinas. The measurements were averaged from at least 2 quadrants per retina and were normalised to the area of the quadrants. Data represents mean ± SEM from n=32 WT and 24 ARHGAP18⁻/⁻ mice. ns, non-significant; t-test.
Fig 4.14. Schematic for re-analysis of the central plexus.

A. A macro was designed for the automated re-analysis of the central plexus (Appendix A.1.2). The whole analysed retina was split into the individual parameters and re-merged with an altered colour scheme. A 380 x 380 μm region between two large vessels (arteries and veins) was selected and the individual parameters were re-analysed.
**Fig 4.15.** ARHGAP18<sup>−/−</sup> mice have increased vascularisation of the central plexus.

A. Confocal images of isolectin B4 stained quadrant plexi of P6 WT and ARHGAP18<sup>−/−</sup> retinas (top panels) and the respective re-analysed central plexi (bottom panels). Black = vessel area; yellow crosses = loops; red dots = single branch points; green dots = double branch points. Bar 250 μm (top panels), 100 μm (bottom panels).

B-D. Quantification of the vessel density (B), branch points (C), and loops (D) in the central plexus. The measurements were averaged from at least 2 quadrants per retina. Data represents mean ± SEM from n=32 WT and 24 ARHGAP18<sup>−/−</sup> mice. * P<0.05, ** P< 0.01; t-test.
Fig 4.16. ARHGAP18−/− mice have increased numbers of sprouts and filopodial extensions.
A. Confocal images of isolectin B4 stained angiogenic front of P6 WT and ARHGAP18−/− retinas. The sprouts were counted manually and indicated by the red dot. Bar 200 μm.
B. Quantification of the number of sprouts per mm of vessel length. The measurements were averaged from at least 2 quadrants per retina. Data represents mean ± SEM from n=32 WT and 24 ARHGAP18−/− mice. **** P<0.0001; t-test.
C. High magnification confocal images of the angiogenic sprouts. The EC sprouts from ARHGAP18−/− mice have more abundant and longer filopodial extensions. Bar 50 μm.
Fig 4.17. Pericyte coverage is unchanged in ARHGAP18⁻/⁻ retinas.
A. Confocal images of VE-cadherin (red) and NG2 (green) (pericytes) stained central plexi of P6 WT and ARHGAP18⁻/⁻ retinas. Scale 100 μm.
B. Analysis of pericyte coverage in A. A macro was designed for the automated analysis of pericyte coverage (Appendix A.1.3). The VE-cadherin stained EC junctions were filled to create an outline of the vessel area (red). The pericyte stain was thresholded (green) and overlaid onto the vessel image. Pericyte coverage represents the area in which the pericyte overlaps with the vessel (yellow) and is represented as a percentage of the total vessel area (red).
C. Quantification of pericyte coverage in WT and ARHGAP18⁻/⁻ retinas. Data represents mean ± SEM from n=5 WT and 5 ARHGAP18⁻/⁻ mice. Number represents the P value, non-significant; t-test.
Fig 4.18. ARHGAP18 knockdown promotes Akt activation following VEGF stimulation.
A. HUVECs were transfected with control or ARHGAP18 siRNAs, starved, stimulated with 50 ng/mL VEGF-A and whole cell lysates collected at the indicated time points. Immunoblots of p-Akt, and p-ERK1/2 to examine responsiveness to VEGF. ARHGAP18 immunoblotting confirmed knockdown. Total Akt and tubulin were used as loading controls. Representative of n=3 independent experiments.
Fig 4.19. ARHGAP18 knockdown promotes expression of TC genes.
A-E. HUVECs were transfected with control (■) or ARHGAP18 (■) siRNAs, starved, and stimulated with or without 50 ng/mL VEGF-A for 6 h. Total RNA was isolated, reverse transcribed and the mRNA level of ARHGAP18 (A), Dll4 (B), Flk1 (C), Flt4 (D), and Flt1 (E) relative to the normalising gene ACTB determined by qT-PCR. Data represents the mean ± SEM from n=3-4 independent experiments. ns, non-significant; * P<0.05; ** P<0.01; t-test. Statistical tests were performed on log2(fold change) values.
**Fig 4.20. ARHGAP18 knockdown ECs have increased preference for the TC position.**

A. Schematic of the tip cell competition assay. HUVECs were transfected with control or ARHGAP18 siRNAs for 1 d, labelled with CellTracker dyes and Hoescht, and mixed at 1:1 ratios. The actual distribution following mixing was determined and the cells were used to establish spheroid sprouts. The imaged sprouts were then assessed for the distribution of cell populations within the sprout and at the TC position.

B. Confocal image of mixed spheroid sprouts following 24 h stimulation with 25 ng/mL VEGF-A. Control cells (red) were labelled with CellTracker Orange and ARHGAP18 siRNA cells (green) labelled with CellTracker Green. Nuclei (blue) were labelled using Hoescht. Inset location indicated by dashed line. Bar 100 μm.

C-D. Quantification of the percentage contribution of sictrl (red) and siARHGAP18 (green) cells in sprouts (C) and in the TC position (D). Data represents the mean ± SEM from n=4 independent experiments. **, P<0.01; ****, P<0.001; t-test.
CHAPTER 5

ARHGAP18 - A RhoC GAP in Junctional Integrity
5.1. **INTRODUCTION**

In the previous chapter we established that the loss of ARHGAP18 results in a TC and hypersprouting phenotype. The main aim of this chapter was to identify the mechanism in which ARHGAP18 regulates EC sprouting.

We describe here that ARHGAP18 localizes to the junctions of angiogenic ECs *in vitro* and *in vivo*. This is associated with changes in junctional stability as ARHGAP18 transiently relocalises to the EC membrane periphery following junctional destabilisation. We postulate that this relocalisation is due to the GAP function of ARHGAP18, which we identify is targeting RhoC. Furthermore, loss of ARHGAP18 results in a contractile phenotype with active serrated EC junctions. The EC junctions have an established role regulating TC selection and vascular patterning. We establish here that ARHGAP18 regulates EC sprouting via its associated GAP dependent effects in regulating the EC junctions.

5.2. **RESULTS AND DISCUSSION**

5.2.1. **ARHGAP18 IS PREDOMINANTLY LOCALISED IN THE CYTOSOL OF RESTING ECs**

In order to gain insight into the mechanism of ARHGAP18 function, its localization was first assessed. We first optimized the IF staining in monolayer HUVECs using two different antibodies raised against the ARHGAP18 peptide: rabbit polyclonal antibodies, and mouse monoclonal antibodies (clone 2A3). We first tested the efficacy of staining of the two antibodies using formaldehyde fixed or methanol-acetone (1:1) fixed cells (Fig 5.1). Fixation with formaldehyde resulted in generally poor IF staining, with no notable difference in the signal between the control and ARHGAP18 cells (Fig 5.1 A). Furthermore, staining with the polyclonal antibodies resulted in non-specific staining of
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the cell nuclei. On the other hand, the methanol-acetone fixed cells stained with the monoclonal antibody had clear cytosolic signals, which were absent in the ARHGAP18 knockdown cells (Fig 5.1 B, top panels). Staining with the polyclonal antibodies did show a difference in staining, but were relatively weak. Subsequent ARHGAP18 IF staining on monolayer cells were performed using the monoclonal antibodies on methanol-acetone fixed cells.

The cells stained for ARHGAP18 were imaged by confocal microscopy to examine the localization of ARHGAP18 in the cell. We observed that ARHGAP18 was predominantly in the localized in the cytosol, and was present in the form of small puncta (Fig 5.2 A). There was some localization of ARHGAP18 in the cell nuclei, although the significance of this is unknown. However, in these resting ECs there was a distinct lack of ARHGAP18 in the cell junctions that had been stained with VE-cadherin. The ARHGAP18 knockdown ECs had substantially less staining, thus confirming the specificity of the antibody staining (Fig 5.2 A and B). Furthermore, staining with an isotype control antibody also had a lack of notable staining (Fig 5.2 B). Recent studies in the Vascular Biology laboratory have confirmed that ARHGAP18 is localized as small puncta throughout the cell cytosol (Lovalace, et al. in review). Using structured illumination microscopy, we have also observed that there is an abundance of ARHGAP18 puncta that align to the microtubules, and that this localisation may have implications in regulating microtubule stability as loss of ARHGAP18 leads to destabilized microtubule organization (Lovelace, et al. in review).

5.2.2. ARHGAP18 IS LOCALISED TO THE JUNCTIONS OF ANGIGENIC SPROUTS

It was hypothesized that since the absence of ARHGAP18 promotes a hypersprouting and TC phenotype, there would be a differential expression of ARHGAP18 in the TCs and
SCs. In order to test this, we examined the localization/expression of ARHGAP18 in *in vitro* sprouts that were stimulated with VEGFA and FGF-2 (Fig 5.3). VEGFA and FGF-2, as mentioned earlier, result in two distinct sprouting phenotypes with abundant but weak sprouts in the VEGF-induced sprouts, and fewer, longer and more stable sprouts in the FGF-2 induced sprouts. Notably, and surprisingly, we observed that ARHGAP18 is distinctly localized to the junctions of both the VEGF (Fig 5.3 A) and FGF-2 (Fig 5.3 B) sprouts. While the TCs and SCs still had abundant localization of ARHGAP18 in the cytosol, there was enriched localization of ARHGAP18 that co-localised with the EC junctions. On the other hand, contrary to the hypothesis, it did not appear that the expression of ARHGAP18 was different between the TCs versus the SCs in the spheroid sprouts.

**5.2.3. ARHGAP18 IS LOCALISED TO THE JUNCTIONS OF CAPILLARY AND ANGIOGENIC EC IN THE DEVELOPING RETINAL VASCULATURE**

We next aimed to confirm these observations using the *in vivo* retina model of angiogenesis. We first optimized the staining protocol using both the rabbit and mouse antibodies. Staining with the rabbit antibody resulted in a generally weak signal, and most notably the rabbit Ig control had substantial non-specific staining (Fig 5.4 A). Ideally, a rabbit antibody would be preferred as there are significant complications with the use of mouse antibodies to detect mouse antigens. However, the rabbit antibody had poor staining and therefore the mouse antibody staining protocol was optimized. We used a mouse on mouse blocking protocol to prevent detection of the endogenous Ig. In the absence of the endogenous Ig block, addition of the secondary antibody alone was able to detect substantial non-specific signals, which were further amplified in the presence of the ARHGAP18 or control primary antibodies (Fig 5.4 B, left panels). However, using a double amount of the mouse on mouse blocking reagent, coupled with an anti-mouse
secondary antibodies containing only the Fab fragments (lacking the Fc chains, and hence unable to bind the endogeneous Fc receptors), we were able to observe significant staining in the ARHGAP18 antibody stained retinas, that was absent in the isotype control (Fig 5.4 B, right panels). We confirmed that the staining was specific by using identical staining conditions for WT and ARHGAP18−/− retinas and observed that the signal in the knockout retinas was substantially reduced (Fig 5.4 C).

Examination of the staining of the WT retina revealed that there was highest expression of ARHGAP18 in the EC (Fig 5.5). However, we could also see expression of ARHGAP18 in the retinal axons and astrocytes (Fig 5.5 B, yellow and blue arrows) and the macrophages at the angiogenic front. Notably, we also observed that there was differential expression of ARHGAP18 in the different vessel subtypes, with higher expression of ARHGAP18 in the arteries and angiogenic front compared to the veins (Fig 5.5 A and B). Most importantly, there was abundant expression of ARHGAP18 in the EC junctions, as indicated by VE-cadherin staining (Fig 5.5 C), and this was more pronounced in the ECs in the capillary network and at the angiogenic front compared to the larger arterial and venous vessels (Fig 5.5 B).

It was hypothesised that the EC at the angiogenic front would be underexpressing ARHGAP18, as its loss promotes the hypersprouting phenotype. In fact, here we observe that ARHGAP18 is more highly expressed in the cells at the angiogenic front. This is consistent with two studies that examined the global gene expression profile of TCs versus SCs, and found that ARHGAP18 was upregulated 4 fold (del Toro et al., 2010) and 2.1 fold (Strasser et al., 2010) in the TCs. Furthermore in in vitro monolayer ECs stimulated with VEGF, we observe a 2 fold upregulation of ARHGAP18 at the 12 h and 24 h time points (Chang et al., 2014). These suggest that, while underexpression of ARHGAP18 promotes hypersprouting, there is an upregulation of ARHGAP18 in the
angiogenic ECs. We hypothesise that this expression is likely to occur as a negative feedback to control the extent of angiogenic sprouting. Most importantly however is the finding that ARHGAP18 relocalises to the EC junctions, and that this occurs more prominently in those of the angiogenic vessels compared to the larger more stable vessels.

5.2.4. ARHGAP18 transiently localises to the junctions of monolayer ECs following thrombin destabilisation

To address the possibility that ARHGAP18 is involved in stabilisation of vessels, we assessed the localization of ARHGAP18 in EC monolayers stimulated with thrombin, where the junctions are dynamically remodeled (Rabiet et al., 1996). This was first tested by expressing mCherry-ARHGAP18, (described above in section 3.2.11), and assessing the localisation of the tagged protein. By live-cell microscopy, we noticed that there was a rapid and transient relocalisation of the fusion protein at ~10 min following thrombin stimulation (data not shown). To confirm this, we stimulated ECs with thrombin and assessed the localisation of endogenous ARHGAP18 over 60 min (Fig 5.6 A). In resting EC, ARHGAP18 was distinctly absent from the EC junctional periphery, as indicated by staining with β-catenin. Within 2 min of thrombin stimulation, ARHGAP18 was localized to the fine filopodial extensions. At 5 min and 10 min following thrombin stimulation, a time where the junctions are zippered and disrupted (also termed active) and the monolayer highly permeable (Gamble et al., 2000), ARHGAP18 was distinctly localized to junctional edges of cell contacts, with a maximal relocalisation at 10 min. By 30 min and 60 min, where the junctions return to a straight and mature (also termed inactive) characteristic, ARHGAP18 was absent from the junctional edges and returned to the cytosol.

5.2.5. ARHGAP18 is a RhoC GAP that regulates EC junctional integrity

5.2.5.1. ARHGAP18 is a RhoC GAP
RhoGAPs are recruited to the cell membrane where they act to catalyse the inactivation of RhoGTPases. We hypothesized that this recruitment of ARHGAP18 to the junctions serves to inhibit the activity of its target RhoGTPase. ARHGAP18 has been previously shown to be a RhoA GAP in epithelial cells (Maeda et al., 2011), and as a Rho1 (RhoA homolog in Drosophila) GAP in Drosophila S2 cells (Neisch et al., 2013). In contrast to these studies, ARHGAP18 knockdown did not alter RhoA activation in basal or thrombin stimulated ECs (Fig 5.7 A). ARHGAP18 silencing also did not alter Rac-1 (lamellipodia), Cdc42 (filopodia) or RhoJ, the EC specific RhoGTPase (Fig 5.7 C-E). However, ARHGAP18 knockdown did have a small but reproducible and significant effect on promoting RhoC activation in both basal and thrombin stimulated EC (Fig 5.7 B). Notably, we observe highest activation of RhoC at 2 min following thrombin stimulation (Fig 5.7 F), and ARHGAP18 is recruited following this, thus suggesting that ARHGAP18 recruitment acts to restore basal RhoC activity levels.

To confirm this effect on RhoC, we examined the levels of active RhoA and RhoC following ARHGAP18 overexpression. As described in section 3.2.11, we designed a retroviral system for ARHGAP18 overexpression that did not induce EC senescence. However, given the time constraints for the resubmission of the manuscript, and that the retroviral production, and viral transduction and selection required a lot of time, a different approach was used to overexpress ARHGAP18 at low, non-senescence inducing levels. Using the adenovirus, we titred the levels of adenovirus to be 1/10th the normal levels and increased the efficiency of transduction by the addition of polybrene, a polycation that is normally used in retroviral transduction. This resulted in efficient transduction of the ECs (~70-80% compared to ~50% without polybrene) (Fig 5.8 A) and low level overexpression of ARHGAP18 (Fig 5.8 B) that did not induce EC senescence. We chose not to aim for EC senescence as it is associated with profound changes in the
cytoskeleton (large flattened morphology) that is known to be associated with changes in the activity of the RhoGTPases. With these conditions, we examined the active levels of RhoA and RhoC and found that while RhoA was unchanged (Fig 5.8 C), we observed a slight but significant suppression of RhoC activity (Fig 5.8 D), hence confirming the subtle effects of ARHGAP18 knockdown.

### 5.2.5.2. **ARHGAP18 regulates EC junctional integrity**

RhoC belongs to the Rho subfamily, that includes RhoA and RhoB and shares 92% sequence similarity with RhoA. One of the key roles of Rho is in cell contractility and in the regulation of EC junctional integrity (Papatriantafyllou, 2012; Sahai and Marshall, 2002). To determine whether this hyperactivation of RhoC induced by ARHGAP18 knockdown replicated the known functions of Rho, we assessed the integrity of the EC junctions and the arrangement of the actin cytoskeleton. While control cells generally had a linear arrangement of VE-cadherin, the ARHGAP18 knockdown cells had a relatively open zipper-like VE-cadherin distribution at the cell junctions (Fig 5.9 A, red arrows, and Fig 5.2 A), which is characteristic of weak remodeling junctions (Huveneers et al., 2012; Ngok et al., 2012). Furthermore, the ARHGAP18 knockdown EC displayed an increased amount of stress fibres (Fig 5.9 A, yellow arrows), indicating a more contractile phenotype. This matches the contractile phenotype observed during cell migration, where we observed disrupted proximal junctions (section 4.2.3, Fig 4.5 C), and is also consistent with Rho regulating the trailing edge of migrating cells (Ridley, 2001).

We next assessed the dynamic remodeling of the junctions following thrombin treatment (Fig 5.10 A). Prior to thrombin, we already observed that loss of ARHGAP18 results in the disrupted phenotype described above. Following thrombin treatment for 2 and 10 min, the ARHGAP18 knockdown cells have an exacerbated response to thrombin with further contractility and an increased formation of intercellular gaps. Furthermore, while the
control cells start to recover by 60 min (intact junctions) and completely return to baseline by 120 min (cortical F-actin arrangement), the ARHGAP18 knockdown cells still remain notably disrupted at these time points with substantial actin stress fibres and serrated EC junctions. To translate this to a functional readout, the integrity of the monolayers were assessed using a FITC-dextran permeability assay. Control or ARHGAP18 knockdown cells were cultured on transwell inserts and stimulated with or without thrombin in addition to FITC-dextran. A low concentration (0.3 U/mL) of thrombin was used to better extrapolate the differences between the cell populations, as the normal concentration (1 U/mL) results in a dramatic loss of barrier integrity in both populations. Consistent with the junctional phenotype, ARHGAP18 knockdown EC displayed increased basal and thrombin-induced FITC-dextran permeability (Fig 5.10 B).

5.2.5.3. The ARHGAP18 junctional phenotype is dependent on RhoC

We next aimed to determine whether the junctional phenotype induced by ARHGAP18 knockdown was through the hyperactivation of RhoC. In order to do this, we chose a rescue approach whereby ARHGAP18 knockdown cells were transfected with siRNAs targeting RhoC. As a control, and to confirm our effects are through RhoC and not the previously reported RhoA, we also assessed the effects of RhoA knockdown. Firstly, we tested the efficacy of a double knockdown protocol, in which cells were first transfected with ARHGAP18 siRNAs then followed with the Rho siRNAs the next day. By assessing the expression of ARHGAP18, RhoA and RhoC by immunoblotting, it was confirmed that the procedure worked with efficient knockdown of each of the Rho proteins in combination with ARHGAP18 (Fig 5.11 A). Interestingly, knockdown of either RhoA or RhoC results in an upregulation of the other respective Rho protein and likely occurs as a compensatory mechanism for the loss of the original Rho.
We chose the junctional and cytoskeletal morphology as the readout of the rescue assay, as this was a well defined phenotype of ARHGAP18, and is an established function of the Rho proteins (Fig 5.11 B). Double control knockdown resulted in a normal cell phenotype with mainly straight junctions and limited actin stress fibres. Surprisingly, in the control cells, knockdown of RhoA resulted in more serrated VE-cadherin localisation, whereas RhoC had more mature junctions compared to control. Strikingly, knockdown of RhoC in ARHGAP18 knockdown cells was able to revert the disrupted phenotype of ARHGAP18 knockdown alone, and rescue the phenotype to that of control cells with predominantly cortical actin with relatively straight junctions. In contrast, RhoA knockdown in ARHGAP18 silenced cells exacerbated the phenotype with further disruption of the junctions and cytoskeleton.

These results conclusively indicated that ARHGAP18 acts as a RhoC GAP to regulate EC junctions and cell contractility. Despite the small magnitude of ARHGAP18’s regulation of RhoC activity, this was sufficient to induce a disrupted cell phenotype that could be rescued by modulating RhoC levels. One of the possible reasons for the small magnitude of regulation is due to the high level of redundancy that occurs within the GAP proteins, where there are over 80 GAPs that regulate the 20 RhoGTPases. In this instance, the consequences of ARHGAP18 loss can be limited by the compensatory functions of the other GAPs. While we have described here that ARHGAP18 regulates RhoC and not RhoA, Rac1, Cdc42, or RhoJ, we do not dismiss the possibility that ARHGAP18 may be regulating another RhoGTPase that we have not tested. However, our results clearly indicate that ARHGAP18 does not target the previously reported RhoA, but acts to inhibit RhoC in ECs. Notably, such epithelial-endothelial differences in RhoGAP specificity have also been demonstrated in other RhoGAP family members, such as ARHGAP24/VasGAP/FliGAP, which targets Rac1 in podocytes (Akilesh et al., 2011),
Rac1 and Cdc42 in HeLa cells (Lavelin and Geiger, 2005), and Rho in ECs (Su et al., 2004). While RhoC belongs to the same family as RhoA, it is functionally distinct. Most importantly, it has been shown that RhoC promotes, whereas RhoA inhibits cell invasion and migration (Bellovin et al., 2006; Hakem et al., 2005; Heasman and Ridley, 2008; Simpson et al., 2004; van Golen et al., 2000b; Wheeler and Ridley, 2004). These functional differences have been attributed to the activation of the downstream activators, such as ROCK, Dia (Sahai and Marshall, 2002), and formins (Kitzing et al., 2010; Vega et al., 2011). In particular, while RhoA and RhoC both activate ROCK, RhoC has higher affinity for ROCK (Sahai and Marshall, 2002). Further, overexpression of RhoC, but not RhoA, results in ROCK-dependent disruption of the AJs, while RhoA activation of Dia results in stabilization of the AJs (Sahai and Marshall, 2002). Consistent with this, we observe that RhoA knockdown disrupts the cell junctions, whereas knockdown of RhoC stabilizes the AJs and rescues the disrupted phenotype of ARHGAP18 knockdown.

### 5.2.6. **In vivo studies of junctional regulation**

#### 5.2.6.1. Loss of ARHGAP18 results in disrupted VE-cadherin distribution in the retinal capillary ECs

We next aimed to confirm the *in vitro* effects of ARHGAP18 on junctional integrity *in vivo*. We first used the retinal angiogenesis model, which we have previously found to result in a hypersprouting phenotype, and assessed the EC junctions by VE-cadherin staining. While the junctions of the capillary ECs in the WT mice were predominantly straight, the junctions in the ARHGAP18*−/−* mice had a relatively serrated staining of VE-cadherin (Fig 5.12, red arrows), consistent with the *in vitro* findings.

#### 5.2.6.2. ARHGAP18 in vascular integrity

We next investigated whether the junctional phenotypes and the *in vitro* permeability results translated to vascular leak *in vivo*. In the zebrafish model, we observed that there
was significant hindbrain, yolk sac and cardiac oedema (Fig 4.7 C), and this was unlikely due to changes in the lymphatics. To test this in a mouse model, we first used the Miles assay, whereby Evan’s blue was injected intravenously, VEGF-A injected into the skin of mice, and quantifying the amount of dye leakage. Confoundingly, we observed that the \textit{ARHGAP18}\textsuperscript{-/-} mice had a significantly reduced amount of vascular leak (Fig 5.13 A). However, there are some caveats with the use of the Miles assay, in particular, the assay requires hemodynamics for the distribution of the dye (Nagy et al., 2008). In addition to hyperpermeability, VEGF-A also causes vasodilation, which increases the blood flow in the microvascular beds and in turn can increase solute flux across the vessel wall (Nagy et al., 2008). Furthermore, the assay can be affected by changes in the density of the capillary beds. As we have not investigated the changes in hemodynamics associated with ARHGAP18 loss, these potential effects may be the cause of the conflicting results.

In collaboration with Dr. Arby Abtin, Immune Imaging Laboratory, Centenary Institute, we next investigated the junctional integrity using a mouse model of acute ear inflammation. The ears of \textit{WT} or \textit{ARHGAP18}\textsuperscript{-/-} mice were treated topically with the irritant, croton oil (Shwaireb, 1995) and then assessed for changes in oedema and neutrophil infiltration. After 24 h treatment, the \textit{ARHGAP18}\textsuperscript{-/-} mice had significantly increased oedema, as measured by the ear thickness (Fig 5.14 A). These ears were then harvested for flow cytometric quantification of neutrophils based on a CD45\textsuperscript{+} Ly6G\textsuperscript{+} CD11b\textsuperscript{+} profile (Abtin et al., 2014). The gating strategy is depicted in Fig 5.14 B. Briefly, single live cells were gated on CD45\textsuperscript{+}, then the double Ly6G\textsuperscript{+} CD11b\textsuperscript{+} cell population selected. Despite the single cell gate, this still results in cell doublets, and these populations were taken into account when quantifying the absolute numbers of infiltrated neutrophils. In the absence of croton oil, there was minimal infiltration of neutrophils in \textit{WT} mice, but there were significantly more neutrophils in the \textit{ARHGAP18}\textsuperscript{-/-} mice (Fig
5.14 C and D). Following croton oil treatment, there was a ~2.4 fold increase in the number of infiltrated neutrophils. These results indicate that there was increased vascular leak into the ear following inflammatory stimulus, and increased neutrophil infiltration into the inflammatory site. However, one major limitation of this result is that the \textit{ARHGAP18}\textsuperscript{-/-} mouse is a global knockout, and therefore we are unable to determine whether the increased neutrophil infiltration is due to the effects of ARHGAP18 on neutrophil or endothelial function. This would be resolved following the generation of the EC specific KO as described above in chapter 7.

\subsection*{5.2.7. Loss of ARHGAP18 promotes tumour growth and vascularisation}

The results suggested that ARHGAP18 is important dualistically in limiting the sprouting phenotype and maintaining junctional integrity. The tumour vasculature is characterized by an abundance of tortuous and leaky vessels. Therefore, the development of the tumours and the structure of the tumour vasculature were assessed by Yang Zhao, PhD student in the Vascular Biology laboratory. B16F10 melanoma cells were injected subcutaneously into the flank of WT and \textit{ARHGAP18}\textsuperscript{-/-} mice and monitored for the development of the tumours. The tumours in the \textit{ARHGAP18}\textsuperscript{-/-} mice developed significantly more rapidly compared to the WT mice (Fig 5.15 A). Furthermore, the tumours were harvested and found to be more highly vascularized in the \textit{ARHGAP18}\textsuperscript{-/-} mice (Fig 5.15 B and C). Ideally, it would be of much interest to determine whether these tumour vessels were more leaky, as would be expected.

\section*{5.3. Summary and conclusions}

It has been recently established that the EC junctions play a major role in the dynamic selection of TCs and vascular patterning (Bentley et al., 2014). We have identified here that the hypersprouting phenotype is closely associated with the role of ARHGAP18 in
regulating the EC junctions. During sprouting, we observe that ARHGAP18 is distinctly localized to the junctions of angiogenic ECs, and we associate this with its function as a GAP protein. Here its effects on RhoC act to maintain junctional integrity to suppress the angiogenic response. In the absence of ARHGAP18, the translocation of ARHGAP18 to the membrane to regulate RhoC is prevented, resulting in elevated RhoC activity, and junctional disruption. Based on other studies, these active junctions are highly associated with a TC phenotype, DLL4 expression, high shuffling capacity and an overall hypersprouting phenotype (Bentley et al., 2014). A further detailed overview of the proposed mechanism and pathway is discussed in chapter 7.
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FIGURES
Fig 5.1. Optimisation of ARHGAP18 staining in HUVECs.
A-B. HUVECs transfected with control or ARHAGP18 siRNAs were cultured on chamber slides, fixed with formaldehyde (A) or methanol-acetone (B), stained with mouse monoclonal or rabbit polyclonal antibodies against ARHGAP18 and imaged by widefield fluorescence microscopy. NT = non-siRNA transfected cell. Cells fixed with formaldehyde poorly stain for ARHGAP18. The monoclonal is more efficient at staining for ARHGAP18, as there is clearer distinction of the fluorescence signal in control and knockdown cells. Bar 50 μm.
Fig 5.2. ARHGAP18 is localised predominantly in the cytosol of resting ECs.

A. Confocal images of monolayer ECs transfected with control or ARHGAP18 siRNAs stained for ARHGAP18 (green), VE-cadherin (red) and nuclei counterstained with DAPI (blue). ARHGAP18 is predominantly localised in the cytosol with some nuclei staining but is notably absent from the periphery of resting ECs. Bar 25 μm.

B. Validation of ARHGAP18 staining. Control or ARHGAP18 siRNA transfected ECs were stained with antibodies against ARHGAP18 or an isotype control antibody. ARHGAP18 siRNA transfected ECs show reduced staining and the signal is absent in the isotype control. NT = non-transfected cell. Bar 50 μm
Fig 5.3. ARHGAP18 is localised to junctions of angiogenic sprouts.
A-B. EC spheroids were stimulated with VEGF (A) or FGF-2 (B), fixed and stained for ARHGAP18 (red) and junctional markers (VE-cadherin or PECAM) (green), nuclei counterstained with DAPI (blue) and imaged by confocal microscopy. ARHGAP18 localises to the junctions of the angiogenic sprouts (arrows). Bar 50 μm.
Fig 5.4. Optimisation of ARHGAP18 staining in mouse retina.
A. Retinas from P6 WT mice were stained with rabbit polyclonal antibodies raised against ARHGAP18, rabbit isotype control or a no primary antibody control and imaged by confocal microscopy.
B. Retinas from P6 WT mice were pre-incubated with or without an endogenous Ig block, stained with mouse monoclonal antibodies raised against ARHGAP18, mouse isotype control or a no primary antibody control and imaged by confocal.
C. Retinas from P6 WT or ARHGAP18−/− mice were stained with the ARHGAP18 monoclonal antibody (red) and VE-cadherin (green) to visualise the vasculature and imaged by confocal.
Fig 5.5. ARHGAP18 is localised to the junctions of capillary and angiogenic EC in the developing retinal vasculature.

A. Low magnification image of P6 WT retinas stained for ARHGAP18 (red) and NG2 (green) (pericytes). A=artery, V=vein, CP=capillary plexus, AF=angiogenic front. Bar 500 μm.

B. Localisation of ARHGAP18 in the different vessel subtypes. ARHGAP18 is also expressed in the retinal axons (yellow arrows) and astrocytes (blue arrows). ARHGAP18 (red) colocalises with VE-cadherin (green) in ECs in the capillary plexus and angiogenic front (white arrows) but not in the larger arterial or venous vessels. Bar 25 μm.

C. High magnification of ARHGAP18 staining in the capillary plexus showing colocalisation with VE-cadherin (arrows). Bar 10 μm.
Fig 5.6. ARHGAP18 transiently localises to the junctions of monolayer ECs following thrombin stimulation.

A. Confocal images of HUVECs stained for ARHGAP18 (green), β-catenin (red) and nuclei counterstained with DAPI following stimulation with thrombin (1 U/mL) for the indicated times. ARHGAP18 localises to the retracting EC extensions at 2 min, to the EC junctional periphery at 10 min and returns to the cytosol at 30 and 60 min following thrombin simulation. Bar main, 25 μm; magnified, 10 μm.
Fig 5.7. ARHGAP18 knockdown increases basal and thrombin-stimulated RhoC activity, but does not alter the activity of other Rho GTPases.

A-E. HUVECs transfected with control (□) or ARHGAP18 (■) siRNAs were stimulated with or without thrombin (1 U/mL) and lysed. Active RhoA (A), RhoC (B), Rac1 (C), Cdc42 (D), and RhoJ (E) levels were determined by GLISA. Data represents the mean ± SEM from n=5 (A and B), 3 (C), 2 (D and E) independent experiments. ns, non-significant; *, P<0.05; t-test.

F. Time course of RhoC activation following thrombin treatment. HUVECs were stimulated with thrombin (1 U/mL) and the levels of active RhoC determined by GLISA at the indicated time points. RhoC is maximally activated following 2 min of thrombin stimulation.
Fig 5.8. ARHGAP18 overexpression suppresses RhoC but not RhoA activity.

A. Efficiency of HUVECs transduced with empty vector (AdEV) and AdARHGAP18 adenoviruses for 24 h in the presence of polybrene.

B. Confirmation of ARHGAP18 overexpression. Immunoblots of ARHGAP18 and actin as the loading control from HUVECs transduced with AdEV and AdARHGAP18.

C-D. HUVECs transduced with AdEV (□) or AdARHGAP18 (■) adenoviruses were stimulated with or without thrombin (1 U/mL) and lysed. Active RhoA (C) and RhoC (D) levels were determined by GLISA. Data represents the mean ± SEM from n=3 independent experiments. ns, non-significant; *, P<0.05; t-test.
Fig 5.9. ARHGAP18 silencing induces stress fibre formation and junctional disruption *in vitro*. A. Confocal images of HUVECs transfected with ctrl or ARHGAP18 siRNAs and stained for VE-cadherin (green) and F-actin (red) and nuclei counterstained with DAPI (blue). Relative to ctrl, ARHGAP18 knockdown EC display more pronounced stress fibres (yellow arrows) and zippered appearance of VE-cadherin (red arrows). Bar 25 μm.
Fig 5.10. ARHGAP18 silencing exacerbates the response and delays the recovery to thrombin stimulation and promotes basal and thrombin-induced vascular leak.

A. Confocal images of HUVECs transfected with control or ARHGAP18 siRNAs, stimulated with thrombin (1U/mL) for the indicated times, fixed and stained for F-actin (red), VE-cadherin (green) and nuclei counterstained with DAPI (blue). ARHGAP18 knockdown cells display increased junctional disruption and cell retraction following thrombin stimulation and are delayed in the return to the resting state. Bar 25 μm.

B. HUVECs transfected with control (grey) or ARHGAP18 (black) siRNAs were seeded in transwells, stimulated with (dashed line) or without (solid line) thrombin (0.3 U/mL) in the presence of FITC-dextran. The FITC-dextran in media from the bottom chamber was collected, measured at the indicated time points and data normalised relative to 0 min. Data represents the mean ± SEM from n=8 experiments. * P<0.05, **** P<0.0001; two-way ANOVA, Sidak’s post-test (at 60 min).
Fig 5.11. RhoC, but not RhoA, knockdown rescues the junctional and cytoskeletal phenotype induced by ARHGAP18 knockdown

A. Confirmation of double knockdown of ARHGAP18 and RhoA or RhoC. HUVECs were transfected with ARHGAP18 or control siRNAs for 1 d, then transfected with RhoA, RhoC or control siRNAs for an additional 2 d. The lysates were collected and immunoblotted for ARHGAP18, RhoA, RhoC and the loading control actin.

B. RhoC, but not RhoA rescues the ARHGAP18 knockdown phenotype. Confocal images of HUVECs double transfected with the indicated siRNAs, fixed and stained for VE-cadherin (green), F-actin (red) and nuclei counterstained with DAPI (blue). Knockdown of RhoA exacerbates the junctional and cytoskeletal phenotype, while knockdown of RhoC reverts the ARHGAP18 phenotype to that of the control. Bar 25 μm.
Fig 5.12. Loss of ARHGAP18 alters VE-cadherin junctional distribution in the retinal capillary EC.
A. Confocal images of retinal capillary EC from P6 WT and ARHGAP18<sup>−/−</sup> retinas fixed and stained for VE-cadherin. Relative to the tight distribution of VE-cadherin in the WT EC junctions, ARHGAP18 loss resulted in more diffuse distribution of VE-cadherin (red arrows). Bar 10 μm.
Fig 5.13. Loss of ARHGAP18 results in decreased vascular leak in vivo.
A. WT and ARHGAP18⁻/⁻ mice were subjected to a Miles assay. The mice were injected intravenously with Evan's blue for 30 min then injected intradermally with PBS or VEGF-A (10 ng) for 30 min. The skin was dissected and the Evan's blue eluted and measured. Data represents the mean ± SEM from n=17 mice per group. **, P<0.01; t-test.

Performed by Ying Lu
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A

![Bar graph showing ear thickness (mm) for Vehicle and Croton Oil treatments.](image)

B

![Flow cytometry plots for Vehicle and Croton Oil treatments.](image)

C

![Flow cytometry plots for CD45, FSC-A, Ly6G, and CD11b for Vehicle, WT, and GAP18^{-/-} samples.](image)

D

![Bar graph showing neutrophil count for Vehicle and Croton Oil treatments.](image)
Fig 5.14. *ARHGAP18*−/− mice have increased vascular oedema and neutrophil infiltration in response to croton oil

A. Loss of ARHGAP18 results in increased ear oedema following inflammatory stimulus. *WT* and *ARHGAP18*−/− mice were treated topically with 2.5% croton oil or vehicle control for 24 h and the ear thickness measured using calipers. Data represents the mean ± SEM from n=9 mice per group. *, P<0.05; t-test.

B. Gating strategy for the identification of neutrophils by flow cytometry. The cell populations were first gated to remove doublets and dead cells. The CD45+ population was then gated for the double Ly6G+ CD11b+ population, which represent the neutrophils. The neutrophils were regated by size to identify the single and double cell populations.

C. Representative flow cytometric plots of *WT* and *ARHGAP18*−/− populations treated with vehicle or croton oil. Numbers represent the percentage of the population.

D. Quantification of infiltrated neutrophils in *WT* and *ARHGAP18*−/− mice treated with vehicle or croton oil for 24 h. Data represents the mean ± SEM from n=9 mice per group. ***, P<0.001; *, P<0.05; t-test.

Performed in collaboration with Dr. Arby Abtin and Dr. Angelina Lay
Fig 5.15. ARHGAP18 loss promotes tumour growth and vascularisation.
A. Loss of ARHGAP18 promotes tumour growth. Tumour volumes were determined in WT and ARHGAP18/- mice following subcutaneous injection of B16F10 melanoma cells. Data represents the mean ± SEM from n=5 mice per group. ****, P<0.0001; *, P<0.05; two-way ANOVA, Sidak’s post-test.
B. Loss of ARHGAP18 promotes tumour vascularisation. Representative confocal images of tumour sections from WT and ARHGAP18/- mice stained for CD31 (red). Bar 50 μm.
C. Quantification of tumour vascularisation in B. Data represents the mean ± SEM from n=8 WT and 7 ARHGAP18/- mice. *, P<0.05; t-test.

Performed by Yang Zhao
CHAPTER 6

REGULATION OF ARHGAP18 TRANSLOCATION
6.1. INTRODUCTION

In the previous chapter, we established that one of the crucial mechanisms that regulate ARHGAP18 function is in its relocalisation to EC junctions. In vitro this occurs as a very rapid response to thrombin stimulation and we postulate that this occurs via post-translational modifications of ARHGAP18. This final chapter is aimed at elucidating the molecular mechanisms that regulate this and focuses on two aspects: novel protein-protein interactors and phosphorylation. The data presented in this chapter is relatively preliminary and will require a substantial amount of further investigation.

6.2. RESULTS AND DISCUSSION

6.2.1. ARHGAP18 DOES NOT INTERACT WITH THE ERM PROTEINS

We first assessed whether there were any potential protein-protein interactions that could regulate ARHGAP18 translocation. Using protein interaction databases, we identified a single potential partner, MPP6, an RNA binding protein (string-db.org). By yeast two-hybrid screening, the vascular biology laboratory identified a novel putative target in Bif-1/Endothelin B1 (Mai Tran, unpublished data), a protein involved in membrane curvature. It remains to be determined as to whether ARHGAP18 and Bif1 interact by coIP and the overall importance of this protein. Assessment of the protein domains of ARHGAP18 revealed that there was a single RhoGAP domain, and no notable protein interaction domains (e.g. SH2, SH3, proline-rich, PDZ) (see section 1.6.1.1).

The recent publication by (Neisch et al., 2013) in Drosophila recently provided some insight into ARHGAP18 function and translocation. It was found that Conundrum, the ARHGAP18 orthologue, localises to the cell cortex, the specialized layer where the plasma membrane and the cortical actin cytoskeleton interact (Bretscher et al., 2002), where it acts as a Rho1 GAP. This was found to be dependent on the interaction with the
ezrin-radixin-moesin (ERM) protein, moesin, which is the sole ERM gene expressed in Drosophila. These ERM proteins act as anchors that interact with the plasma membrane and the actin cytoskeleton and can control Rho activation (Bretscher et al., 2002; Fehon et al., 2010). We therefore hypothesized that a similar situation may occur in mammalian cells. In ECs in particular we argued that during thrombin stimulation, ARHGAP18 translocates to the cell cortex, and that this is dependent on the ERM proteins.

To determine whether there was an involvement of the ERM proteins, we took two approaches: colocalisation by IF, and interaction by coIP. Firstly, we examined whether there was colocalisation of the two proteins in the presence and absence of thrombin treatment for 10 min, which described above results in the translocation of ARHGAP18 to the cell membrane. Notably, staining with the ERM antibody, which detects all three family members showed a predominantly cortical actin-like localisation of the proteins, with organization into the filament structures (Fig 6.1 A). However, this was not similar to ARHGAP18 in the presence nor the absence of thrombin treatment. Staining with the moesin antibody revealed a more punctate localisation of moesin, but was absent from the membrane following thrombin stimulation (Fig 6.1 A, right panels).

To confirm this negative result, we examined the potential interaction between the ERM proteins and ARHGAP18 by coIP. ARHGAP18 was pulled down from total cell lysates, consisting of a pool of non-treated and thrombin treated cells, and immunoblotted for the ERM proteins. While there was efficient pulldown, which was also quite specific based on the absence of the protein in the isotype control antibody pulldown, there was no evidence of any of the ERM proteins (Fig 6.1 B). We confirmed that the ERM proteins were able to be detected by the antibody using the WCL. Comparison of the protein sequences suggests that the lack of interactions is highly likely to be intrinsic differences between the Drosophila and mammalian ARHGAP18 proteins (Fig 6.1 C). While
alignment of the whole protein shows a 34% sequence similarity to the human ARHGAP18 sequence, there is much higher similarity (55%) in the GAP domain. On the other hand, the moesin minimal interaction domain appears to be a Drosophila specific domain as the similarity is significantly lower, at 28% which is even lower than the whole protein alignment. Furthermore, phylogenetic analysis of RhoGAP proteins has also demonstrated that Conu displays higher homology to ARHGAP28 and ARHGAP40 than to ARHGAP18 (Porazinski et al., 2015). (Neisch et al., 2013) also describes the presence of an N-terminal sterile alpha motif (SAM) domain, which like the GAP domain is more highly conserved between the species. SAM domains exhibit diverse protein-protein interactions, including self-associations, the binding to SAM and non-SAM domains and also in binding RNA (Kim and Bowie, 2003), which gives little insight into the function of ARHGAP18.

6.2.2. ARHGAP18 COLOCALISES AND CO-IMMUNOPRECIPITATES WITH CORTACTIN

We next examined the potential interaction with the cortical actin binding protein cortactin. Cortactin has been shown regulate cell migration via its interaction with the actin cytoskeleton (Katsube et al., 2004) and regulators of actin polymerization such as N-WASP, ARP2/3 and MLC kinase (Desmarais et al., 2009; Dudek et al., 2002; Kowalski et al., 2005; Oser et al., 2009; Weaver et al., 2002). Cortactin localizes to the cell periphery following stimulation with S1P and thrombin (Vouret-Craviari et al., 2002) and has been further shown to interact with RhoGAP proteins, such as BPGAP1 (Lua and Low, 2004). We therefore hypothesized that the translocation of ARHGAP18 to the cell periphery is mediated via its interaction with cortactin and that this is crucial in regulating the function of ARHGAP18 as a RhoGAP and in controlling junctions and sprouting.
In resting cells, cortactin was localized predominantly in the cytosol like punctate dots, much like ARHGAP18 (Fig 6.2, left panels). Interestingly, following treatment with thrombin, we observed that cortactin also translocates to the junctional periphery, and shows abundant colocalisation with ARHGAP18 (Fig 6.2 A, white arrows). However, we also observe that there were also cells which had cortactin translocating to the junctions despite an absence of ARHGAP18 colocalisation (Fig 6.2A, yellow arrows).

Next, we examined whether there was an interaction by co-IP. Strikingly, pulldown of ARHGAP18 revealed an abundance of cortactin that was immunoprecipitated, which was absent from the control antibody (Fig 6.2 B). Given that ARHGAP18 transiently localizes to the cell junctions following thrombin stimulation, it was next assessed whether the interaction between cortactin and ARHGAP18 is dynamically altered during thrombin destabilization. Interestingly, the interaction between ARHGAP18 and cortactin was not changed (Fig 6.2 C). This potentially indicates that ARHGAP18 exists as a protein complex with cortactin that is shuttled to and from the membrane during the thrombin-induced dynamic remodeling.

To confirm this interaction, a pulldown of cortactin was next performed to determine whether ARHGAP18 could be co-immunoprecipitated. While ARHGAP18 could be detected, it was surprisingly being detected at a lower molecular weight, that being ~73 kDa (Fig 6.2 D). While we and (Maeda et al., 2011) have previously identified a smaller isoform of ARHGAP18, this would be the first indication of a potential functional significance of this isoform. One potential explanation is that ARHGAP18 undergoes post-translation cleavage in order to exist in a complex with cortactin. In fact, this may suggest that most of the ARHGAP18 exists in a dormant state, which requires cleavage to allow interaction with cortactin and/or expose the active domains of ARHGAP18. In support of this, the Vascular Biology laboratory have recently discovered that cells
overexpressing different truncations of ARHGAP18 have vastly different cell phenotype (Mai Tran and Julie Hunter, unpublished data). While the full-length ARHGAP18 and C-terminal truncations have minimal effects on the cell phenotype, a N-terminal ARHGAP18 truncation (expressing amino acids 296-663) results in a prominent rounded cell morphology with extensive cell protrusions. This is also dependent on the RhoGAP domain, as introduction of the R365A mutation into the GAP domain prevents this phenotype. Thus, this potentially suggests that the N-terminal region of ARHGAP18 acts as an inhibitor of its GAP function, possibly by obscuring the active site or interaction of the domain with the downstream GTPase, and that the cleavage of this region is required for its function (Dr. Aaron McGrath and Dr. Mika Jormakka, unpublished data). While this is interesting, it would require extensive further study to extrapolate the intricacies of the system.

6.2.3. ARHGAP18 TRANSLOCATION TO THE JUNCTIONAL PERIPHERY IS DEPENDENT ON CORTACTIN

We next aimed to determine whether the translocation of ARHGAP18 or cortactin was dependent on the respective protein. Given its interaction, and especially since cortactin appeared to bind to the ‘functional’ isoform of ARHGAP18, it was hypothesized that cortactin is required for the trafficking of ARHGAP18 to the membrane periphery. In order to investigate this, we examined the localisation of ARHGAP18 following thrombin stimulation in the presence or absence of cortactin. Firstly, knockdown of cortactin by siRNA transfection was optimized in HUVECs. Interestingly, like ARHGAP18, we observed that transfection with one of the siRNAs, siCTTN1, resulted in significant cell elongation, that was absent with the other two siRNAs tested (Fig 6.3 A). We therefore attributed these effects with this siRNA as non-specific effects, and therefore siCTTN1 was not used for downstream experiments. Transfection with all three siRNAs resulted in
efficient knockdown of cortactin, and two of the three siRNAs notably resulted in downregulation of ARHGAP18 (Fig 6.3 B), which may or may not be due to off-target effects of the siRNAs.

We next investigated the thrombin-induced localisation of ARHGAP18 and cortactin following ARHGAP18 or cortactin knockdown. Cortactin knockdown was achieved either individually (siCTTN2 or siCTTN3) or through a pool of the two siRNAs. The effects of the individual siRNAs were similar to that of the pooled siRNA, but had more inefficient knockdown and hence the pooled siRNA results are the only ones shown. In the control knockdown cells, we observed that the cells had a contracted cell phenotype with notable localisation of both ARHGAP18 and cortactin in the cell periphery (Fig 6.4 A, left panels). In the ARHGAP18 knockdown cell, we observed that despite the lack of ARHGAP18 expression, there was still an abundance of cells that had relocalised cortactin (Fig 6.4, middle panels, white arrows). A single non-transfected cell served as a control that clearly shows translocated ARHGAP18 that co-localises with cortactin. On the other hand, knockdown of cortactin resulted in an absence of ARHGAP18 translocating to the cell periphery (Fig 6.4, right panels). Furthermore, consistent with the immunoblots, knockdown of cortactin also downregulated expression of ARHGAP18. This downregulation of ARHGAP18 by immunofluorescence staining was also observed in siCTTN3 transfected cells (data not shown), suggesting that cortactin knockdown may indeed be downregulating ARHGAP18 and not be a result of off-target siRNA effects. Overall, this indicates that cortactin serves as a critical factor to traffic ARHGAP18, whereas ARHGAP18 is not essential for cortactin movement.

6.2.4. **Phosphorylation of ARHGAP18**

6.2.4.1. **Identification of putative phosphorylation sites**
Phosphorylation of proteins is a major mechanism that regulates protein function and localisation. We used Phosphosite (phosphosite.org) to identify putative ARHGAP18 phosphorylation sites (Fig 6.5 A). This gathers phospho-protein data from other publications, aligns the protein sequence from the mouse and human and identifies conserved serine, threonine and tyrosine residues of known phospho motifs. In the human sequence, 8 potential sites were identified that were all notably excluded from the GAP domain, but congregated at the N- and C-termini of ARHGAP18. Furthermore, out of these 8 putative sites, 3 of them (S66, T154, S610) were of significant interest as they contain the RXXS*/T* motif, which is a substrate for several kinases including Akt, PKA, PKC and also ROCK (Kang et al., 2007). Furthermore, of these 3 sites, two of them also contain the S*/T*Q motif, which is a substrate for ATM/ATR phosphorylation (Stokes et al., 2007). ATM/ATR are important in the context of DNA damage and oxidative stress, and may be implicated in the regulation of ARHGAP18 in oxidative stress-induced senescence (Coleman et al., 2010; Powter et al., 2015). However, this would be the subject of further study, but not in the context of this project.

6.2.4.2. The ARHGAP18 is phosphorylated following thrombin stimulation

In order to determine whether the RXXS*/T* sites were phosphorylated, HUVEC lysates were immunoprecipitated using the ARHGAP18 or control antibodies and immunoblotted using the phosphor-ser/thr Akt substrate antibody. This antibody specifically recognizes the RXXS*/T* motif only when phosphorylated at the ser or thr positions with arg at the -3 position. While there was an abundance of phosphorylated proteins in the WCL samples, the ARHGAP18 pulldown samples specifically identified a single ~73 kDa protein, that was absent in the isotype control pulldown (Fig 6.5 B). Notably, this is the same size as that in the cortactin pulldown (Fig 6.2 A), which we speculate is due to post-translational cleavage. We next investigated whether the phosphorylation of the
ARHGAP18 motif was altered by thrombin stimulation. Remarkably, we observed a dynamic hyper-phosphorylation of this motif following thrombin stimulation with a maximal phosphorylation at 10 min before returning to baseline at 60 min (Fig 6.5 C). This interestingly is the same time point at which there is maximal translocation of ARHGAP18 to the cell membrane (Fig 5.6 A). Therefore, we hypothesized that this phosphorylation is required for the translocation of ARHGAP18. In order to definitively confirm this, we would need to mutate the potential phospho sites, however this is outside the scope of this thesis and would be for future investigation.

6.2.4.3. The thrombin-induced phosphorylation of ARHGAP18 is not dependent on Akt

We next sought to identify the kinase that was regulating the phosphorylation of ARHGAP18 and to determine whether this was driving ARHGAP18 translocation. Firstly the effects of Akt were tested by treatment with or without the PI3K inhibitor, LY294002. Notably in basal cells there was reduced phosphorylation of ARHGAP18 following LY294002 treatment (Fig 6.6 A). However, in thrombin-stimulated cells, there was hyperphosphorylation of ARHGAP18. WCL were run as a control for LY294002 efficacy, and notably indicated that Akt is in fact hypoactivated following thrombin stimulation. To confirm this, the activation of Akt and ERK were assessed in a time course following thrombin stimulation (Fig 6.6 B). While ERK displays transient activation, Akt is markedly hypoactivated following thrombin stimulation. This therefore indicates that it is highly unlikely that Akt is the kinase that phosphorylates ARHGAP18 following thrombin stimulation. To confirm this effect, we assessed ARHGAP18 translocation in the presence or absence of the inhibitor, and found no difference in ARHGAP18 translocation following Akt inhibition (Fig 6.6 C).
6.2.4.4. **ROCK inhibition suppresses phosphorylation of ARHGAP18 and prevents ARHGAP18 relocalisation**

One other kinase that phosphorylates RXXS*/T* motifs is ROCK (Kang et al., 2007). ROCK is activated downstream of the Rho and drives disruption of the AJs through phosphorylation of MLC. In order to determine whether ROCK mediates phosphorylation of ARHGAP18, we immunoprecipitated cell lysates treated with thrombin in the presence or absence of the ROCK inhibitor, Y27632, and assessed the phospho motif (Fig 6.7 A). We observed that there was substantially less phosphorylation of the motif in all the time points. The phosphorylated MLC2 indicates that ROCK is highly activated at 10 min, and is effectively inhibited by Y27632. However, despite the reduction in the phosphorylated ARHGAP18 motif, there was still substantial phosphorylation at 10 min. This likely indicates that the motif is still being phosphorylated by another kinase, that is not ROCK or Akt. This could potentially be PKA or PKC, which are also regulated following thrombin stimulation (Aslam et al., 2010; Rahman et al., 2001) and have been found to phosphorylate a majority of ROCK substrates (Kang et al., 2007). However, we have not examined the contributions of these kinases to ARHGAP18 phosphorylation.

We next tested the effects of ROCK inhibition of the actin cytoskeleton and cell junctions. As has been previously reported (van Nieuw Amerongen et al., 2000), ROCK inhibition completely prevents thrombin mediated disruption of the cell junctions and formation of the actin stress fibres (Fig 6.7 B). Furthermore, ROCK inhibition completely prevented the translocation of ARHGAP18 to the cell periphery (Fig 6.7 C).

6.3. **Summary and Conclusions**

ARHGAP18 dynamically translocates to unstable EC junctions, such as EC monolayers stimulated with thrombin or the remodeling angiogenic vessels, where we postulate it acts
as a RhoC GAP to inhibit RhoC activity and thus promote junctional stability. In this chapter, we have identified two potential mechanisms that regulate the translocation of ARHGAP18: its interaction with cortactin and phosphorylation of ARHGAP18.

### 6.3.1. Cortactin is a novel interacting partner of ARHGAP18

The preliminary results suggest that cortactin serves as a critical interactor of ARHGAP18 to actively translocate ARHGAP18 to the cell periphery. We are able to observe this interaction through co-localisation and co-IP experiments and show that knockdown of cortactin effectively inhibited ARHGAP18 translocation. Further experiments are required to substantiate and confirm this possibility.

One major caveat of these experiments is that the translocation of ARHGAP18 and cortactin is a relatively poor readout. Not all cells respond in the same manner and at the same time following thrombin. It is widely assumed that the translocation of ARHGAP18 to the membrane serves to inhibit RhoGTPase activation, in this case being RhoC. Therefore, a further readout of ARHGAP18 function following cortactin knockdown would be to measure RhoC activation, which would provide a quantitative readout. Further, the co-IP experiments using these antibodies can potentially have non-specific effects. Ideally, the co-IP experiments would be performed in ECs that express tagged versions (e.g Flag, His) of ARHGAP18 and cortactin, and pulldowns performed using antibodies against these specific tags. Moreover, by expressing truncations and mutations of ARHGAP18 and cortactin, the ultimate goal would be to establish the domains that are important for their interaction and function.

It has been demonstrated that Src and Rac1 are critical regulators of cortactin translocation (Tehrani et al., 2007; Vouret-Craviari et al., 2002; Weed et al., 1998). Both Src and Rac1 are activated following VEGFA stimulation and have important functions in regulating angiogenic sprouting and junctional regulation (Gavard and Gutkind, 2006).
Therefore, it would be of interest to investigate whether Src and/or Rac1 signaling also regulates ARHGAP18 trafficking and to determine the potential implication of Src-Rac1-cortactin-ARHGAP18 signaling in sprouting angiogenesis.

6.3.2. Phosphorylation

Here we have identified putative phosphorylation sites that may be critical in the function of ARHGAP18. In particular, the RXXS*/T* motif present at three sites of ARHGAP18 is of significant interest, as we show that this is phosphorylated in a time-dependent manner that coincides with the translocation to the membrane. While this motif can be phosphorylated by several different kinases, we have elucidated that Akt and ROCK inhibition can inhibit basal phosphorylation, while ROCK inhibition can suppress, but not completely prevent, thrombin-induced phosphorylation. This is likely due to the influences of the other kinases such as PKA or PKC. However, we demonstrate that ROCK inhibition prevents ARHGAP18 translocation to the membrane. This may indicate that ROCK regulates phosphorylation of one or more of the ARHGAP18 RXXS*/T* motifs, which act as the critical signal to drive its relocalisation. This likely indicates that thrombin induces RhoC-ROCK activation, which leads to AJ disruption. ROCK thereby phosphorylates and recruits ARHGAP18 to restore basal RhoC activity to prevent excessive junctional disruption. The proposed mechanism is described further in chapter 7.

There are some major limitations of these preliminary experiments. Firstly, we are unable to determine whether the phosphorylation is the active driver of ARHGAP18 recruitment. Secondly, while ROCK prevents ARHGAP18 translocation, we are unable to establish whether this is due to its kinase effects or as a result of inhibiting junctional disruption. There is a possibility that ROCK is not directly involved in the recruitment, but that by preventing junctional disruption, it is preventing the recruiting signal for ARHGAP18. To
address both of these concerns, it would be for future study to investigate the effects of the individual phosho ser/thr sites. This could be achieved by constructing ARHGAP18 mutations, such as substituting the phosphor ser/thr with alanine to prevent phosphorylation and examining the contribution of each to ARHGAP18 translocation. Further, it would be of future interest to characterise these mutants in the context of RhoC activity and in regulating EC sprouting.
CHAPTER 6.
REGULATION OF ARHGAP18 TRANSLOCATION

FIGURES
A. ARHGAP18 does not colocalise with the ERM proteins. Confocal images of HUVECs stimulated with or without thrombin (1 U/mL) for 10 min, fixed and stained for Ezrin/Radixin/Moesin (ERM) or moesin (red) and ARHGAP18 (green) and nuclei counterstained with DAPI (blue). ARHGAP18 shows the typical relocalisation to the membrane, but is not associated with ERM or moesin. Bar 25 μm.

B. ARHGAP18 does not interact with ERM or moesin. HUVEC lysates were immunoprecipitated with ARHGAP18 or control antibodies and immunoblotted for ERM and moesin. Whole cell lysate (WCL) run as a positive control for the antibody.

C. The human and mouse ARHGAP18 and Drosophila Conu protein sequences were aligned using ClustalW and the sequence similarity of the whole protein, the GAP domain, the MID and the putative SAM domain are indicated by the values. Relative to the GAP and SAM domains, the MID shows little sequence similarity.

**Fig 6.1. ARHGAP18 does not colocalise or interact with the ERM proteins.**
Chapter 6. Regulation of ARHGAP18 Translocation

A) NT, Thrombin 10min, and Zoom images showing the distribution of Cortactin and ARHGAP18.

B) Western blot analysis of ARHGAP18 IP with WCL, IgG, and ARHGAP18 showing bands at 72 kDa and 70 kDa.

C) Time-course of Cortactin and ARHGAP18 IP with IgG showing bands at 72 kDa and 70 kDa.

D) Western blot analysis of Cortactin and ARHGAP18 IP with WCL, IgG Cortactin showing bands at 78 kDa and 73 kDa.
Fig 6.2. ARHGAP18 colocalises with cortactin at the junctional periphery, and co-immunoprecipitates with cortactin in a thrombin-independent manner.
A. Confocal images of HUVECs stimulated with or without thrombin (1U/mL) for 10 min, fixed and stained for cortactin (red) and ARHGAP18 (green) and nuclei counterstained with DAPI (blue). ARHGAP18 shows abundant colocalisation with cortactin at the junctional periphery (white arrows) but is also absent in some cell junctions (yellow arrow). Bar 25 μm.
B. ARHGAP18 co-immunoprecipitates with cortactin. HUVEC lysates were immunoprecipitated using ARHGAP18 or isotype control antibodies and immunoblotted for cortactin. WCL were run as a positive control for the antibody.
C. Thrombin does not alter the ARHGAP18-cortactin interaction. HUVECs were stimulated with thrombin (1U/mL) for the indicated times, lysed, immunoprecipitated with ARHGAP18 or control antibodies and immunoblotted for cortactin.
D. HUVEC lysates were immunoprecipitated using cortactin or control antibodies and immunoblotted for ARHGAP18. WCL were run as a positive control for the antibody. Cortactin immunoprecipitation resulted in the detection of the smaller (~73 kDa) isoform of ARHGAP18.
Fig 6.3. Optimisation of cortactin siRNA knockdown.
A. Brightfield images of HUVECs transfected with control or the individual cortactin siRNAs for 3 d. Transfection with siCTTN1 results in a significant alteration in cell phenotype that is absent in the other cortactin siRNAs. Bar 50 μm.
B. Confirmation of cortactin knockdown. Lysates from cells transfected with control or cortactin siRNAs were immunoblotted for cortactin, ARHGAP18 and actin as the loading control. All siRNAs resulted in cortactin knockdown. ARHGAP18 is notably downregulated by two of the cortactin siRNAs.
Fig 6.4. Cortactin relocation is not dependent on ARHGAP18, but is required for ARHGAP18 relocation.

A. Confocal images of cells transfected with control, ARHGAP18 or cortactin siRNAs, stimulated with thrombin (1U/mL) for 10 min, fixed and stained for ARHGAP18 (green), cortactin (red) and nuclei counterstained with DAPI (blue). Control cells show abundant colocalisation of ARHGAP18 and cortactin at the junctional periphery (arrows). Knockdown of ARHGAP18 still results in cortactin relocation (arrows) but knockdown of cortactin shows an absence of ARHGAP18 relocation. Bar 25 μm.
Fig 6.5. ARHGAP18 contains putative RXXS*/T* phosphorylation sites that are phosphorylated in a thrombin-dependent manner.

A. Identification of putative ARHGAP18 phosphorylation sites using Phosphosite. ARHGAP18 contains three RXXS*/T* motif sites at S66, T154 and S610 and two sites with the S*Q motif at S66 and S610.

B. Pulldown of putative phospho-ARHGAP18. HUVEC lysates were immunoprecipitated using ARHGAP18 or isotype control antibodies and immunoblotted using the pS/T Akt substrate antibody which is raised against the RXXS*/T* motif. WCL was run as a positive control for the antibody.

C. Phosphorylation of the putative phospho-ARHGAP18 is regulated by thrombin in a time-dependent manner. HUVECs were stimulated with thrombin (1U/mL) for the indicated times and cell lysates were immunoprecipitated using ARHGAP18 antibodies and immunoblotted for the RXXS*/T* motif. Phosphorylation is increased following thrombin, at a maximal at 10 min and restored to basal levels at 60 min.
Fig 6.6. The putative phospho-ARHGAP18 is not regulated by Akt activation.

A. Inhibition of Akt reduces basal but not thrombin-induced ARHGAP18 phosphorylation. HUVECs were pre-treated with or without the LY294002 (30 μM, 30 min) then stimulated with or without thrombin (1U/mL) for 10 min. Cell lysates were immunoprecipitated using ARHGAP18 antibodies and immunoblotted for the RXXS*/T* motif. WCL were immunoblotted for p-Akt to confirm Akt inhibition. Akt inhibition results in reduced basal phospho-RXXS*/T* but is increased following thrombin treatment.

B. Akt is inhibited following thrombin stimulation. HUVECs were stimulated with thrombin for the indicated time points and WCL immunoblotted for p-Akt and p-ERK1/2. While ERK1/2 is hyperphosphorylated following thrombin stimulation, Akt is hypophosphorylated.

C. Akt inhibition does not prevent ARHGAP18 relocalisation following thrombin stimulation. HUVECs were pre-treated with or without LY294002 (30 μM, 30 min) then stimulated with thrombin (1U/mL) for 10 min. Cells were stained for ARHGAP18 (green) and nuclei counterstained with DAPI (blue) and imaged by confocal microscopy. ARHGAP18 relocalises following thrombin in the presence or absence of the Akt inhibitor. Bar 25 μm
Fig 6.7. ROCK inhibition suppresses phosphorylation of ARHGAP18 and prevents ARHGAP18 relocalisation.

A. ROCK inhibition suppresses, but does not completely inhibit, phosphorylation of ARHGAP18. HUVECs were pre-treated with or without the Y27632 (2.5 μM, 30 min) then stimulated with or without thrombin (1U/mL) for the indicated times. Cell lysates were immunoprecipitated using ARHGAP18 antibodies and immunoblotted for the RXXS*/T* motif. WCL were immunoblotted for p-MLC2 to confirm downstream ROCK activation and inhibition. ROCK inhibition results in reduced phospho-RXXS*/T* at all time points, but still results in substantial activation at 10 min.

B. Confirmation of ROCK inhibitor efficiency on actin and junctional phenotypes. HUVECs were pre-treated with or without the Y27632 (2.5 μM, 30 min), stimulated with or without thrombin (1U/mL) for 30 min, stained for VE-cadherin (green), F-actin (red) and nuclei counterstained with DAPI (blue) and imaged by confocal microscopy. ROCK inhibition prevented the thrombin-induced stress fibres and junctional disruption. Bar 25 μm.

C. ARHGAP18 relocalisation to the junctional periphery is dependent on ROCK activity. HUVECs were pre-treated with or without 2.5 μM Y27632 for 30 min, stimulated with or without thrombin for 10 min, stained for ARHGAP18 (green), and nuclei counterstained with DAPI (blue) and imaged by confocal. ROCK inhibition prevented ARHGAP18 translocation to the EC junctions. Bar 25 μm.
CHAPTER 7

FINAL DISCUSSION
CHAPTER 7. FINAL DISCUSSION

Tight regulation of angiogenesis is important in establishing and maintaining normal tissue vascularization. This involves highly regulated changes in EC behavior to coordinate the processes of vessel sprouting, fusion, lumen formation, stabilization and remodeling. Aberrant regulation can drive over- or under-production of angiogenesis, which contributes to pathologies such as cancer and ischemia, respectively. Here we have identified ARHGAP18 as a negative regulator of the initial steps of sprouting angiogenesis, in regulating junctional homeostasis and suppressing EC sprouting. Loss of ARHGAP18 promotes activation of RhoC and active serrated EC junctions, and promotes a TC-like hypersprouting and migratory phenotype. In pathogenesis, this results in a profound increase in tumour vascularization and growth.

In the stable vessels, quiescent ECs are interconnected with strong VE-cadherin mediated cell-cell adhesions. VE-cadherin functions not only to stabilize the interactions but also acts to suppress VEGFA signaling (Fig 7.1 A). Following the sensing of VEGFA in the microenvironment, VEGFR2 signaling induces junctional disruption by phosphorylating VE-cadherin and promotes pro-angiogenic signaling via downstream activation of a multitude of signaling pathways. This initiates TC formation, which acts to direct vessel sprouting towards the VEGFA gradient. VEGFA signaling also induces activation of Notch signaling in adjacent cells to laterally inhibit the TC phenotype and induce a SC phenotype, which support the extension of the elongating sprout. The TC and SC phenotypes however are transient and constant re-regulation of VEGFR-Notch signals and differential EC junction dynamics drive the competition and shuffling of cells within the sprout.
ARHGAP18 was initially identified for its role in regulating tube formation, where it was downregulated during the migratory and upregulated during the stabilization phases (Coleman et al., 2010). Further, knockdown of ARHGAP18 prevented the formation of stable EC sprouts. This suggested potential roles of ARHGAP18 in regulating cell migration and/or stabilization. Using multiple *in vitro* and *in vivo* models, we describe that ARHGAP18 is a regulator of both angiogenic sprouting and junctional integrity. Loss of ARHGAP18 results in increased spheroid sprouting *in vitro*, zebrafish ISV sprouting, *ex vivo* aortic ring sprouting and vascularization of the post-natal mouse retina. This sprouting phenotype is associated with a TC-like phenotype, with increased formation of vascular sprouts, filopodia, vessel branching and expression of the TC-enriched genes DLL4, Flk1 and Flt4. The increased signaling through the VEGFRs, but not the inhibitory VEGFR1, drives Akt hyperactivation, which has been demonstrated to be responsible for driving DLL4 expression (Liu et al., 2003). DLL4 acts to regulate lateral inhibition of TC phenotype in adjacent SCs through the activation of Notch signaling. Furthermore, the expression of the VEGFRs, DLL4 and Notch signaling all regulate the shuffling capacity of ECs within the sprout, such that cells expressing high levels of VEGFR activity and DLL4 expression are highly motile and have an increased propensity to occupy the TC position (Bentley et al., 2014). Consistent with this, we observe that, using a chimeric spheroid sprouting model, ARHGAP18 knockdown cells have a significantly increased frequency to occupy the leading position.

We postulate that ARHGAP18 is a negative regulator of sprouting through its Rho GAP function in regulating RhoC and junctional integrity. Rho GTPases are recruited to the membrane periphery following stimulation where they activate downstream effectors. We observe that ARHGAP18 is dynamically recruited to the junctional periphery following 10 min of thrombin stimulation, whereas RhoC displays maximal activation at 2 min.
This suggests that ARHGAP18 is recruited to the destabilized EC junctions in order to suppress excessive RhoC signaling (Fig 7.1 B). Consistent with this notion of stabilization, we observe that ARHGAP18 is localized to the EC junctions of the unstable angiogenic vessels but not to relatively stable junctions of the arteries and veins in the retina or in the junctions of quiescent monolayers of EC in vitro. In these stable settings, we postulate that ARHGAP18 may be localized in the cytosol or in association with the microtubules (Lovelace et al., in review). One of the major functions of RhoC is in its regulation of junctional integrity through its downstream activation of ROCK (Abraham et al., 2009; Sahai and Marshall, 2002). We show here that ARHGAP18 loss leads to hyperactivation of RhoC and junctional disruption, while overexpression of ARHGAP18 decreases RhoC activation. We confirmed the action of ARHGPA18 on RhoC by demonstrating that knockdown of RhoC is able to revert the junctional phenotype mediated via ARHGAP18. A recent study has demonstrated that differential VE-cadherin dynamics, in addition to VEGF-Notch-DLL4 regulates angiogenic sprouting by controlling cell rearrangement (Bentley et al., 2014). Consistent with this notion, the disrupted junction phenotype of ARHGAP18 KD is associated with increased cell rearrangement. We postulate that through the hyperactivation of RhoC, ARHGAP18 loss drives VE-cadherin disruption, which in turn promotes VEGFR-Akt signaling to regulate DLL4 expression and angiogenic sprouting. However, while we have not directly demonstrated that the effects of ARHGAP18-RhoC signaling on EC sprouting, it has also been shown that RhoC regulates VEGF signaling and Akt activation (Liu et al., 2012; Ruth et al., 2006). To date the role of RhoC on cell migration is quite controversial and is thought to be due to direct effects through downstream effectors such as ROCK or indirect effects through activation of Rac1 (Del Galdo et al., 2013; Kitzing et al., 2010;
Vega et al., 2011). This effect of ARHGAP18-RhoC and RhoC itself on angiogenic sprouting would be for future investigation.

During sprouting angiogenesis, we, and others observe that ARHGAP18 is upregulated in the TCs. Further, ARHGAP18 is upregulated at the late time points following VEGFA stimulation. This suggests that rather than being a direct inducer of sprouting, ARHGAP18 acts as a negative feedback regulator to control the extent of sprouting angiogenesis. Given the correlation between VEGFR-Notch signaling, differential EC junctions and ARHGAP18 expression, it would be interesting to determine whether the expression or localization of ARHGAP18 changes in a single cell nature within the cells of the sprouting vessel. We would postulate that there would first be VEGFR signaling to RhoC that disrupts the EC junctions then relocalisation and upregulation of ARHGAP18 to restrict excessive RhoC-mediated junctional destabilization (Fig 7.1 B). It would therefore be very interesting to determine what controls the expression and localization of ARHGAP18 in this environment. One potential factor is the transcription factor YAP. YAP activity is regulated in a cell contact dependent manner by VE-cadherin (Choi et al., 2015). It has recently been demonstrated that YAP knockdown triggers downregulation of ARHGAP18 and this plays an important role in regulating Rho during 3D morphogenesis (Porazinsiki et al., 2015). Therefore, we predict that during the cycles of active serrated EC junctions, YAP is internalized and acts as a TF to upregulate ARHGAP18 (Fig 7.1 B). Another mechanism of regulating ARHGAP18 function is through its localization. Our preliminary experiments identified two potential mechanisms to control localisation: through its phosphorylation and its interaction with cortactin. We observed phosphorylation of putative RXXS*/T* motifs present on ARHGAP18 that correlated with its dynamic localisation. We identified that this is potentially mediated by ROCK, a kinase that is downstream of Rho, as ROCK inhibition reduced phosphorylation of
ARHGAP18 and prevented ARHGAP18 relocalisation. We postulate that RhoC signaling to ROCK phosphorylates ARHGAP18 that drives its recruitment to the membrane where it serves as a negative feedback regulator to prevent excessive RhoC-ROCK signaling (Fig 7.1 B). However, these phosphorylation studies will require substantial further studies, such as mutation of the putative phospho motifs, to definitely prove such a mechanism. The other mechanism to regulate localisation is through its interaction with cortactin, an actin and Rho GAP binding protein that regulates cell migration. We demonstrated that cortactin and ARHGAP18 interact via coIP and that cortactin and ARHGAP18 appeared to colocalise to similar regions of the junctional periphery following junction destabilization. Further, knockdown of cortactin prevented the recruitment of ARHGAP18 to the periphery. Interestingly, it appeared that cortactin interacted with a smaller isoform of ARHGAP18, which was also the isoform of the putative phosphorylated version of ARHGAP18. These suggest that post-translation modifications of ARHGAP18 may be crucial in regulating its function. We have further evidence to suggest that N-terminal truncations of ARHGAP18 result in gross morphological changes, which suggests that the N-terminal region may be acting as a negative regulator of ARHGAP18 function, and that the binding to other proteins or its phosphorylation are the key mechanisms to relinquish this inhibition. We have also recently observed that ARHGAP18 colocalises with the microtubules and that knockdown of ARHGAP18 affects microtubule stability (Lovelace et al., in review). These microtubules may act as a mechanism to traffic ARHGAP18 to the cell periphery. However, these studies will require a substantial amount of further experiments to demonstrate the critical regions and/or domains that regulate its interaction with other proteins and localization.
Another future direction is to examine the influence of ARHGAP18 overexpression. While we have demonstrated an importance of ARHGAP18 in regulating EC senescence, it would be also interesting to determine whether ARHGAP18 overexpression results in changes that are opposite to that of the knockdown. Using our current retroviral model, we are only able to overexpress ARHGAP18 to very small magnitudes and are unable to direct this to its RhoGAP function, as most of the overexpressed protein remains non-functional in the cytosol. In order to address this, we would create a lentiviral vector (which we have proved is much more efficient) to express a myristolated-ARHGAP18 that targets it to the membrane, as has been demonstrated by others (Porazinski et al., 2015). Using this, we would be able to assess the impact of ARHGAP18’s RhoGAP function on sprouting angiogenesis and its potential membrane interactors.

To investigate the sprouting effects *in vivo*, we are currently developing an inducible ARHGAP18 transgenic mouse line (TRE-ARHGAP18-IRES-GFP). This would allow us to generate endothelial-specific ARHGAP18 overexpressing mice (by crossing with VE-cadherin-tTA mice). Further, while we presume that the effects of ARHGAP18 knockout are mediated through the endothelial population (based on the other *in vitro*, and *ex vivo* results and the lack of phenotypes in other cell populations), we are also addressing this through the generation of an EC specific knockout mouse. To do this, we are using the current global knockout mouse and crossing the mouse with a global flippase (FLP) mouse. This utilizes the FLP recombinase, which recognizes the FLP recombinase target (FRT) sequences in the knockout cassette (Fig 4.9 A), to remove the En2 splice acceptor and restore the WT status. The second step is to cross this mouse with an endothelial specific Cre mouse (e.g. the VE-cadherin-Cre (Alva et al., 2006)) which then floxes out the loxP sites that flank exon 2 (Fig 4.9 A). This could be alternatively manipulated by crossing with the VE-cadherin-CreERT2 transgenic mouse (Monvoisin et al., 2006),
which would allow for tamoxifen-inducible EC specific deletion of ARHGAP18. This would further also permit for future studies into the later steps of angiogenesis, such as vascular remodeling.

One major unanswered question is the role of ARHGAP18 during chronic Ras activation. We established that chronic Ras-MAPK-ERK signaling elicits a dramatic downregulation of ARHGAP18 in addition to its induction of a partial EndMT phenotype. However, restoration of ARHGAP18 was unable to affect the Ras phenotype in terms of gross morphology and EndMT marker expression. Since we have now defined a role of ARHGAP18 in regulating sprouting and EC junctions, it is worth investigating whether ARHGAP18 acts as a critical intermediate protein to these Ras phenotypes. However, given the extensive changes in morphology and cytoskeletal rearrangement, we would not predict that restoration of a single RhoGAP would dramatically affect the phenotype. We would postulate that this is regulated by a coordinated regulation of multiple Rho signaling pathways, regulators and effectors. ARHGAP18 may just be one of these, and easily compensated for by other RhoGAPs. Nevertheless, this would be for future investigation. One of the implications of aberrant Ras signaling in the vascular system is in vascular malformations and tumours. Mutations of TIE2 also result in venous malformations and is also linked with a downregulation of ARHGAP18 (Uebelhoer et al., 2013). This highlights the difference between the physiological and pathological environments where upregulation of ARHGAP18 during physiological angiogenesis acts to restrict excessive sprouting by maintaining junctional integrity, while the loss of ARHGAP18 may act as a driver of vascular pathology. The direct consequences of ARHGAP18 loss in the context of vascular diseases will be of much interest.

In conclusion, understanding the role of ARHGAP18 in the vasculature and in pathology is of major importance, given the possibility that targeting the RhoGTPase cycle is a
therapeutic opportunity (van der Meel et al., 2011). We have demonstrated that ARHGAP18 is a negative regulator of angiogenesis via its control of EC junctional integrity and sprouting. Loss of ARHGAP18 drives excessive vessel hypersprouting and tumour development consistent with an important role for this regulatory protein in stabilisation of the vasculature.
CHAPTER 7.
FINAL DISCUSSION

FIGURES
A. Resting stable ECs

Resting ECs have stable EC junctions mediated by VE-cadherin, which further acts to inhibit VEGFR2 activation to promote EC quiescence. In stable ECs, ARHGAP18 is localised in the cytosol or in association with microtubules.

B. Angiogenic or destabilised ECs

In response to angiogenic or destabilising stimuli, RhoC activates ROCK which disrupts the AJs and induces actomyosin contractility. AJ disruption breaks down the stable EC-EC interactions and facilitates VEGFR signaling to promote a pro-angiogenic phenotype. ROCK additionally phosphorylates ARHGAP18, which in combination with cortactin is recruited to the cell periphery. This serves to inactivate RhoC to prevent excessive ROCK signaling. ARHGAP18 is also upregulated, which is potentially mediated via YAP. VE-cadherin disruption facilitates YAP translocation to the nucleus where it acts as a TF for ARHGAP18.
APPENDIX

AND

REFERENCES
APPENDIX

A.1. FIJI IMAGING MACROS

A.1.1. DAPI COUNTING MACRO

//Macro for the automated determination of cell nuclei in a whole image field
//-----------------------------------------------------------------------------------------------
//Macro Created by Garry Chang 2010-July-21
//-----------------------------------------------------------------------------------------------
//Input image: DAPI stained cell nuclei, only as a DAPI channel image

run("8-bit");
runt("Subtract Background...", "rolling=1000");
runt("Brightness/Contrast...");
runt("Enhance Contrast", "saturated=0.5");
runt("Apply LUT");
runt("Gaussian Blur...", "sigma=5");
setAutoThreshold();
//runt("Threshold...");
setAutoThreshold();
setThreshold(0, 64);
runt("Convert to Mask");
runt("Convert to Mask");
runt("Analyze Particles...", "size=0-Infinity circularity=0.00-1.00 show=Outlines display exclude clear record");
A.1.2. RETINA VASCULARISATION MACRO

//Macro for the subanalysis of Wimasis analysed whole quadrant Retinas
//Note that the quality of the data depends on the initial analysis
//This serves as a macro to pull out existing data in a smaller area
//-------------------------------------------------------------------
//Macro Created by Garry Chang 2013-April-14
//-------------------------------------------------------------------

//Image order:
//1. Junctions
//2. Loops
//3. Vessels

//Rename images for generality
selectImage(3);
run("Invert");
rename("vessels")
selectImage(2);
rename("loop");
run("Channels Tool...");
run("Yellow"); //Makes loops yellow
run("RGB Color");
selectImage(1);
file=getTitle();
rename("junction");
//Need to adjust colour balance of colours to increase signal:noise
setMinAndMax(120,130,4);//Red
setMinAndMax(40,180,2);//Green
imageCalculator("Add create","loop","junction");

//Select ROI
waitForUser("Select ROI"); //Select point will form a 250x250 pixel rectangle from top left
getSelectionBounds(x,y,width,height);
makeRectangle(x, y, 250, 250);
run("Copy");
newImage("crop","RGB Black",250,250,1);
run("Paste");
selectAllWindow("crop");
run("Duplicate...","title=crop-blue");
run("Duplicate...","title=crop2-green");
run("Duplicate...","title=crop3-red");
run("Duplicate...","title=crop4-yellow");

selectAllWindow("vessels");
makeRectangle(x, y, 250, 250);
run("Copy");
newImage("vesselcrop","8-bit Black",250,250,1);
run("Paste");
selectAllWindow("vesselcrop");
imageCalculator("Average create","crop","vesselcrop");
rename("Merge");
saveAs("PNG","/Users/garryhoi-kaichang/Desktop/Retina Test/"+file);
rename("Merge");
//===================================================================
//VESSEL DENSITY===================================================================

selectAllWindow("vesselcrop");
setAutoThreshold("Default");
run("Analyze Particles...","size=30-Infinity circularity=0.00-1.00 show=Nothing display");
selectWindow("Merge");
waitForUser("Vessel Density");

// VESSEL DENSITY

run("Clear Results");

// LOOP & Junction COUNTING

selectWindow("crop4-yellow");
// Color Thresholder 1.46r
// Color Threshold Yellow
min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
run("HSB Stack");
run("Convert Stack to Images");
selectWindow("Hue");
rename("0");
selectWindow("Saturation");
rename("1");
selectWindow("Brightness");
rename("2");
min[0]=34;
max[0]=51;
filter[0]="pass";
min[1]=0;
max[1]=255;
filter[1]="pass";
min[2]=104;
max[2]=255;
filter[2]="pass";
for (i=0;i<3;i++){
    selectWindow(""+i);
    setThreshold(min[i], max[i]);
    run("Convert to Mask");
    if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++){
    selectWindow(""+i);
    close();
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);
// Colour Thresholding--------
//Counts particles
run("Analyze Particles...", "size=30-Infinity circularity=0.00-1.00 show=Nothing display exclude");
//Next Channel

//Pause for data input to excel

selectWindow("Merge");
waitForUser("Loops");
run("Clear Results");

// Colour Threshold Red
selectWindow("crop3-red");
min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
run("HSB Stack");
run("Convert Stack to Images");
selectWindow("Hue");
rename("0");
selectWindow("Saturation");
rename("1");
selectWindow("Brightness");
rename("2");
min[0]=25;
max[0]=225;
filter[0]="stop";
min[1]=0;
max[1]=255;
filter[1]="pass";
min[2]=104;
max[2]=255;
filter[2]="pass";
for (i=0;i<3;i++) {
    selectWindow(""+i);
    setThreshold(min[i], max[i]);
    run("Convert to Mask");
    if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++) {
    selectWindow(""+i);
    close();
}
selectWindow("Result of 0");
selectWindow("Result of Result of 0");
rename(a);
// Colour Thresholding----------
//Counts particles
run("Analyze Particles...", "size=10-26 circularity=0.00-1.00 show=Nothing display exclude");

//Pause for data input to excel
selectWindow("Merge");
waitForUser("Single Branches");
run("Clear Results");

//Count merged red branches...are still doubles-sorted on size
selectWindow("crop3-red");
run("Analyze Particles...", "size=27-50 circularity=0.00-1.00 show=Nothing display exclude");

//Colour Threshold Green
selectWindow("crop2-green");
min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
run("HSB Stack");
run("Convert Stack to Images");
selectWindow("Hue");
rename("0");
selectWindow("Saturation");
rename("1");
selectWindow("Brightness");
rename("2");
min[0]=70;
max[0]=100;
filter[0]="pass";
min[1]=0;
max[1]=255;
filter[1]="pass";
min[2]=104;
max[2]=255;
filter[2]="pass";
for (i=0;i<3;i++) {
    selectWindow(""+i);
    setThreshold(min[i], max[i]);
    run("Convert to Mask");
    if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++) {
    selectWindow(""+i);
    close();
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);

// Colour Thresholding-------------

// Data for double red and the greens are pooled together
run("Analyze Particles...", "size=10-Infinity circularity=0.00-1.00 show=Nothing display exclude");

// Pause for data input to excel
selectWindow("Merge");
waitForUser("Double Branches");
run("Clear Results");

// Colour Threshold Blue
selectWindow("crop-blue");
min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
run("HSB Stack");
run("Convert Stack to Images");
selectWindow("Hue");
rename("0");
selectWindow("Saturation");
rename("1");
selectWindow("Brightness");
rename("2");
min[0]=160;
max[0]=190;
filter[0]="pass";
min[1]=0;
max[1]=255;
filter[1]="pass";
min[2]=104;
max[2]=255;
filter[2]="pass";
for (i=0;i<3;i++) {
    selectWindow(""+i);
    setThreshold(min[i], max[i]);
    run("Convert to Mask");
    if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++)
    selectWindow(""+i);
    close();
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);
// Colour Thresholding--------
//Counts particles
run("Analyze Particles...", "size=10-Infinity circularity=0.00-1.00 show=Nothing display exclude");

//Pause for data input to excel
selectWindow("Merge");
waitForUser("Triple Branches");
run("Clear Results");

//==================LOOP & Junction COUNTING===================

//Close image
run("Close All");
A.1.3. Pericyte coverage macro

//Macro for the analysis of pericyte coverage in mouse retinas
//Images of NG2 and VE-cadherin stained retinas as the input, using whole confocal stacks

//Macro Created by Garry Chang 2014-January-23

file=getTitle();
run("Z Project...", "start=1 stop=100 projection=[Max Intensity]");
run("Subtract Background...", "rolling=50");
run("Duplicate...", "title=Merge duplicate channels=1-2");
selectImage(2);
run("RGB Color");
setMinAndMax(5,150,4);//Red
setMinAndMax(10,180,2);//Green
saveAs("PNG", "/Users/garryhoi-kaichang/Desktop/Pericyte Test/"+file);
close();
selectWindow("Merge");
run("Gaussian Blur...", "sigma=1");
run("Split Channels");
selectImage(3);
rename("pericyte");
selectImage(4);
rename("VEC");

//VEC vasculature
selectWindow("VEC");
run("Brightness/Contrast...");
waitforUser("Set MinMax");/SET MIN MAX----20,30
run("Apply LUT");
run("Make Binary");
run("Dilate");
run("Close-");

//Pericytes
selectWindow("pericyte");
run("Brightness/Contrast...");
waitforUser("Set MinMax");/SET MIN MAX----10-80
run("Apply LUT");
setThreshold(30, 255);
run("Convert to Mask");

//Merge Images
run("Merge Channels...", "c1=VEC c2=pericyte create keep");
run("RGB Color");
a="_analysed"
saveAs("PNG", "/Users/garryhoi-kaichang/Desktop/Pericyte Test/"+file+a);
run("Color Threshold...");

//Colour Threshold
waitforUser("Set Yellow-30/50")/Yellow: 30-50
run("Measure");

waitforUser("Set Yellow-60/90-Pass")//Red+Yellow: 60-90 Pass
run("Measure");
waitforUser("Get area")
run("Close All");
REFERENCES


References


References


References


References


References


References


