

2015-04-20

# Characterizing the role of Rasa1 in vascular development in zebrafish

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Davari, P. (2015). Characterizing the role of Rasa1 in vascular development in zebrafish (Master's thesis, University of Calgary, Calgary, Canada). Retrieved from <https://prism.ucalgary.ca>. doi:10.11575/PRISM/25307

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UNIVERSITY OF CALGARY

Characterizing the role of Rasa1 in vascular development in zebrafish

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

CALGARY, ALBERTA

APRIL, 2015

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## Abstract

Vascular malformation syndromes arise during embryonic development and can have devastating effects on the quality of life of patients. Capillary malformations present with the overgrowth and permanent dilation of the capillaries under the skin but are often accompanied by fast-flow arteriovenous malformations in other internal organs. Recently, mutations in the GTPase activating protein RASA1 were found to be one cause of capillary malformation-arteriovenous malformation (CM-AVM).

I set out to model CM-AVM in the zebrafish and found that loss of *rasa1* leads to a large arteriovenous malformation that connects the artery and vein without intervening capillaries. In wild-type zebrafish embryos, a distinct aorta and a caudal vein plexus are visible in the tail region. However, in *rasa1a* morphants, there is an enlarged single vessel instead of a plexus, and this vessel fails to develop sprouts. Arteriovenous shunts develop commonly in this region.

I next examined the mechanism by which *rasa1* functions. I found that *rasa1a* genetically interacts with *rras* but not farnesyl- modified *ras* (*rat sarcoma*) genes. *rasa1a* also functions in parallel pathway to *rras2*. Finally, I showed misregulation of *vegfc* (*vascular growth factor c*) and *vegfr3* (*vascular growth factor receptor 3*) in *rasa1a* morphants, suggesting *rasa1a* loss leads to misregulation of venous signaling pathways.

## **Acknowledgements**

I would like to express my sincere gratitude to my supervisor, Dr. Sarah Childs, for her continuous guidance, encouragement, and support during my Master's program. Her ideas, feedbacks, and vision helped me shape my research career. She has always inspired me with her positive attitude. Without her guidance and support, this dissertation would not have been possible.

I am also thankful to all my colleagues and friends in Childs' lab: Michela Goi, Corey Arnold, Tom Whitesell and Nicole Munsie for their friendship and help. I would like to specially acknowledge Dr. Jae-Ryeon Ryu for her support and guidance all throughout my research project. I would also like to thank my committee members, Dr. Sarah McFarlane and Dr. Paul Mains for their mentorship and support.

Heartfelt thanks go to my husband, Majid, for all his understanding, support and patience during the work of this thesis. His love made my journey to finish this thesis easier.

My deepest gratitude goes to my mom and dad, Rowshank and Mansour, and my sisters, Parastoo and Paron, who always had their love and support for me.

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## List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AVF	arteriovenous fistulas
AVM	arteriovenous malformation
BMP	bone morphogenic protein
CM	capillary malformation
CVP	caudal vein plexus
DA	dorsal aorta
DLAV	dorsal longitudinal anastomotic vessel
ECM	extracellular matrix
EGF	epidermal growth factor
FTase	farnesyl transferase
FTI	farnesyl transferase inhibitor
GDI	guanine dissociation inhibitor
GEF	guanine nucleotide exchange Factor
GGTase	geranylgeranyl transferase
GGTI	geranylgeranyl transferase inhibitor
ISV	intersegmental vessel
LEC	lymphatic endothelial cell
LM	lymphatic malformation
LPM	lateral posterior mesoderm
PCV	posterior cardinal vein
PDGF	platelet-derived growth factor
Shh	Sonic hedgehog
SIVP	sub intestinal venous plexus

## **Chapter One: Background**

A precisely developed vascular system is essential in vertebrates to carry blood for delivery of gases, nutrients and removal of wastes as well as for the systemic functions of endocrine and immune systems. In vertebrates, the vasculature is comprised of three main vessel systems. The arterial system transports blood from the heart, with larger arteries dividing in to progressively smaller arteries and capillaries. Conversely, the venous system collects the blood from capillaries and carries the blood back to the heart through progressively larger veins. Finally, the lymphatic system of vessels collects interstitial fluid from the tissues and returns it to the venous system in a continuous circulatory loop. In air-breathing organisms, there is a second circulatory loop which carries the blood from the heart to the lungs and returns the oxygenated blood back in to the heart.

The vessels themselves consist of two major cell types. Endothelial cells (ECs) form a single cell layer that lines the inner surface of all the blood vessels. In contrast pericytes and vascular smooth muscle cells, collectively known as mural cells, support the endothelium by adhering to the abluminal side of the vessel. Mural cells regulate vascular stability.

Vasculogenesis and angiogenesis are two principal processes by which new blood vessels are formed. Vasculogenesis is defined as the coalescence and differentiation of precursors (angioblasts) into endothelial cells to form a vessel de novo. Following the formation of the primitive vessels by vasculogenesis, subsequent vessels are formed by angiogenesis, a process by which new vessels are generated from pre-existing ones. The vessels then undergo remodeling and the vasculature is refined based on the structure and metabolic needs of the tissue. Some

unnecessary or transient vessels also regress later during morphogenesis to establish a functional circulatory system in the embryo.

## **1.1 Development of vasculature**

### ***1.1.1 Vasculogenesis: coalescence and differentiation of angioblasts into endothelial cells to form a vessel de novo***

In vertebrates, hematopoietic precursors and angioblasts are thought to be in close association and derived directly after gastrulation from a common mesodermal precursor termed the hemangioblast. In avian species and mammals, hemangioblasts develop extraembryonically in yolk sac blood islands whereas in zebrafish embryos they develop in the intermediate cell mass derived from the lateral posterior mesoderm (LPM)(Isogai et al., 2001) . During early development, the *ETS* family of transcription factors is required for specification of angioblasts and induction of endothelial specific markers such as *VEGFR2 (VASCULAR GROWTH FACTOR RECEPTOR 2)* (Sumanas and Lin, 2006). In fact, VEGF signaling is believed to be necessary for specification of angioblasts in vertebrates. However, there is no single specific transcription factor regulating endothelial specification. Instead a combination of multiple factors with overlapping expression patterns and redundant functions controls this process (De Val and Black, 2009).

In zebrafish embryos, from 9- 10 hours post fertilization (hpf) angioblasts start to migrate from the lateral posterior mesoderm towards the midline where they aggregate to form the first embryonic vessels, the dorsal aorta (DA) and the posterior cardinal vein (PCV) (Poole and Coffin, 1989).

Angioblasts migrate in two waves to the dorsal midline. The first-wave angioblasts contribute to the formation of the dorsal aorta while the second-wave angioblasts contribute to the vein. In fact, arterial and venous cells of the DA and the PCV originate from two distinct populations of angioblasts located medially and laterally respectively within the LPM (Kohli et al., 2013). Thus, the specification of arterial-venous fates initiates early, before the migration of angioblasts medially. Similar to zebrafish, in higher vertebrates such as mouse and chick, arteries form earlier than veins. However, both DA and PCV are initially separate bilateral pairs. Each pair fuses during later stages of development to form a single DA and PCV in the midline (Garriock et al., 2010).

Studies in multiple vertebrate including zebrafish, *Xenopus*, and mice have revealed that Sonic hedgehog (Shh), Vascular Endothelial Growth Factor (VEGF), and Notch signaling pathways are critical in the proper specification of arterial- venous fate (Udan et al., 2013). In zebrafish embryos, sonic hedgehog expression in the floor plate and the neural tube activates *VEGFA* expression in the ventral part of the somites close to the midline (Liang et al., 2001). *VEGFA* expression consequently activates VEGFA receptor (*VEGFR2*) in a subset of angioblasts located more medially to the midline. This leads to the activation of Notch signaling through its ligands DELTA (*dll1 and dll4*) and subsequently increases levels of the artery specific marker, *ephrinB2* (*EphrinB2*) as the angioblasts start to migrate. Lateral angioblasts are more distant from the source and exposed to a lower concentration of VEGFA and Shh signaling, and therefore specify as venous cells and express the receptor EphB4 (Fig. 1.1). The transcription factor CoupTFII is also important for venous specification (Pereira et al., 1999).

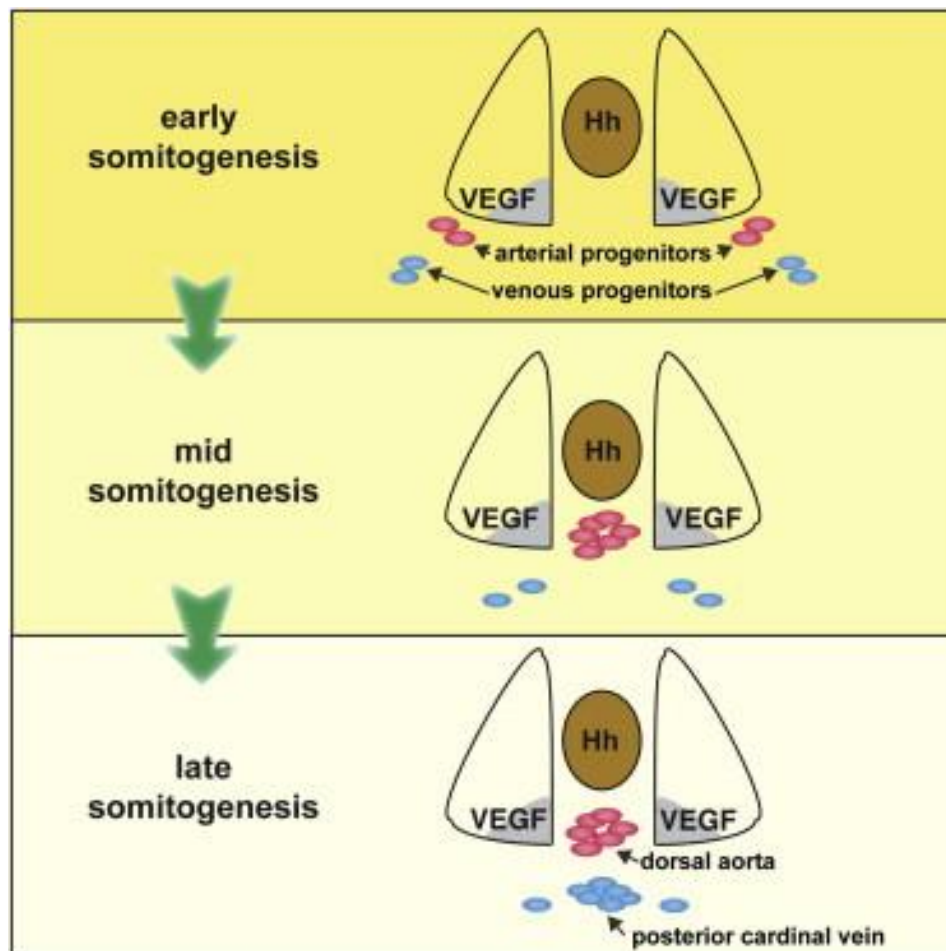
### ***1.1.2 Angiogenesis: Formation of new vessels from pre-existing vessels***

#### **1.1.2.1 Sprouting angiogenesis**

Following the formation of the primitive vessels by vasculogenesis, subsequent vessels are formed by angiogenesis. As development proceeds, this process becomes increasingly important in the formation of so many vessel structures such as intersegmental vessels (ISV), vessels of the heart, central nervous system (CNS), yolk sac, limbs and developing retina. In adults angiogenesis happens in tumor growth, wound healing, and in response to hypoxia (Udan et al., 2013).

The process of sprouting angiogenesis associates with several key events. This process initiates with the activation of endothelial cells by specific growth factors. Following this step, leading endothelial cells at the distal end of the sprouts take on the role of endothelial “tip cells”. Tip cells are critical for the emerging and guidance of the sprouts (Siekman and Lawson, 2007b; Gerhardt et al., 2003). These cells extend filopodia and lamellipodia which are sensitive to growth cues. The endothelial cells located at the base of vascular sprouts behind the tip cells form endothelial “stalk cells”. These cells do not extend processes. After the sprouts grow into the tissue, they fuse and anastomose with each other to form a closed circulatory loop.

In zebrafish embryos, blood circulation begins at approximately 24 to 26 hpf. At this time, the DA and PCV are located medially in the trunk, rather than as a pair of lateral vessels as in many other vertebrates (Isogai et al., 2001). The intersegmental vessels (ISVs) of the trunk are some of the earliest formed angiogenic vessels common to all vertebrates.



**Figure 1.1 Schematic of artery- vein specification in zebrafish embryos.**

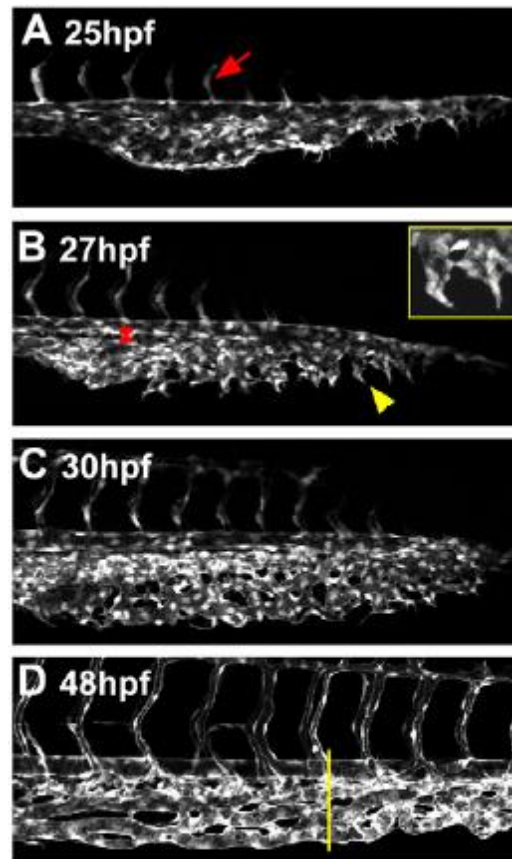
By early somitogenesis angioblasts are positioned in bilateral lines within the LPM. Sonic hedgehog expression in the floor plate and the neural tube activates *VEGFA* expression in ventral part of the somites close to the midline. *VEGFA* expression induces the expression of arterial marker in a group of angioblasts located more medially. Medial angioblasts (arterial precursors) migrate to the midline in the first phase where they differentiate as arterial cells. Lateral angioblasts are more distant from the source of *VEGFA*; therefore take on a venous identity. *Figure adopted from (Kohli et al., 2013), used by permission of the publisher.*

Time lapse imaging of vessel development in zebrafish shows a two- step process for the formation of ISVs. During the first step, the primary sprouts emerge from the DA at around 22 hpf and form the intersegmental arteries (Isogai et al., 2003). The new sprouts grow dorsally in between the somite boundaries and once they have reached the dorsal level of the neural tube, they connect with each other and form the dorsal longitudinal anastomotic vessel (DLAV). Secondary sprouts begin to emerge from posterior cardinal vein around 32 hpf. These sprouts will either connect to an existing intersegmental artery to form an intersegmental vein, or they will grow up to the level of the horizontal myoseptum and form the parachordal vessels. Lymphatic endothelial cell (LEC) progenitors arise from the parachordal vein; therefore they have a venous origin. The lymphatic sprouts grow and extend rostrally and caudally across the entire length of the trunk and form a continuous thoracic duct just ventral to the dorsal aorta (Yaniv et al., 2006). Primary sprouts contribute to the formation of both functional arteries and veins later during embryogenesis, but they are considered as arterial sprouts as they emerge exclusively from the DA and express arterial markers.

The parts of the DA and PCV caudal to the cloaca are designated as caudal artery and caudal vein, which is the main focus of my study. The caudal artery continues to the most caudal end of the embryo where it turns back to empty into the caudal vein. At around 25 hpf, coinciding with the initiation of blood circulation, the morphogenesis of the caudal vein begins. The endothelial cells of the caudal vein form sprouts and send filopodia ventrally. Following the active sprouting, the cells migrate ventrally and fuse (anastomose) with the neighboring vessels to form the primordial “caudal vein plexus” (CVP). Between 30 to 36 hpf, the sprouting

angiogenesis slows down but the CVP continues to mature and serves as a transient niche for hematopoietic stem cells (Torregroza et al., 2012). By 2 days post fertilization (dpf), the active sprouting of the CVP has stopped. Over the next week of development, the plexus becomes gently remodeled and regresses down to a single and defined ventral vessel (Choi et al., 2011) (Fig. 1.2). Taken together, different stages of CVP morphogenesis from sprouting and migration to remodeling, pruning and regression of venous derived endothelial cells, makes the CVP an excellent model for venous angiogenesis, and a good complement to the well-studied arterial segmental vessel model.





**Figure 1.2 Normal development of the zebrafish caudal vein plexus.**

(A-E) Confocal images of the caudal vein plexus of zebrafish embryos at different developmental stages. (A) Ventral sprouting of venous endothelial cells. The red arrow shows an ISV. (B) Ventral migration of venous endothelial cells. The yellow arrowhead shows venous sprouts. (C) Anastomosis (fusion) of the ventral sprouts. (D) Remodelling (pruning) of the caudal vein plexus. (E) Regression of the caudal vein plexus. *Figure adopted from (Choi et al., 2011), used by permission of the publisher.*

#### 1.1.2.1.1 Arterial sprouting Angiogenesis

The distinct timing of arterial and venous sprouting indicates the differential regulation of artery and vein angiogenesis. The VEGF family of proteins is required for blood vessel development in vertebrates. *Vegfa* mutant mouse embryos exhibit a severe reduction in the number of endothelial cells and fail to form vessels in early stages (Carmeliet et al., 1996). However, loss of specific splice variants of *Vegfa* in mice inhibits the formation of arteries and blocks the expression of arterial specific markers during later development (Lawson and Weinstein, 2002). *Vegfa* signals through two endothelial cell-specific receptor tyrosine kinases, *Vegfr1* and *Vegfr2*. *Vegfr2* acts as a typical receptor tyrosine kinase in response to a *Vegfa* gradient and subsequently affects endothelial cell proliferation, migration, survival and permeability. *Vegfr2*- deficient mice show defects similar to loss of *Vegfa*. In contrast, *vegfr1* can be alternatively spliced to a short soluble form (*sVegfr1*) that consists only of the extra cellular binding domain that acts as a decoy receptor (Zygmunt et al., 2011).

This soluble receptor, sVEGFR1 has the potential to act as a sink for VEGFA, and thus indirectly inhibits VEGFA signaling. *Vegfr1*- deficient mice exhibit overproliferation of endothelial cells due to excessive activity of *Vegfa*. Moreover, local guidance of endothelial sprouts requires *sVegfr1* expression in the stalk cells that neighbor tip cells. Upregulation of *sVegfr1* in stalk cells provides a decoy for *Vegfa* and increases the sharpness of the *Vegfa* gradient close to tip cells and therefore directs the vessel outgrowth perpendicular to the existing vessel (Chappell et al., 2009). Zebrafish *vegfr1* morphants (treated with Morpholino Oligonucleotide) display increased

number of tip cells and excessive branching of the segmental arteries (Krueger et al., 2011; Zygmunt et al., 2011)

Consistent with *Vegfa*- deficient mice, reduction of *vegfa* in zebrafish embryos causes arterial- specific defects. *vegfr2-deficient* embryos also display severe vascular defects (Covassin et al., 2006). The dorsal aorta and the caudal vein do not segregate completely (Herbert et al., 2009), and the formation of intersegmental arteries fails (Lee et al., 2007). Therefore *vegfa* is critical in the differentiation and the formation of arteries.

Unlike the VEGF family of proteins, Notch signaling generally acts to suppress angiogenesis. Notch signaling is evolutionary conserved and is the key pathway in regulating the specification of tip versus stalks cells, as well as artery and vein identity. Activation of Notch signaling promotes stalk cell fate at the expense of tip cell fate. Thus, inhibition of Notch signaling leads to a dramatic increase in the number of endothelial tip cells during retinal angiogenesis in mouse and within segmental arteries in zebrafish (Siekman and Lawson, 2007a; Suchting et al., 2007).

During tip cell selection in angiogenesis, a feedback loop between VEGFA and Notch signaling establishes a distribution of tip and stalk cells within the VEGFA activated endothelium (Lobov et al., 2007; Geudens and Gerhardt, 2011). In zebrafish embryos, *Vegfa* is secreted from the somites and activates *Vegfr2* in the tip cells of intersegmental vessels. Activation of *Vegfr2* subsequently up regulates Notch activity through Notch receptor Dll4 in the neighboring stalk cells (Geudens and Gerhardt, 2011). Therefore, a functional VEGFA/ Notch regulatory feedback loop is essential for the identification of tip and stalk cells.

#### 1.1.2.1.2 Venous Sprouting Angiogenesis

Much less is known about the signaling pathways in venous angiogenesis. Vegfc and its receptor Vegfr3, the third Vegf receptor, are critical for lymphatic development and venous angiogenesis. Lymphatic vessels do not develop in mice mutants deficient for Vegfc ligand or the receptor and consequently they exhibit lymphedema (Makinen et al., 2001). *VEGFR3* mutations in human also lead to inherited lymphedema (Karkkainen and Petrova, 2000). Vegfr3 also plays a critical role in blood angiogenesis. In mouse embryos, *Vegfr3* is expressed in intersegmental vessels and retinal vasculature during angiogenesis. Consistent with its expression pattern, inhibition of *Vegfr3* leads to the reduction of angiogenic sprouts in blood vessels (Tammela et al., 2008). In zebrafish embryos, *vegfc* is strictly required for lymphangiogenesis and venous development (Hogan et al., 2009). *vegfc* is expressed in the hypochord and in circulating erythrocytes, while its receptor *vegfr3* is expressed in the posterior cardinal vein (PCV), intersegmental vessels (ISV) and Caudal vein plexus (CVP) (Helker et al., 2013; Covassin et al., 2006). Loss of *Vegfc* or its receptor leads to a complete absence of segmental veins. However, initial formation of segmental arteries can occur independently of Vegfc signaling (Covassin et al., 2006). *vegfc* knockdown also inhibits the formation of lymphangioblast buds (Hogan et al., 2009).

Notch ligand *dll4* expression is restricted to arterial endothelial cells in zebrafish embryos. Therefore, Notch activation in arterial cells suppresses Vegfc signaling in arteries; however venous endothelial cells which do not express *dll4* respond to Vegfc/vegfr3 signaling and grow venous sprouts (Hogan et al., 2009).

Arterial and venous endothelial cells display distinct molecular identities during early developmental stages. Unlike the arterial sprouts, ventral venous sprouts from the caudal vein follow independent cues to form the caudal vein plexus in zebrafish embryos. In *vegfr2* morphants, formation of segmental arteries is blocked, however venous sprouts still form a primitive caudal vein plexus which is comprised of a dorsal and ventral vein with interconnecting vessels (Herbert et al., 2009). Conversely, over expression of *noggin3*, an endogenous inhibitor of the Bone Morphogenic Protein (BMP) signaling pathway results in the formation of a caudal vein plexus with decreased number of sprouts and improper interconnections, but no ISA defects. Consistent with these results, over expression of *bmp2b* ligand and BMP receptor type I (*bmpr1*) leads to the formation of ectopic venous sprouts in the CVP while the patterning of segmental arteries remains largely unaffected. These observations suggest that Bmp functions as a vein-specific pro-angiogenic cue during early vascular development. In mammals, BMP functions as a pro-angiogenic cue as well. Inhibition of the BMP signaling, blocks the branching of retinal vessel networks, whereas over expression of the BMP induces ectopic branching (Wiley et al., 2011).

The HMG- CoA reductase (HMGR) pathway influences venous angiogenesis. The HMGR pathway plays a role in the production of cholesterol and isoprene derivatives, which are the substrates for protein prenylation. Protein prenylation is a post transcriptional modification which is important for several cellular processes such as cell migration and cell proliferation. Inhibition of HMGR pathway using the small molecule apixone blocks caudal vein plexus angiogenesis in zebrafish embryos by modulating protein prenylation (Choi et al., 2011). Notably,

members of the Ras family of GTPases are modified by prenylation (discussed later in this chapter).

### **1.1.3 Vascular Lumen Formation**

The formation of vascular lumens by endothelial cells is required to support blood flow. A lumen can be generated *de novo* within a single cell or between cells. The mechanism of *de novo* lumen formation *in vitro* and *in vivo* shares common features. In endothelial cell cultures lumen formation occurs via either endothelial cell hollowing or endothelial cord hollowing. During cell hollowing each cell generates intracellular vacuoles independently. The vacuoles fuse to form an intracellular lumen and the two cells form junctions. Alternatively, in cord hollowing, endothelial cells make lumens after they establish contact with each other. During this process vesicles fuse within plasma and expand a large intercellular lumen that subsequently fuses to the plasma membrane of the cell.

*De novo* formation of blood vessel lumens shares conserved processes with lumen formation of other epithelial tubes. As mouse is not a good *in vivo* model for this process, it has been best studied *in vivo* in zebrafish during sprouting angiogenesis and vessel anastomosis in ISV and in an anastomosing brain artery. The lumen formation in these cells occurs via cord hollowing similar to endothelial cell cultures. Later the lumen can be extended and connected to other vessels by plasma membrane invagination or cell rearrangements, depending on the presence or absence of the blood flow, respectively. Membrane invagination extends the lumen into the inside of the cell by adding membrane material through vesicles in to the apical membrane. If the blood flow is not present, endothelial cells slide against each other and apical

membranes later fuse to form a lumen. It has also been suggested that lumen formation may also occur through cell hollowing in zebrafish. Lumen formation in other *in vivo* models, such as the *Drosophila melanogaster* tracheal system and the *Caenorhabditis elegans* excretory system, shares common biological features. In the *D. melanogaster* tracheal system a lumen is created between two cells via chord hollowing, while the anastomosis of two branches occurs via plasma membrane invagination. The intracellular lumen in *C. elegans* excretory cells is formed via cell hollowing by coalescence of vesicles that then fuse to the plasma membrane to form the branches of the excretory system. The common sequence of cell biological processes during lumen formation depends on the interactions between extracellular matrix and integrin receptors, which requires RHO GTPase family members RHOA, RAC1 and CDC42 (Sigurbjornsdottir et al., 2014).

#### **1.1.4 Vascular Remodeling and Maturation: Adjusting structural changes in response to changing conditions and tissue/organ demands**

Vascular remodeling is the process by which the primitive vasculature (plexus) matures to an efficient network of vessels with optimal blood flow. Blood flow and viscous force are important in vessel remodeling and maturation *in vivo*. *In vitro* studies show that fluid shear stress can induce changes in endothelial cell migration, proliferation, alignment and apoptosis and therefore affect vascular remodeling. However, mechanisms *in vivo* remain to be determined. Endothelial cell-cell adhesion is critical for proper networking and remodeling of vessels. A complex network of transmembrane adhesive proteins maintains the contact between

endothelial cells. Tight junctions and adherens junctions are the main types of adhesive structures in these cells.

After vascular remodeling is completed, vessel stability is achieved through recruitment of mural cells to the vessel wall. Later during development, mural cells become very important for the maintenance of vascular integrity and enable the vessels to endure the increasing pressure. Ultimately, a simple plexus can be remodeled quite differently to adopt a pattern depending on the tissue/ organ structure and function (Udan et al., 2013).

## **1.2 Vascular Malformations**

Vascular malformations are blood vessel abnormalities which generally arise from defective embryonic development. They can be classified into two groups: fast-flow malformations and slow-flow malformations. Capillary (CM), venous (VM), and Lymphatic malformations (LM) are considered as slow-flow malformations, whereas arteriovenous malformations (AVM) and arteriovenous fistulas (AVF) are considered as fast-flow anomalies. Complex combinations of these anomalies are also present.

Capillary Malformations (CM) are the most common type of vascular malformation and are usually referred to as “port-wine stain” because they usually occur on the skin or mucosa as a red-colored lesion that may become darker and thicker over time (Fig. 1.3). CMs are present at birth with the incidence of 0.3% in the birth population. CMs are mostly inherited or they can be a part of vascular malformation syndromes such as ParkesWeber syndrome, Sturge-Weber Syndrome, and Klippel-Trenaunay syndrome (Revenu et al., 2013). ParkesWeber syndrome is a combination of CAs with arteriovenous fistulas (AVF) which are small abnormal connections



between arteries and veins. Klippel-Trenaunay syndrome is a complex combination of lymphatic, venous and capillary malformations that leads to an enlargement of the affected body part. Sturge-Weber Syndrome is also a complex vascular malformation characterized by scattered CMs all over the body and vascular malformations involving the eye and the brain (Bayrak-Toydemir and Stevenson, 1993). CMs on the surface of the skin or mucosa are not life-threatening, but can cause psychological problems.



**Figure 1.3 Diversification of phenotypes associated with *Rasa1* mutations.**

(A-E) Multifocal CMs. (F) Nose AVM. (G) Hand AVM. (F) Parkes Weber syndrome.

*Figure adopted from (Revencu et al., 2013), used by permission of the publisher.*

There are no treatments for CMs currently, however, laser therapy may be considered if the patient requires treatment.

Arteriovenous malformations (AVM) are fast-flow and the most dangerous type of all vascular malformations. AVMs are characterized by abnormal connections between arteries and veins, lacking the small capillaries in between. The direct connection between the artery and the vein is called a shunt. AVMs are more common in the central nervous system, but can also involve the skin and other tissues such as bone and muscle. These abnormalities are often present at birth; however some can remain quiescent till the second or third decade of life. Most people with neurological AVMs (those located in the central nervous system) experience no significant symptoms and the disorder is usually discovered incidentally, however in some individuals, the neurological damage reaches a critical point and can be life-threatening. AVMs in the brain and spinal cord can cause hypoxia, hemorrhage and/ or tissue displacement, with hemorrhage being the greatest potential danger. Currently there are no drug treatments for AVM. Surgical treatments such as catheter embolization and surgical resection are often very difficult, and multiple treatments are required when patients show progression of their AVM (NIH National Institute of Neurological Disorders and Stroke, 2014).

The combination of multiple CMs with fast-flow vascular malformations is called capillary malformation- arteriovenous malformation (CM-AVM). The capillary stains associated with CM-AVM are distinguishable from those of common CM, and the clinical diagnosis of CM-AVM is based on characterizing the CM lesion. About 30% of the patients with a clinical CM-AVM

diagnosis have fast-flow malformations including AVM, AVF and ParkesWeber syndrome (Revencu et al., 2008).

CM-AVM is inherited in an autosomal dominant pattern; however the variability of the phenotype as well as the localization and multi-focal nature of the lesions is likely due to somatic second hits, indicating the lesions are caused by a biallelic somatic loss of function. RAS p21 protein activator 1 (*RASA1*; *p120-RasGAP*) is the causative gene in the autosomal dominant form of CM-AVM (Revencu et al., 2013).

Based on three large cohort studies, more than 320 human patients with *rasa1* mutation have been reported. Among these patients CMs, AVMs, AVFs and ParkesWeber syndrome are common phenomenon (Eerola et al., 2003; Revencu et al., 2008; Revencu et al., 2013). According to a recent study by Revencu et al in 261 patients with *Rasa1*- related disorders, 68 different mutations were identified. Of these individuals with *rasa1* mutation, over 97% had visible CMs, about 24% were diagnosed with intracranial or extracranial AVMs/ AVFs, and 8% showed ParkesWeber phenotype. However, given that comprehensive imaging is preferentially performed in the head region of the patients the occurrence of AVMs/AVFs in *RASA1*- related disorders might be underestimated. This interesting work highlighted that superficial CMs can be an indication of an underlying, and more serious, AVM (Revencu et al., 2013).

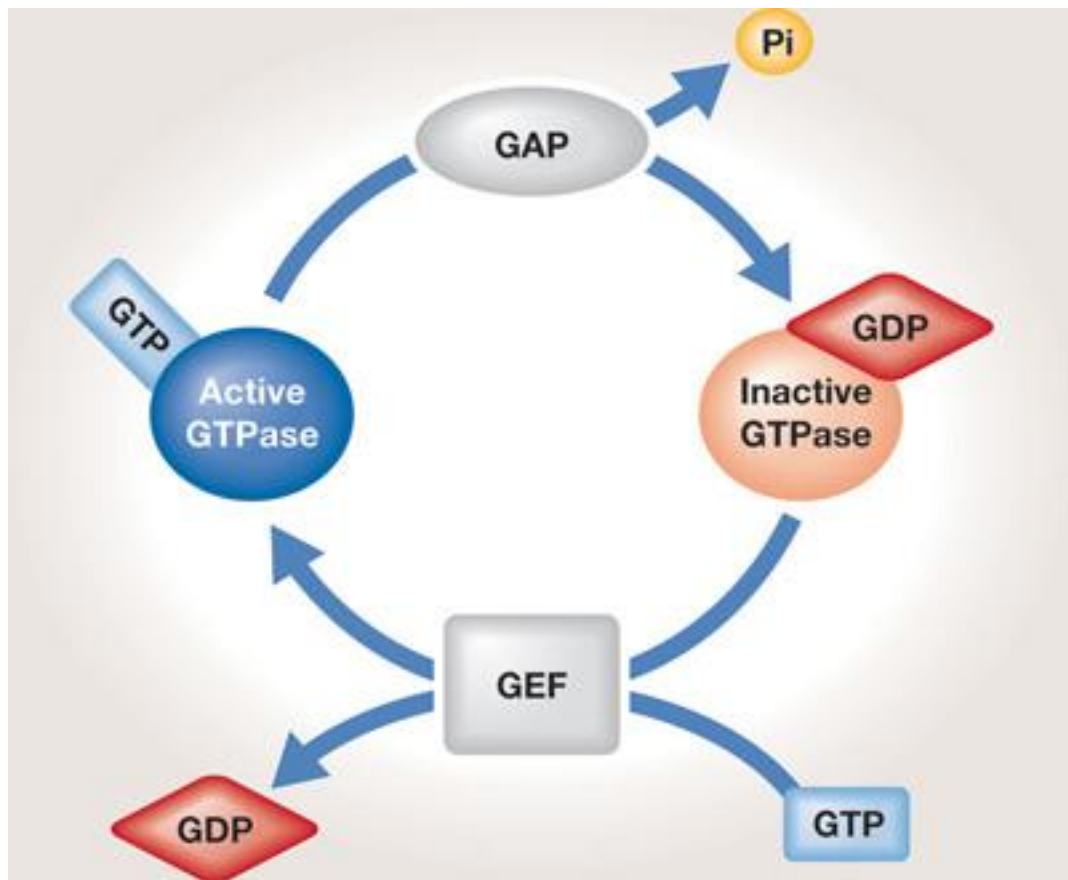
*RASA1* is best known as a suppressor of Ras function through enhancing the intrinsic GTPase activity of RAS proteins (see below). Mutations in oncogenic RAS proteins that lead to constitutively active RAS, have found in a large number of human cancers, but not in vascular malformations. Since CM-AVM is an overgrowth of vessels, repression of Ras signaling by *RASA1*

is a plausible mechanism by which malformations might develop; however, this has not been explored *in vivo* (Eerola et al., 2003). My project will examine the control of Ras signaling by *rasa1* in the vasculature.

### **1.3 The Ras GTPase Superfamily**

#### **1.3.1 Biochemistry and regulation of small GTPases**

Small guanosine triphosphatases (GTPases) are central regulators in signal transduction pathways in almost every aspect of cell dynamics. They function as molecular switches that are activated by extracellular stimuli to regulate intracellular signaling including actin dynamics and gene transcription, which consequently controls fundamental cell processes. Small GTPases have a common biochemical activity. Hydrolysis of GTP leads to the formation of GDP. The GDP-bound state is inactive, whereas the GTP-bound form has a high affinity for downstream effectors. Small GTPases exhibit low intrinsic GTP hydrolysis activity, therefore the GTP/GDP cycling requires regulatory proteins such as the Guanine nucleotide Exchange Factors (GEFs) to facilitate GDP dissociation and stimulate the formation of the active GTP-bound form. GTPase Activating Proteins (GAPs) enhance GTP hydrolysis activity and promote the formation of GDP (Fig. 1.4). Guanine dissociation inhibitors (GDIs) are also work in opposition to GEFs by inhibiting the release of GDP. They are also required to form soluble complexes to assist dissociation from membrane surfaces of a class of lipid-modified small GTPases, which carry a farnesyl or geranylgeranyl group in their C-terminus.



**Figure 1.4 Regulation of small GTPases**

GTPase-activating proteins (GAPs) accelerate the rate of GTP hydrolysis, promoting the inactive GDP-bound state of GTPases whereas guanine nucleotide exchange factors (GEFs), induce the dissociation of GDP and binding of GTP, promoting the active GTP-bound state of GTPases.

*Figure adopted from (Duran and Hall, 2012), used by permission of the publisher.*

The superfamily of Ras GTPases is one of the largest GTPase families. Classic Ras proteins are the most well-known members of this group and control several fundamental cell processes such as growth and differentiation. The Ras superfamily is comprised of over 100 proteins and is divided into five main protein families according to functions and sequence homologies: Ras, Rho, Rab, Ran, and Arf (Fig. 1.5) (Wennerberg et al., 2005).

### **1.3.2 Post-transcriptional modifications of the Ras superfamily of GTPases**

Many Ras superfamily proteins are post-translationally modified by lipid modifications which assist their association with cellular membranes. The majority of Ras superfamily proteins bear a CaaX motif at their C-terminal composed of: Cysteine-aliphatic residue-aliphatic residue-variable amino acid. Prenylation is a covalent lipid modification of C-terminal cysteine residues of CaaX motifs by the farnesyl transferase (FTase) and geranylgeranyl transferase (GGTase), which catalyze the addition of a 15-carbon farnesyl or 20-carbon geranylgeranyl lipid group respectively. Prenylation promotes the interactions of modified proteins and cellular membranes due to the hydrophobicity of the lipid membrane. Prenylation is required for most of Ras, Rho and Rab family members. However, some Ras superfamily members lack the C-terminal motif and yet still associate with membranes by alternative mechanisms (Zhang and Casey, 1996).

Since Ras GTPases are a major contributor to the formation of cancer, prenylation is a major target for the development of anti-cancer drugs. Farnesyltransferase inhibitors (FTIs) and geranylgeranyl transferase inhibitors (GGTIs) target FTases and GGTases, respectively, and lead to improper functioning of Ras proteins (Sebti and Hamilton, 2000).

### ***1.3.3 Sub-families of the Ras superfamily of GTPases***

Ras (rat sarcoma) genes were first characterized as human oncogenes and are mutated somatically in a large proportion of cancers (22% for Kras, 8% for Nras and 3% for Hras; Prior et al., 2012). Kras, Nras and Hras are generally involved in cell proliferation, cell morphology, cell differentiation and apoptosis. The members of the closely related Rho (Ras homologous) family mainly participate in determining cell morphology, actin regulation, cytoskeletal organization and cell polarization. RhoA and the other family members Rac1 and CDC42 function in lumen formation. While RhoA promotes the formation of actin fibers, Rac1 and CDC4 promote the formation of lamellipodia and filopodia respectively. Rab (Ras-like proteins in brain) proteins are the largest subfamily of Ras superfamily. Rab family members are mostly involved in vesicular transport and protein trafficking. Rab proteins facilitate the formation and the budding of vesicles as well as the fusion and release of the vesicular content. Like the Rab family, Arf (ADP-ribosylation factor) family members are also associated with vesicular transport. Arf1, the best characterized member of the family, is important for the formation of vesicular coats which facilitate vesicle formation and release. There is only one identified member of Ran (Ras-like nuclear) family in eukaryotes, which is primarily involved in nucleocytoplasmic import and export of RNA and proteins transport (Wennerberg et al., 2005) (Table 1.1). For my research project I have focused on Ras subfamily of proteins and associated regulators.

#### **1.3.3.1 Ras Protein Subfamily**

Most Ras subfamily members are relatively small proteins of about 183 to 340 amino acids, and there are 35 Ras family genes in humans. Ras subfamily proteins are functionally



distinct, but can cross-talk with their close relatives (Colicelli, 2004). The Ras subfamily includes the classic Ras oncoprotein branch (Hras, Kras, and Nras), Rras branch (Rras, Rras2/TC21, and Rras3/Mras), Rap branch (Rap1A, 1B, 2A and 2B), Ral branch (RalA and RalB), and Rheb. Rit and Rin branches are also included in the Ras family due to the similarity in the effector domains. Based on the ability of the mutants to cause growth transformation, the Ras subfamily can be divided into two main groups: Ras oncoproteins, Rras, Rit, and Rin proteins, which show transforming activity in cell culture versus Rap, Ral and Rheb proteins, which do not. Most Ras family members are farnesylated, with the exception of Rras and some Rap and Ral proteins that require geranylgeranylation. Here I will focus on 3 main branches: Ras oncoproteins branch, Rras branch, and Rap branch (Reuther and Der, 2000).

#### 1.3.3.1.1 RAS oncogene proteins (KRAS, NRAS, and HRAS)

In agreement with their role in human cancers, Ras classic proteins are mostly known for their role in normal mitogenic signaling pathways. Kras, but not Nras and Hras, is essential for murine growth and development (Johnson et al., 1997). It is also the most mutated Ras family member in cancer (Prior et al., 2012). Kras is required for differentiation of erythroid progenitors during fetal liver erythropoiesis in mice, and Kras deficient mouse embryos display anemia (Braun et al., 2006). Kras knock down in zebrafish results in the mispatterning of ISV and the sub intestinal venous plexus (SIVP) (which vascularizes the gut) as well as hematopoietic defects (Liu et al., 2008b). Hras, on the other hand is involved in the signaling pathways that control integrin function (Kinbara et al., 2003).

Ras proteins have multiple effectors. Ras binding with its effectors Raf1, A-Raf and B-Raf leads to the activation of RAF kinase activity, which in turn activates the mitogen-activated protein kinase (MAPK/ERK) signaling cascade. The activation of MAPK/ERK pathways stimulates transcription factors important for cell growth, cell differentiation, and survival. The B-Raf mutation, which results in constitutive activity, occurs frequently in human cancers, suggesting the important role of this pathway in tumourigenesis (Colicelli, 2004).

Phosphoinositide 3-kinase (PI3K) is another important Ras effector. The interaction between Ras and PI3K activates PI3K catalytic activity, which leads to the phosphorylation of AKT. AKT activation stimulates a number of downstream effectors, such as the mTOR pathway, which promotes cellular growth (Colicelli, 2004). Upregulation of endothelial mTORC1 has been found in CM-AVM patient tissues, as well as in the zebrafish CM-AVM models (Kawasaki et al., 2014).

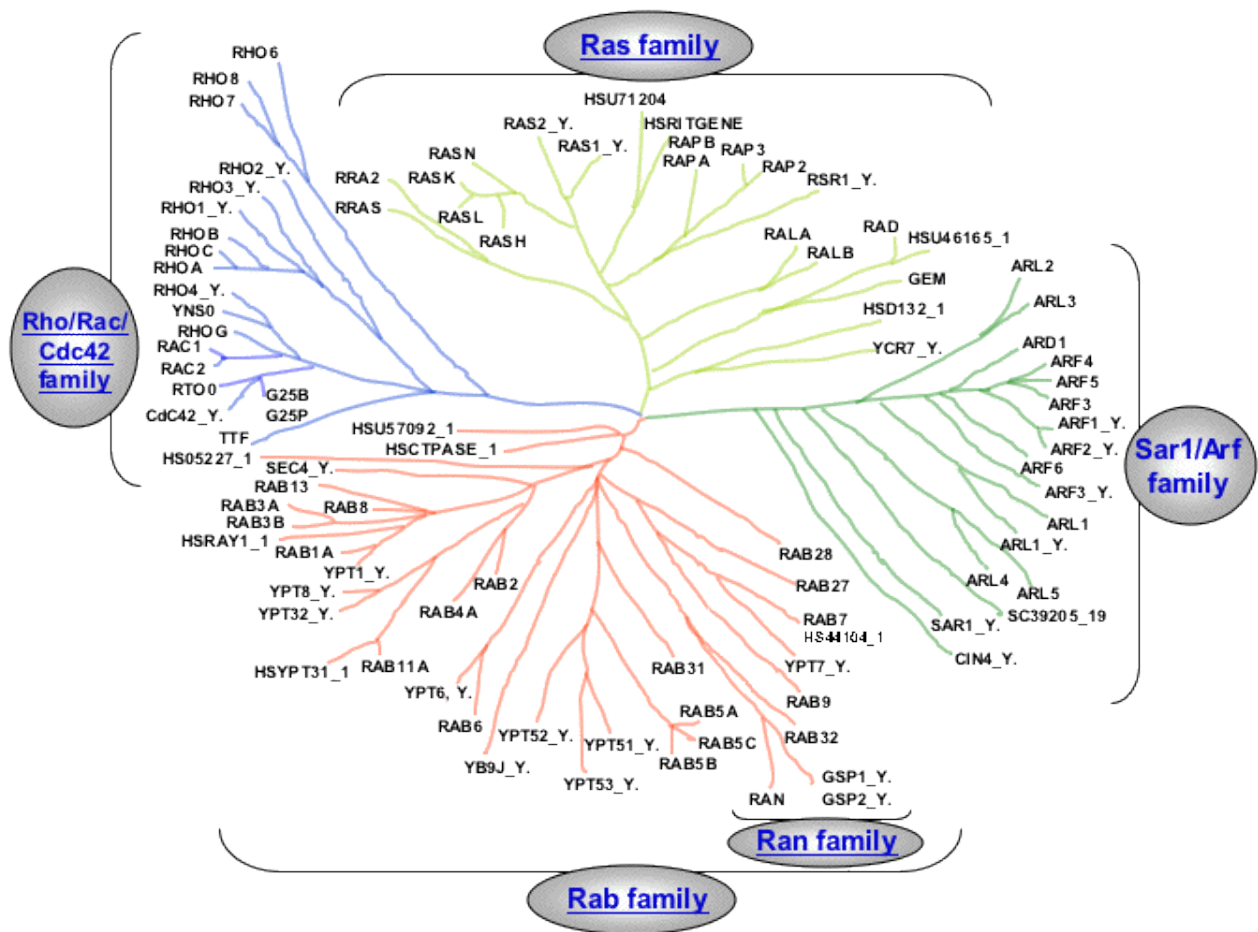
Another effector of Ras and Rap is Ras-interacting protein (Rasip). *Rasip1* deficient vessels in mouse and zebrafish embryos develop hollow tubes that permit circulation of premature erythrocytes, but finally collapse and result in hemorrhage and embryonic lethality (Wilson et al., 2013; Xu et al., 2011), showing a role for Ras in lumen formation.

#### 1.3.3.1.2 RRAS (Related to RAS) proteins (RRAS, RRAS2, RRAS3)

Like Ras proteins, members of Rras family, including Rras, Rras2, and Rras3 (Mras), can be potently transforming. Activating mutations in Rras2 have been found in some human cancers. Rras predominantly controls integrin function and promotes cell adhesion. Integrins are cell-

surface receptors that can bind to the extracellular matrix (ECM) molecules such as collagen, laminin and fibronectin through their extracellular region. Integrins can also bind to cytoskeleton components inside the cell, and thus transmit signals from ECM to control cytoskeletal reorganization and subsequently affect cell adhesion, migration and growth. Activating mutations of Rras can convert suspension cell lines into highly adhesive cells, whereas Rras dominant-negative expression diminishes cell adhesiveness (Kinbara et al., 2003).

Like Ras proteins, Rras family interacts with PI3K and Raf kinases. Rras proteins also share some GEFs and GAPs with Ras proteins. The overlap in partners between Ras and Rras is likely due to the sequence similarities among members of both branches.



**Figure 1.5 Dendrogram of the Ras Superfamily of proteins. Note that Ras, Rras, Rap, and Ral cluster together in the Ras family.**

*Figure adopted from (Takai et al., 2001), used by permission of the publisher.*

Subfamily	Function
<b>Ras</b>	cell proliferation, cell morphology, cell differentiation, apoptosis
<b>Rho</b>	cell morphology, actin regulation, cytoskeletal organization, cell polarization
<b>Rab</b>	vesicular transport, protein trafficking
<b>Ran</b>	nucleocytoplasmic import
<b>Arf</b>	vesicular transport

**Table 1.1 Summary of subfamilies of Ras superfamily modes of function.**

	Members	Cellular role
<b>Ras</b>	Hras, Kras, Nras	Mitogenesis
<b>Rras</b>	Rras, Rras2/TC21, Rras3/Mras	Integrin-mediated cell adhesion
<b>Rap</b>	Rap1A, 1B, 2A, 2B	Integrin-mediated cell adhesion

**Table 1.2 Summary of the main modes of function of the Ras subfamily main branches (Ras, Rras, and Rap).**

However distinct physiological consequences of Ras vs. Rras activation may be due to some unique effectors contributing to the regulation of each branch (Colicelli, 2004).

#### 1.3.3.1.3 RAP (Ras-Proximal) proteins

Rap proteins are potent activators of integrins thus regulate integrin-dependent cell adhesion and spreading. Embryonic fibroblasts deficient in a Rap GEF (which leads to Rap activation) show decreased attachment to collagen and fibronectin and enhanced migration. Likewise, overexpression of a Rap-specific GAP (which leads to Rap activation) inhibits cell attachment to fibronectin and causes detachment. Unlike Ras and Rras proteins, Rap deficient mutants in cell culture do not show cellular transforming activity. Rather, overexpression mutations of some RAP proteins negatively regulate Ras- dependent transformation. Rap proteins may share some effectors with Ras proteins (Colicelli, 2004).

Both Rap and Rras are important in cellular signaling pathways that control integrin function. For instance, the active form of Rap1 or Rras in macrophages initiates phagocytosis through activation of integrins. However inhibition of Rap1 blocks phagocytosis in macrophages, whereas inhibition of Rras fails to do so. On the other hand, overexpression of Rap1GAP (which leads to Rap dysfunction) abolishes integrin activation induced by Rras. Thus, Rap and Rras may act in a similar pathway and Rras may need Rap to induce integrin activation. However, Rap can be activated by a variety of growth-promoting stimuli independently of Rras (Kinbara et al., 2003) (Table 1.2).

#### **1.3.4 p120RasGAP (*Rasa1*): The causative gene in Capillary Malformation- Arteriovenous Malformation (CM-AVM)**

As discussed above, GTPase activating proteins (GAP) enhance the endogenous GTPase activity of RAS proteins which leads to the formation of inactive GDP-bound Ras.

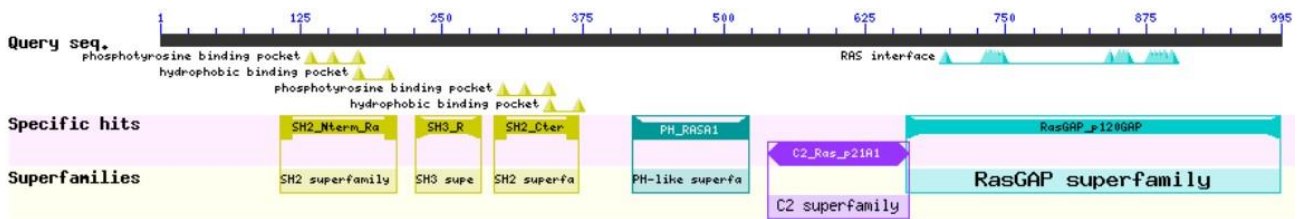
*P120RasGAP (Rasa1)* was the first identified GAP for RAS. In previous studies, *Rasa1* has been classified as a specific GAP for members of Ras sub-family of GTPases (*Kras*, *Nras*, and *Hras*). Since the primary structures of all the three Ras proteins are almost identical, *Rasa1* interacts with all three proteins with no preference *in vitro* (Zwartkruis and Bos, 1999). *Rasa1* also stimulates the GTPase activity of all the members of Rras sub-family including *Rras* (Rey et al., 1994), *Rras2* and *Rras3* in cell culture. *In vitro*, *Rasa1* also interacts with *Rap1a* without enhancing its GTPase activity. Indeed, *Rap1a* can act as a potent inhibitor of Ras GTPase activity, perhaps by limiting the amount of *Rasa1* available for interaction with *Ras* (Frech et al., 1990). However not much is known about the *in vivo* interactions of *Rasa1* with the members of the *Ras* superfamily.

*Rasa1* is a relatively small cytosolic protein, which contains several conserved domains including a C-terminal GAP (GTPase-activating proteins) domain, an N-terminal Src Homology 3 (SH3) domain which is flanked by two Src Homology 2 (SH2) domains, followed by a PH (pleckstrin homology) and a CaLB/C2 (calcium-dependent phospholipid-binding) domain (Fig.1.6).

The catalytic GAP domain of *Rasa1* enhances the hydrolysis of the GTP-bound active form of the Ras to the GDP-bound inactive form. Thus, *Rasa1* can act as a potent Ras suppressor.  $\text{Ca}^{2+}$ -dependent C2 and PH domains have diverse functions and are essential for protein targeting to

couple extracellular signals to intracellular events. Moreover, *Rasa1* can act as a down-regulator of its downstream effectors through its SH domains. Generally proteins with an SH2 domain can associate with phosphorylated tyrosine kinase receptors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and Eph tyrosine kinases, and are involved in signal transduction. *Rasa1* SH2 domains can interact with p190RhoGap (a Gap for Rho family of GTPases), suggesting the co-ordination of Ras and Rho- mediated signaling pathways. The SH3 domain is also required for Ras downstream signaling and is involved in Rho-mediated cytoskeletal reorganization. A *Rasa1*/p190RhoGAP complex is required for reorientation of focal adhesions and for maintaining elongate morphology, independent of Ras activity during directional movement in wounding assays. *Rasa1* deficient cells also display reduced movements and are incapable of establishing cell polarity that can be partly due to loss of the *rasa1*/p190RhoGap complex (Pamonsinlapatham et al., 2009).





**Figure 1.6 Rasa1 binding domains.**

Rasa1 contains N-terminal SH2-SH3 domains, a PH domain, a CA-dependent phospholipid binding domain and a C-terminal RasGAP domain.

Figure adopted from <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>.

*Rasa1* is an essential gene in vertebrates. *Rasa1* homozygous mutant mice fail to organize endothelial cells to form a proper vascular network within the yolk sacs and display blood vessel growth abnormalities that lead to vessel rupture and death by E10.5 (Henkemeyer et al., 1995). Conditional knockout of *Rasa1* in endothelial precursors results in embryonic lethality at the same stage, suggesting that vascular defects are the cause of lethality in *Rasa1* null mice. However, the gene is only required for early angiogenesis as conditional deletion of *rasa1* in mice at or older than 2 months of post natal life results in dilation, hyperplasia and leakage of lymphatic vessels but no blood vessel defects (Lapinski et al., 2012).

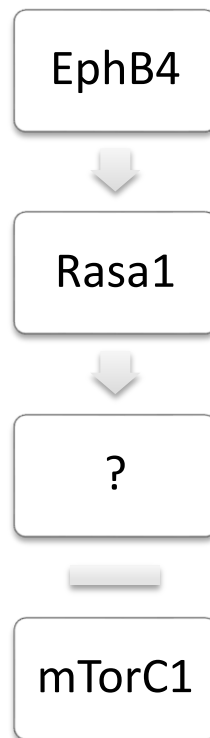
As discussed earlier, mutations of *Rasa1* in humans cause an autosomal dominant form of capillary malformation-arteriovenous malformation (CM-AVM), but adult homozygous patients have not been identified, suggesting homozygous mutation of *Rasa1* may be incompatible with life (Eerola et al., 2003; Revencu et al., 2013; Revencu et al., 2008)

In an effort to define a molecular pathway in which *rasa1* plays a role as a causative gene for CM-AVM, a recent study used morpholino knockdown in zebrafish. In parallel, our lab was independently working on this project, and many of the findings we had with regard to phenotype were similar. My data is described in the results section, while the published data is described here. There are two *Rasa1* homologues in zebrafish: *rasa1a* and *rasa1b*. Kawasaki et al. (2014) showed that *rasa1* is a downstream effector of endothelial *ephb4* receptor in venous development, leading to an enlarged posterior cardinal vein and abolition of the CVP. *rasa1* is presumed to inhibit Ras activation but the details of this part of the pathway were not probed in this publication. The end result of loss of *rasa1* is higher endothelial mTORC1 activity. mTORC

activation is linked with increases in cell size, and thus the enlarged vessel results from an increase in cell size, not cell number. In addition, more segmental veins are formed at the expense of the segmental arteries, suggesting a switch to a more venous fate. Inhibition of both *rasa1* and *ephb4* results in an identical vascular phenotype suggesting the two genes are in the same pathway. *rasa1a* deficient embryos show overactivation of mTORC1 suggesting the deregulation of *ephb4/rasa1/mTORC1* signaling pathway in endothelial cells (Kawasaki et al., 2014) (Fig. 1.7). However, there is no *in vivo* evidence for downstream GTPase effectors of *rasa1* that lead to mTORC1 overactivation. It is also unknown if *rasa1* interacts with other growth factor signaling pathways involved in venous angiogenesis.

#### **1.4 Hypothesis**

I hypothesize that *rasa1* knockdown leads to vascular malformations that arise from unregulated *Ras* GTPase signaling and lead to disturbances in growth factor pathways.



**Figure 1.7 Current possible *in vivo* mechanism for *rasa1*-mediated CM-AVM.**

*ephb4/ rasa1a* interaction is required for inhibition of *mTORC1* activity. Partial inhibition of *rasa1* leads to overactivation of *mTORC1* and subsequently promotes the formation of abnormal vessel connections. However which GTPase(s) is interacting with *rasa1* is still unknown *in vivo* and this component will form the basis of my thesis.

*Model adopted from (Kawasaki et al., 2014).*

## Chapter Two: Materials and Methods

### 2.1 Model system, embryo handling and fixation

Zebrafish (*Danio rerio*) embryos were raised at 28°C in E3 media (see Table 2.1 for all buffers cited) plus 0.003% 1-phenyl 1-2-thiourea (PTU; Sigma, St. Louis, MO) added at 24 hpf to block pigmentation. Embryos were staged according to (Kimmel et al., 1995) in hpf. For *in situ* hybridization and some antibody staining experiments, embryos were fixed in 4% paraformaldehyde in PBS and after a brief wash with PBT; they were stored in 100% methanol in -20°C. Fish strains used include : wild type Tupfel long fin (TL) zebrafish, *Tg(kdrl:mcherry)<sup>ci5</sup>*, *Tg(fli1:nEGFP)y7*, *Tg(kdrl:GFP)<sup>la116</sup>*, and *Tg(gata1:dsRed)<sup>sd2</sup>* (Table 2.2) The *kdrl* and *fli1a* promoters are both expressed in endothelial cells. However, *kdrl* is expressed later, around 5 to 7 somite stage (Liao et al., 1997), while *fli1a* is expressed earlier from 1 to 4 somite stage (Liu et al., 2008a). The *fli<sup>y7</sup>* allele expresses nuclear GFP.

### 2.2 RNA isolation and cDNA synthesis

Total RNA was isolated from zebrafish embryos by collecting embryos at the appropriate developmental stages, removing the buffer and freezing embryos in -80°C until ready to use. For RNA isolation 50-100 zebrafish embryos were homogenized using a 22 gauge needle and a syringe with 600 µL of RLT buffer from the RNeasy Mini Kit (Qiagen, Mississauga, ON) and 6 µL of beta-mercaptoethanol. The RNeasy Mini Kit was used for the rest of the procedure as per the manufacturer's instruction. To make the cDNA, a volume of 1.8 µL DNase I reaction buffer (Thermo Scientific, Wilmington, DE) and 1.8 µL DNase I were added to 1 µg total RNA and

mixture was incubated in the room temperature for 15 minutes. The reaction was then inactivated by adding 1.8  $\mu$ L 25 mM EDTA and placed at 65°C for 10 minutes. For cDNA synthesis, to each tube 2  $\mu$ L of oligo(dT)20 (50  $\mu$ M) (Invitrogen, , Carlsbad, CA) and 4  $\mu$ L of dNTPs (10mM, Invitrogen) were added and incubated at 65°C for 5 minutes and placed on ice. Lastly, 8  $\mu$ L of 5X cDNA synthesis buffer, 2  $\mu$ L of DTT (0.1), 2  $\mu$ L of RNaseOUT (40U/ $\mu$ L, Invitrogen), 2  $\mu$ L of RNase-free water, and 2  $\mu$ L Superscript III (10 U/ $\mu$ L; Invitrogen) were added to each tube and the mixture was allowed to react at 50°C for 1 hour. The reaction was stopped by heating at 85°C for 5 minutes. Finally, 2  $\mu$ L of RNase H (0.1 U/ $\mu$ L; Invitrogen) was added followed by 20 minutes incubation at 37°C. The cDNA was then stored at -20°C for later use.

### **2.3 PCR amplification**

For standard PCR to generate probes for *in situ* hybridization (ISH), Taq DNA polymerase (0.25  $\mu$ L; New England Biolabs, Ipswich, MA) was mixed with the corresponding 5x buffer (2  $\mu$ L), dNTP (1  $\mu$ L, 10 mM; Invitrogen, Carlsbad, CA), and 1  $\mu$ L of each the forward and reverse primers (University of Calgary Core DNA services) to amplify variable volumes of cDNA template. For synthesis of RNA probes from the template DNA, reverse primers incorporated a T7 promoter binding site for *in vitro* transcription of the product. DNA fragments were then separated using gel electrophoresis in 1% agarose in TBE buffer. The DNA fragment was then purified using a QIAGEN PCR purification kit and the manufacturer's instructions (Qiagen, Mississauga, ON). A Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) was used to measure the concentration of the product. All PCR primers used in *in situ* hybridization experiments are listed in Table 2.3.

## 2.4 RNA probe synthesis

*In vitro* transcription for probe synthesis was performed by incubating 0.1 µg/200 bps of the PCR template, T7 RNA polymerase (2 µL; Promega), 5X buffer (4 µL; Promega), 100 mM DTT (2 µL; dithiothritol; Promega), 10X DIG RNA labeling mix (2 µL; Roche Applied Science, Laval), and RNAsin (1 µL; Promega) in a final volume of 20 µL, for 2 hours at 37°C. The DNA template was removed by adding 2 µL of RNase-free DNase (0.1 U/µl; Promega) and the mixture incubated for 15 minutes at 37°C. The mixture was then added to the top of the quick spin column and centrifuged at 2800 rpm at 4°C for 2 minutes. The reaction was terminated by adding 2 µL of 0.2 M EDTA (pH 8). To verify the proper probe synthesis, 1 µL of the probe was run on a 1% agarose gel, and the remaining probe was stored in hybridization buffer at -80 °C.

## 2.5 Wholemount RNA *In situ* Hybridization

*In situ* hybridization (ISH) method is used to visualize gene expression within fixed tissues and cells. ISH was performed using a protocol described by Lauter *et al.* (2011). Fixed embryos were treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 minutes. Then the embryos were rehydrated by washes in 25%, 50% and 75% dilutions of methanol dilute with PBT, each for 5 minutes. After several washes with PBT, embryos were permeabilized by incubation with Proteinase K (10 µg/ml; Promega) at room temperature. The reaction was terminated with two rinses in 5% glycine in PBT. Embryos were then fixed with 4% PFA for 15 minutes at room temperature. To remove residual PFA embryos were washed several times with PBT. For pre- hybridization, embryos were put in 50% hybridization buffer (hyb) and incubated at 60°C for at least one hour. The pre-hyb buffer was then removed and replaced with 1:100 dilution of probe in hybridization

buffer with 5% dextran sulfate. The embryos incubated overnight at 60°C. The day after incubation, probe was removed and the embryos were washed twice with a 50% formamide, 2x SSC, 0.1% Tween solution, each time for 5 minutes at 60°C. Next, they were washed in 2x SSC for 15 minutes at 60°C, followed by 2 x 30 minute high stringency washes in 0.2x SSC at 60°C. In order to decrease the non-specific staining, embryos were blocked using 10% normal sheep serum (NSS) in PBT and incubated for 1 hour at room temperature. Next, the blocking solution was removed and the embryos were incubated for 2 hours in 10% sheep serum in a 1/5000 dilution of anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche Applied Science, Laval, QC) in PBT at room temperature. Next, the antibody was removed followed by two quick washes with PBT and a long wash overnight. The following day, embryos were transferred to 24-well dishes and were washed 4 x 5 minutes in NTT solution. The staining took place after addition of 1 mL of NTT containing 4.5 µL NBT (nitro blue tetrazolium chloride, Roche) and 3.5 µL BCIP (5-bromo-4-chloro-3-indolyl-phosphate, Roche). The embryos were kept in the dark and allowed to react with the color for a variable amount of time (~30 minutes to 4 days). To terminate the color reaction, embryos were fixed with 4% PFA in PBS followed by two quick PBT washes. Embryos were stored in PBT at 4°C for later imaging. For imaging the expression pattern, embryos were mounted in 3% methylcellulose and photographed under white light using a Stemi SV11 microscope and Axiocam HRc digital camera (Carl Zeiss Inc., Thornwood, NY).

## **2.6 Morpholino injection**

All the morpholinos were obtained from Gene Tools LLC (Corvallis, OR) and were diluted in water to 2 mM stocks. Embryos were collected between the 1-4 cell stages and chorions were



removed by soaking the embryos in pronase for ~2 minutes. Embryos were then transferred to 1.5% agarose (Invitrogen) ramps and were injected using a Femtojet injector (Eppendorf AG, Hamburg, Germany) with 1 mm microcapillary needles with filament (World Precision Instruments, Sarasota, FL). A Flaming/Brown micropipette puller (Sutter Instruments Co., Novato, CA) was used to pull the needles to the point. Embryos were observed under a dissection microscope (Leica Microsystems, Richmond Hill, ON). The diameter of the injected liquid was measured at a specific magnification and was converted to ng using the volume injected and the reagent concentration. Embryos were then transferred to 100 mm glass dishes (VWR, Mississauga, ON) and allowed to grow in E3 buffer at 28.5°C according to standard zebrafish protocol. All the morpholinos used in this thesis and the optimal dosages are listed in Table 2.4.

## 2.7 Microinjection

To verify the successful knockdown function of the splice blocking *rasa1a* morpholino, cDNA was made from both wild type and morphant. The cDNA used as a template for a PCR reaction. Two primers were used flanking the splice site targeted by the morpholino. Two primers flanking a ~100 bps segment of eukaryotic translation elongation factor 1 alpha1, like1 (*EEF1α1/1*) were also used in the PCR reaction as a control. *EEF1α1/1* is expressed ubiquitously, thus the expression minimally changed even in gene knockdowns that result in severe vascular defects. DNA products were visualized by gel electrophoresis. All of the primers that were used for morpholino efficiency experiments are summarized in Table 2.3.

## **2.8 Immunohistochemistry**

Fixed wild type and *rasa1a* morphants in 100% methanol were collected and incubated in pure acetone for 30 minutes at -20°C for permeabilization. Following 3 × 5 minutes washes in PBT, embryos were blocked for 1 hour with 10% NSS in PBT. Next, the embryos were incubated in 1:100 dilution of the primary antibody (Sigma, Mouse anti ZO-1 and Mouse antiβ-catenin) in PBT at room temperature for at least 2 hours. The embryos were then washed 4 × 15 minutes in 1% NSS/PBT and incubated with the secondary antibody (Sigma, Donkey anti mouse Alexa488) in a 1:500 dilution in 1% NSS/PBT for at least 2 hours at room temperature. Lastly, the embryos were washed 4 × 15 minutes in PBT and kept in darkness at 4°C. For sectioning, embryos were embedded in JB4 (Polysciences, Warrington, PA) and cut into 7 μm sections using a Leica microtome. Embryos were then photographed with Zeiss Axio Lab.A1 Compound Microscope equipped with an AxioCam HRC camera (Carl Zeiss Canada Ltd., Toronto, ON).

## **2.9 Confocal Microscopy**

Embryos were photographed in high resolution using a Zeiss LSM700 confocal microscope. Live embryos were anesthetized in 0.04% buffer Tricaine methanesulfonate (Tricaine; Sigma) in E3 and mounted and oriented properly in 0.75% low melt agarose in E3. Images were taken using the 20x objective lens, and consist of typically several z-stack layers to achieve image depth. Zeiss Zen software was used for image analysis, adjusting levels, compiling z-stacks, measuring maximum migration distance, and producing video in time-lapse imaging. Cell counts were done using the automatic particle counting feature of ImageJ software (Schneider et al., 2012). The images were further modified using Adobe Photoshop.

## 2.10 Chemical inhibitor treatment

Ras farnesyl transferase inhibitor, L-744,832 (Calibiochem, 5 mg; injecting 5mM-3nI; soaking 100  $\mu$ M between 18 to 30hp) was dissolved in DMSO and then diluted in E3 buffer. The embryos were allowed to develop in darkness at 28°C and the phenotype was scored at 30 hpf.

## 2.11 Phenotype scoring and statistical analysis

To quantify venous angiogenesis in the CVP, I counted the number of caudal plexus segments (the area defined between two adjacent somite boundaries) lacking the arterial/venous boundaries and the interconnecting vessels within the venous plexus in lateral sections of both *rasa1a* morphants and their uninjected siblings at 30 hpf. Any segment with these criteria was given a value of 1. The total of 6 segments was counted in each embryo starting at the end of yolk extension. The assay was conducted in double transgenic *Tg (fli1a: neGFP)<sup>y7</sup> (kdr1: mCherry)<sup>ci5</sup>* with green endothelial cell nuclei and red cytoplasm. Embryos with morphological defects were excluded from the analysis. Statistical analysis was conducted using unpaired t-test with Welch's correction (in presence of unequal variances) and ordinary one-way ANOVA with Tukey's multiple comparison test (for pairwise comparison in presence of unequal sample sizes).

Solution	Content
E3	<ul style="list-style-type: none"> <li>- 34.8g NaCl</li> <li>- 1.6g Kill</li> <li>- 5.8g CaCl<sub>2</sub>•2H<sub>2</sub>O</li> <li>- 9.78g MgCl<sub>2</sub>•6H<sub>2</sub>O</li> <li>- H<sub>2</sub>O to a final volume of 2L</li> </ul>
PBS	<ul style="list-style-type: none"> <li>- 8g NaCl</li> <li>- 0.2g Kill</li> <li>- 1.44g Na<sub>2</sub>HPO<sub>4</sub></li> <li>- 0.24g KH<sub>2</sub>PO<sub>4</sub></li> <li>- H<sub>2</sub>O to a final volume of 1L</li> </ul>
PBT	<ul style="list-style-type: none"> <li>- PBS</li> <li>- 1mL Tween 20</li> <li>- H<sub>2</sub>O to a final volume of 1L</li> </ul>
TBE	<ul style="list-style-type: none"> <li>- 10.8g Tris base</li> <li>- 5.5g Boric acid</li> <li>- 4mL 0.5M EDTA</li> <li>- H<sub>2</sub>O to a final volume of 1L</li> </ul>
20×SSC	<ul style="list-style-type: none"> <li>- 175g NaCl</li> <li>- 88.2g Sodium citrate</li> <li>- H<sub>2</sub>O to a final volume of 1L</li> </ul>
NTT	<ul style="list-style-type: none"> <li>- 4mL 1M Tris (pH 9.5)</li> <li>- 4mL 1M NaCl</li> <li>- 40μL Tween 20</li> <li>- 32 mL H<sub>2</sub>O</li> </ul>
Hybridization buffer (hyb)	<ul style="list-style-type: none"> <li>- 50mL formamide</li> <li>- 25mL 20x SSC</li> <li>- 500mg torula yeast RNA</li> <li>- 5mg heparin</li> <li>- 40μL Tween 20</li> <li>- 25 mL RNase-free H<sub>2</sub>O</li> </ul>
10x Phenylthiourea (PTU)	<ul style="list-style-type: none"> <li>-304.4 mg of PTU</li> <li>-1 L E3</li> </ul>

**Table 2.1 Table of reagents**

Transgenic line	Transgene	Origin	Fluorophore
Tg(kdrl:EGFP) <sup>la116</sup>	Kdrl	Choi <i>et al.</i> , 2007	EGFP
Tg(gata1:dsRed) <sup>sd2</sup>	Gata	Traver <i>et al.</i> , 2003	dsRed
Tg(kdrl:mCherry) <sup>ci5</sup>	Kdrl	Proulx <i>et al.</i> , 2010	mCherry
Tg(fli1:nEGFP) <sup>y7</sup>	Fli1	Weinstein Lab	Nuclear EGFP

**Table 2.2 Table of Transgenic lines**

Gene	Forward Primer	Reverse Primer
Rasa1a	ACTGTCGTCCCCAGTAGCTC	tgtaatacgaactcactatacccCTGGCCAACTGTCAATAGCA
Rasa1a MO efficiency	ACTGTCGTCCCCAGTAGCTC	CTGGCCAACTGTCAATAGCA
EF1 $\alpha$	TCAACGCTCAGGTCATCATC	GATGTGAGCAGTGTGGCAATC
BMP2b	TCTTCAACCTTACCTCCATTC	aatttaatacgaactcactataggTGTTCATCGGCACCC
BMP4	TAATCTCAGCAGCATCCCA	aatttaatacgaactcactataggCGGTGCCACAATCCAGT

**Table 2.3 Table of primers**

Morpholino	Sequence	Target	Optimal dosage
Rasa1a <sup>e5i5</sup>	AAAGGGTTAGAGACACATACCACCT	Exon5/ Intron5	12 ng
Rasa1a <sup>i2e3</sup>	CGCAATGATCCTAAAAACACACATC	Intron2/ Exon3	7.9 ng
Rras <sup>e4i4</sup>	CTCAGGAAAACACCTACCTGCCACT	Exon4/ Intron4	5.9 ng
Rras2 <sup>e3i3</sup>	TGAGTGGAACGTTTGCTCACCTTCC	Exon3/ Intron3	7.9 ng
Rap1b <sup>ATG</sup> or <sup>e2i2</sup>	GGACTACTAACTTGTATTCACGCAT	ATG	15.5 ng
	AAATGATGCAGAACTTGCCTTTCTG	Exon2/ Intron2	

**Table 2.4 Table of Morpholinos**

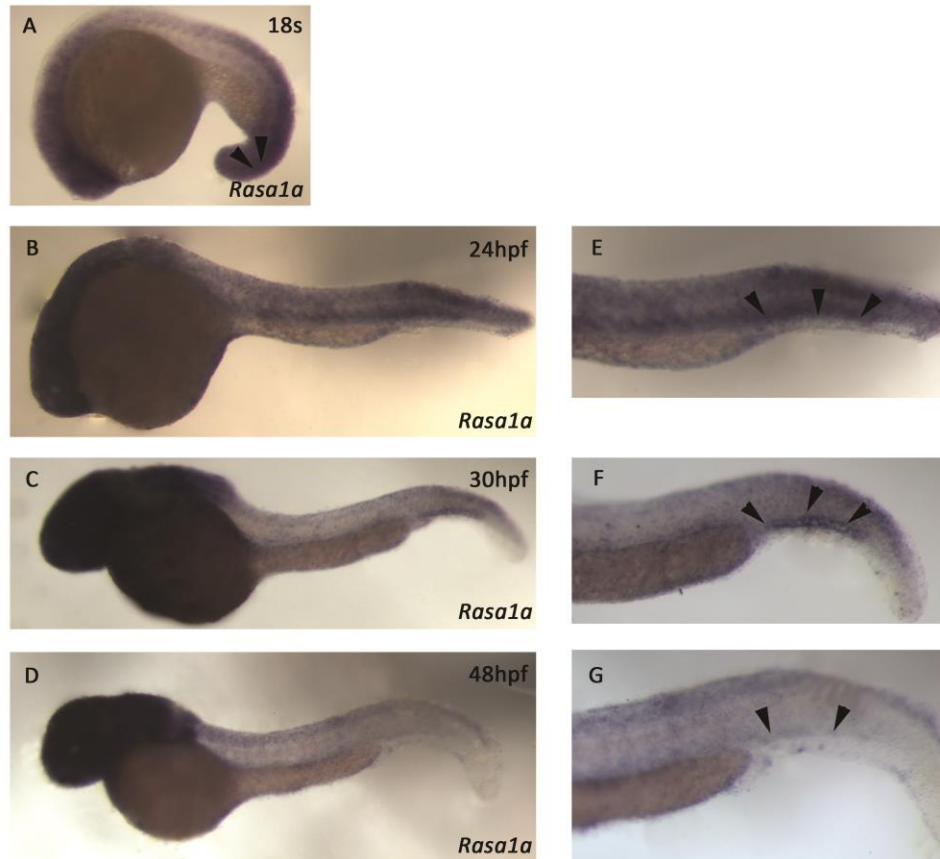
## Chapter Three: Results

**Abstract:** In humans, mutations in *RASA1* have been associated with capillary and arteriovenous malformations. *RASA1* is a GTPase activating protein (GAP) for the Ras signaling pathway; however, its downstream effectors have not been explored *in vivo*. Here I characterize the effect of loss of *rasa1a* in vessels in zebrafish and its downstream effectors. I show that *rasa1a* is expressed ubiquitously in zebrafish from 18 somite stage to 48 hours post fertilization (hpf). I found that knockdown of *rasa1a* results in a single, enlarged vessel, instead of a caudal vein plexus. In addition, *rasa1a* knockdown blocks remodeling and maturation of the caudal vein plexus, resulting in arteriovenous shunts and non-functional blood circulation. Next I used genetic analysis to show that the small GTPase *rras* knockdown partially rescues the *rasa1a* knockdown phenotype, suggesting that *rras* act downstream of *rasa1*. Surprisingly, *rras2* knockdown phenocopies the *rasa1a* knockdown phenotype. Thus, *Rras* appears to interact with *Rasa1*, while *Rras2* appears to act in a parallel pathway to *Rasa1*. I also found differences in expression of *vegfc* and its receptor *vegfr3* in *rasa1* mutants, which are important in venous specification. Although BMP signaling is also essential for venous angiogenesis, *rasa1a* knockdown does not alter the expression levels of BMP ligands, *bmp2b* and *bmp4*. Together, my results indicate that *rasa1a* loss results in vascular malformations, which arise from unregulated *rras* GTPase activity as well as disrupted venous growth factor signaling pathways.

### **3.1 *rasa1a* is expressed ubiquitously in zebrafish from 18s stage to 48hpf**

The expression pattern of *rasa1a* is unknown in zebrafish. To see whether it is expressed in the correct time and location to affect vascular development I used *in situ* hybridization in embryos at 18 somites, 24 hpf, 30 hpf, and 48 hpf. The time period between 18 somites to 48 hpf is critical in CVP development. By around 19hpf (20 somites), endothelial cells of the CVP undergo active sprouting, extend ventral filopodia and develop a complex honey comb-like venous network. By 48hpf the plexus regresses down to form a single ventral vessel (Choi et al., 2011). *rasa1a* appears to be expressed ubiquitously over this time period, although the expression was more enriched in the posterior somites surrounding the caudal vein plexus at 18 somite and 24hpf (Fig. 3.1A, B, and E). By 30hpf, expression of *rasa1a* was apparent in the vicinity of the caudal vein plexus in higher magnifications (Fig 3.1 C, F). *rasa1a* expression diminishes over time and by 48hpf, the time point that venous vessels start to regress, expression of *rasa1* diminished in the CVP region, although its pattern was still ubiquitous (Fig 3.1D, G). The ubiquitous expression pattern of *rasa1a* in zebrafish embryos is consistent with its global expression in various tissues and cell types in mouse and human.





**Figure 3.1: *rasa1a* is expressed ubiquitously in zebrafish from 18s stage to 48hpf**

(A-D) Whole mount *in situ* hybridization of *rasa1a* shows ubiquitous expression. At 18 somites and 24hpf stage *rasa1a* expression is ubiquitous and more enriched in the posterior somites surrounding the caudal vein plexus (CVP; A-B, E). At 30hpf, the expression of *rasa1a* is apparent in the vicinity of the CVP (C, F). At 48hpf, *rasa1a* expression diminishes in the CVP region (D, G). Higher magnification of the CVP at 24 (E), 30 (F), and 48 (G) hpf.

### 3.2 *rasa1a* knockdown phenotype characterization

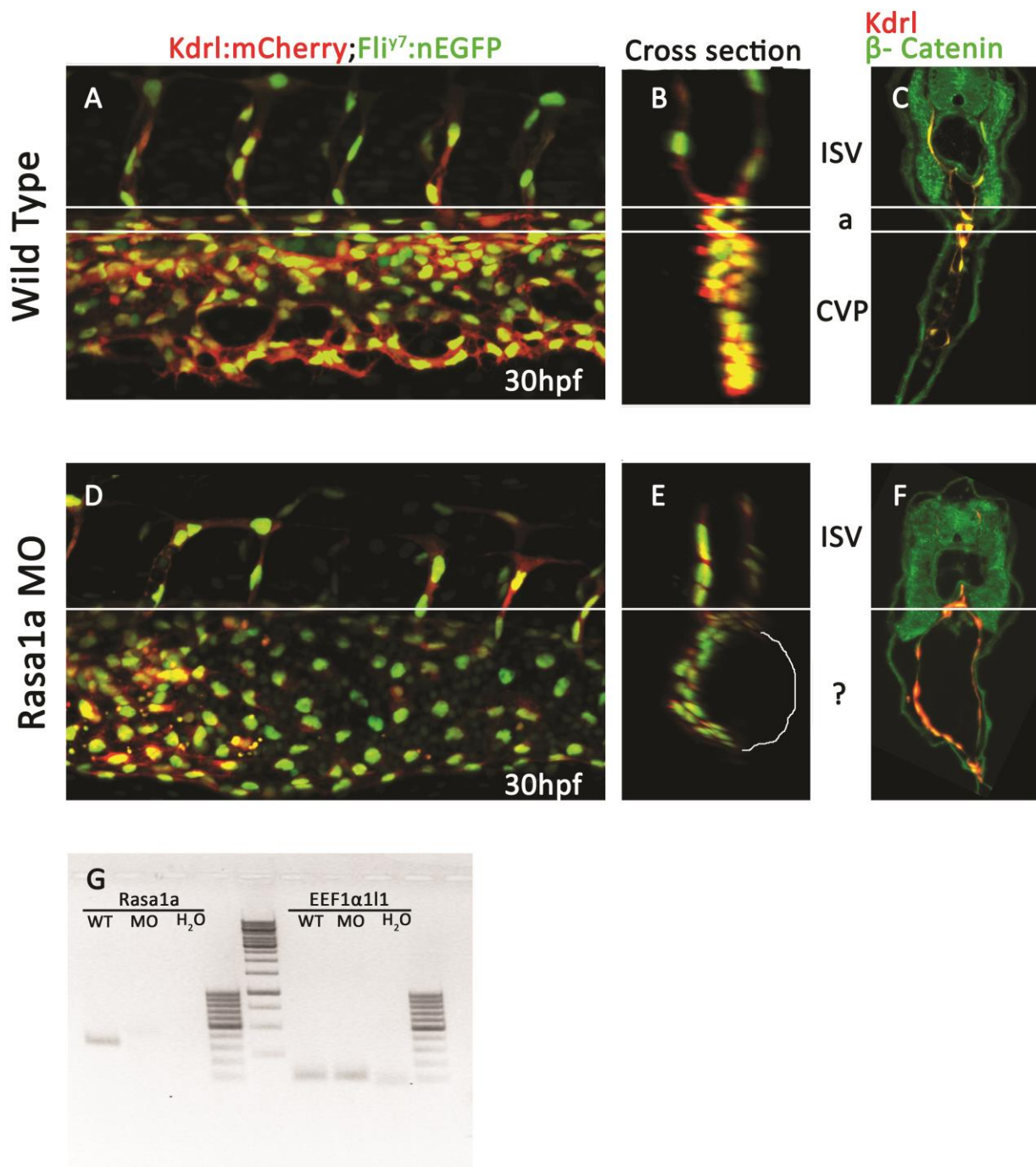
#### 3.2.1 Knock down of *rasa1a* results in an “enlarged single vessel” instead of a caudal venous “plexus”

In order to determine the role of *rasa1a* in development, I used morpholino antisense oligonucleotides (MO), a standard technique to knockdown genes in zebrafish *in vivo*. Two different MOs targeting different splice sites were injected into zebrafish embryos between the 1 and 4 cell stage. The first MO was against the exon 5/ intron 5 splice boundary (*rasa1a*<sup>e5i5</sup>). I was able to verify the mis-splicing of *rasa1a* in a PCR reaction using primers surrounding the targeted exon 5, which should be skipped when the MO is causing mis-splicing. Uninjected controls show the wild-type product (310 bp). In contrast, in *rasa1a* morphants the wild-type band completely disappeared and there were no other accompanying band of the alternatively spliced product, suggesting the nonsense mediated decay of the product (Fig. 3.2 G). Expression of a control gene (Eukaryotic translation elongation factor 1 alpha1, like1; *EEF1α1/1*) is equivalent in both samples suggesting that the RNA is not degraded in morphants. I extracted the wild-type band from the gel and sequenced to show it matched the sequence of the targeted product in the *rasa1a* gene.

Although the expression pattern of *rasa1a* was not endothelial- specific, apparent vascular defects were clearly seen in the CVP at 30hpf. The most obvious defect was the formation of a single enlarged vessel instead of a honeycomb- like plexus in this region (Fig. 3.2 A- F). By 30hpf, endothelial cells in the CVP region of wild-type zebrafish embryos exhibit active sprouting and send out several ventral sprouts (Fig. 3.2 A). However, *rasa1a* morphants did not grow any sprouts (Fig. 3.2 D). Further analysis of the transverse sections of the caudal region also

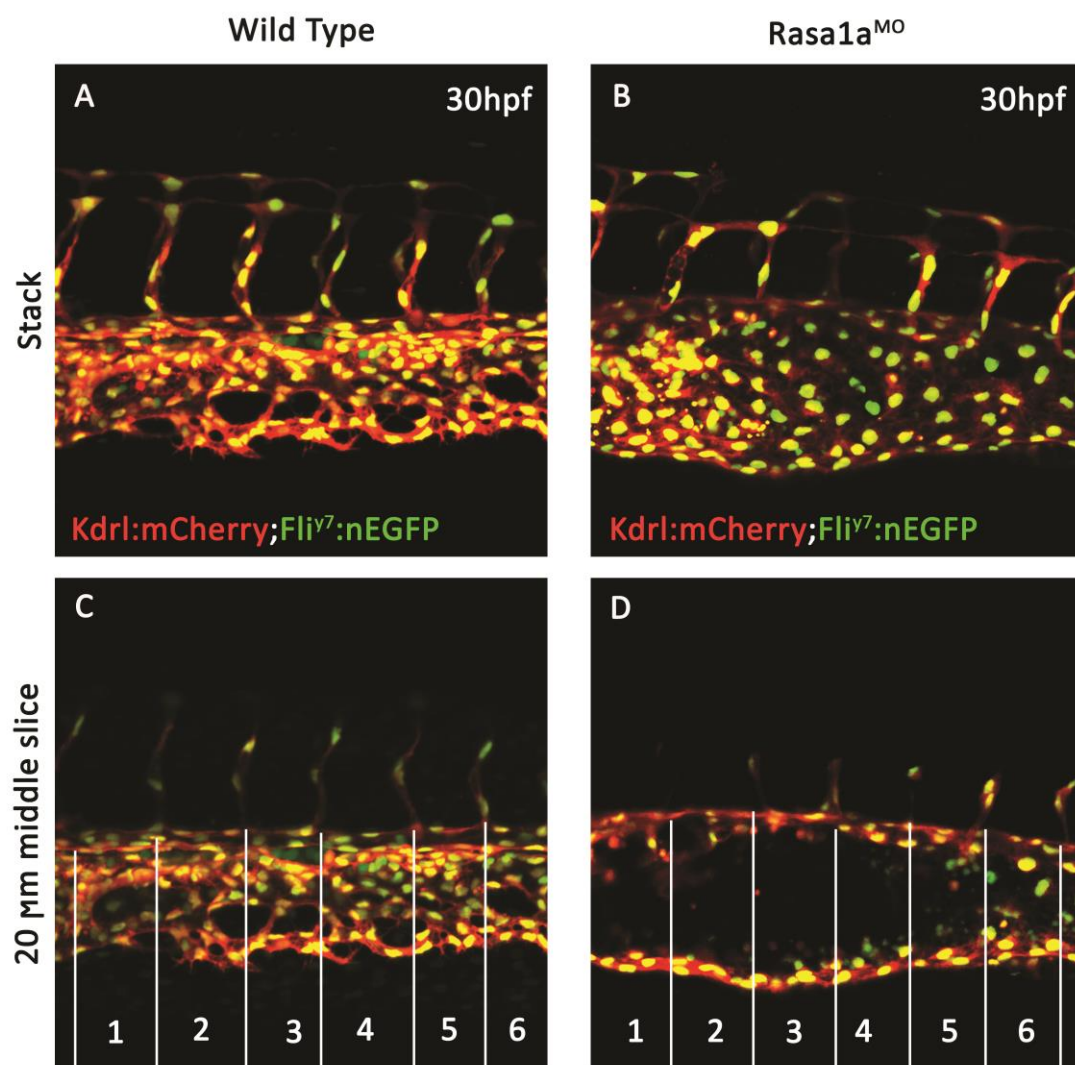
demonstrated a distinct aorta and a venous plexus in wild-type embryos (Fig. 3.2 B), but in *rasa1a* morphants, only an enlarged single vessel was observed and the CVP was lacking interconnecting sprouts (Fig. 3.2 E).

I imaged the caudal region in lateral view using confocal imaging. To help me with scoring the phenotype I developed a metric. The number of caudal plexus segments (the area defined between two adjacent somite boundaries) lacking the arterial/venous boundaries and the interconnecting vessels within the venous plexus were counted in lateral sections and each given a value of 1 in both *rasa1a* morphants and their uninjected wild-type siblings at 30hpf (Fig. 3.3 A-B). 6 segments were counted in each embryo, caudal to the cloaca. At 12 ng of the *rasa1a*<sup>e5i5</sup> MO, 85 % of the embryos showed the caudal plexus phenotype. The average number of caudal plexus segments lacking interconnecting vessels was 4.7 (of 6 segments, n=50) in *rasa1a*<sup>e5i5</sup> morphants as compared to none (of 6 segments, n=49) in their wild type siblings (Figure 3.3E- F; P value< 0.0001 as determined by an unpaired t test with Welch's correction). The second splice morpholino targets the intron2/ exon 3 boundary (*rasa1a*<sup>i2e3</sup>), and has been shown to cause the same vascular phenotype by others (Kawasaki et al., 2014). Although only the *rasa1a*<sup>e5i5</sup> MO was selected for further experimentation, an identical caudal vein plexus phenotype was detectable with *rasa1a*<sup>i2e3</sup> morpholino. The phenotype observed with both morpholinos is completely consistent with a role for *rasa1* as a causative gene in CM-AVM.

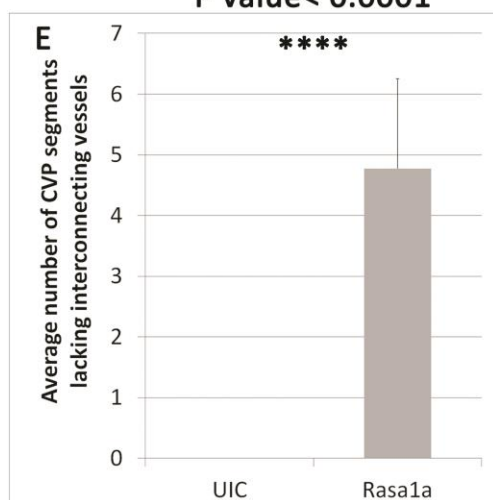


**Figure 3.2 Knock down of *rasa1a* results in an “enlarged single vessel” instead of a caudal venous “plexus”**

Confocal imaging of the CVP in *Tg (fli1a: neGFP)<sup>y7</sup> (kdrl: mCherry)<sup>ci5</sup>* transgenic fish (lateral view; red= endothelial cytoplasm, green= endothelial nuclei) in uninjected controls (A) and *rasa1a* morphants (D). (A) At 30hpf in lateral view normal embryos have a distinct aorta (a), intersegmental vessels (ISV) and caudal vein plexus (CVP) in the tail region. (D) In *rasa1a* morphants at 30 hpf an enlarged, single vessel is formed instead of a plexus. B and E are confocal sagittal projections of the images in A and D respectively. White line in *rasa1a* morphnat cross section show anticipated vessel structure where confocal data is weak due to sample depth. (C and F)  $\beta$ -catenin staining marks all the adherens junctions while Kdrl marks endothelial cells (red). (G) PCR analysis of morpholino knockdown in *rasa1a*- deficient embryos: the wild-type band is absent in morphants, while the *EEF1 $\alpha$ 1/1* band is present at equal levels suggesting specific targeting of *rasa1a* by the e5i5 morpholino.



P value < 0.0001



**F**

	UIC 30hpf	Rasa1a MO e5i5 30hpf
n	49	50
Mean	0	4.7
SD	0	1.4

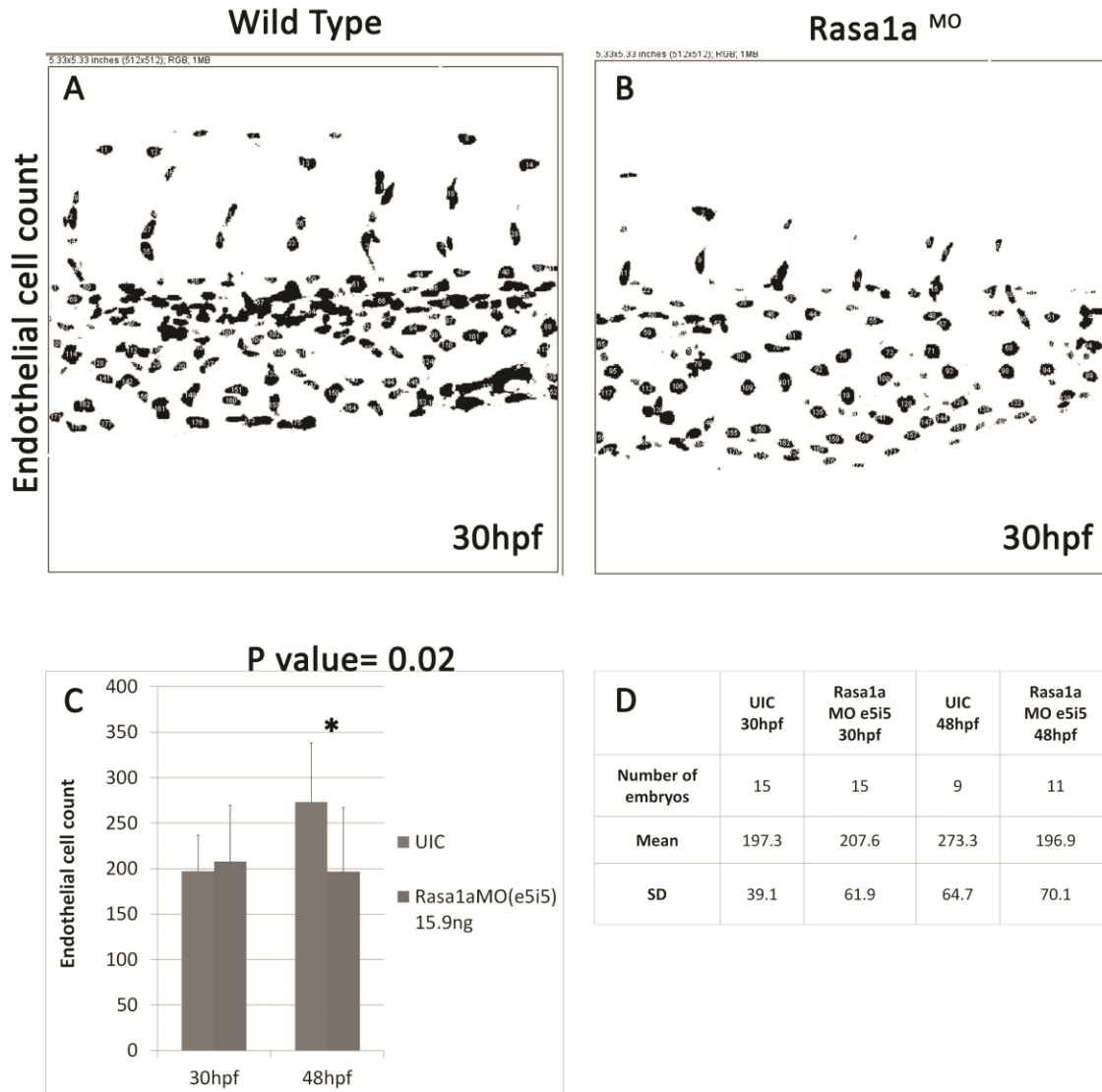
**Figure 3.3 *rasa1a* knockdown results in a single enlarged vessel instead of a plexus**

Confocal images of the CVP (lateral view) in uninjected controls (A) and *rasa1a* morphants (B). 20  $\mu$ m single slice of the CVP in uninjected controls (C) and *rasa1a* morphants (D), used for counting the segments lacking arterial/ venous boundaries and interconnecting vessels within the CVP; segments are numbered 1 to 6. Average number of CVP segments lacking interconnecting vessels is 4.7 in *rasa1a* morphants comparing to 0 in uninjected controls (E-F; P value < 0.0001 as determined by unpaired t- test with Welch's correction, n= number of embryos, two-tailed errors).

### **3.2.2 *rasa1a* knockdown does not affect endothelial cell number**

In order to investigate the cellular changes that result from *rasa1a* knockdown in zebrafish *in vivo*, I first determined whether the enlarged venous plexus is formed as a result of increased cell number. Using the *Tg (fli1a: neGFP)<sup>y7</sup> (kdrl: mCherry)<sup>ci5</sup>* transgenic line in which endothelial cells are labeled with a nuclear eGFP and a cytoplasmic mCherry, I performed an endothelial nuclei count using the automatic particle counting feature of ImageJ. Nuclear counts were obtained in a defined area starting from the end of the yolk extension ( $n=15$  per condition at 30hpf,  $n=9$  uninjected controls (UIC) and  $n=11$  *rasa1a* MO at 48hpf,  $N=3$  experiments; Fig.3. 4A- B). I did not observe any difference between the numbers of endothelial nuclei at 30hpf with an average of 197 cells in uninjected controls as compared to 207 cells in *rasa1a* morphants. However at 48hpf the number of endothelial nuclei decreased in *rasa1a* morphants with significance (P value= 0.02 as determined by unpaired t test with Welch's correction; Fig. 3.4 C- D). The reduction in the cell number at 48hpf could be due to the morpholino toxic effects or endothelial cell apoptosis. Taken together these results suggested that an increase in endothelial cell number may not be responsible for the *rasa1a* knock down phenotype.





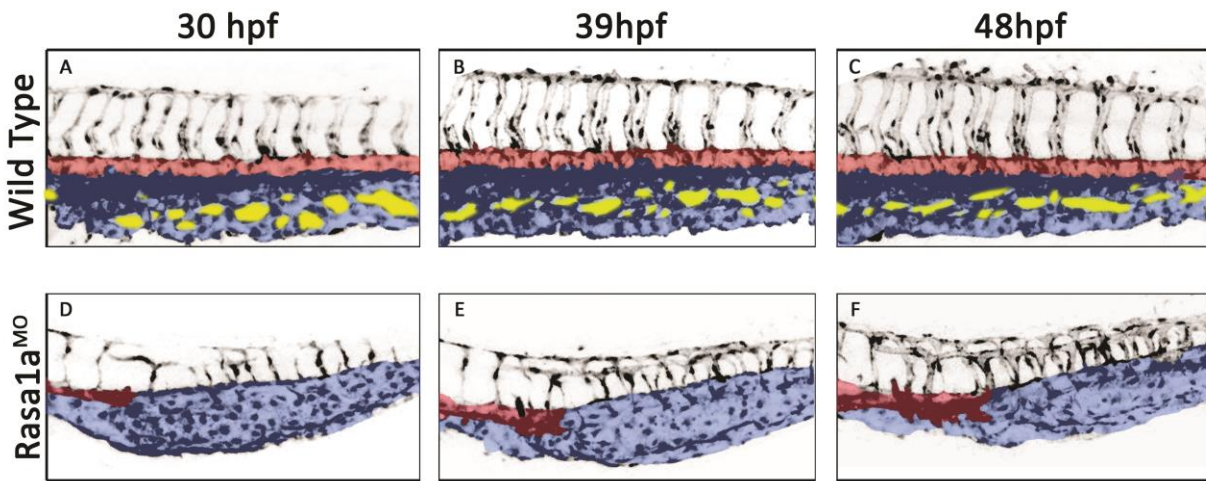
**Figure 3.4 *rasa1a* knockdown does not affect endothelial cell number**

Example of endothelial cells counts using automatic particle counting feature of ImageJ (A-B). *rasa1a* knockdown does not increase the number of endothelial cells in the CVP region at 30hpf (C,D). The number of endothelial cells decrease at 48 hpf (C-D; P value= 0.02 as determined by unpaired t test with Welch's correction, two-tailed errors).

### **3.2.3 *rasa1a* controls remodeling and maturation of the caudal vein plexus**

Between 30 to 36hpf, the angiogenic sprouting of the CVP decreases while the plexus remodels to form an efficient network of vessels. By 48hpf, active angiogenesis has stopped and the plexus remodels to a single vessel. During vascular remodeling, endothelial cell junctions undergo dynamic rearrangements, allowing remodeling of interconnecting vessels within the honey comb- like plexus.

Through measuring the interstitial (negative) space between the interconnecting vessels, I was able to visualize the maturation and remodeling of the caudal vein plexus in control embryos between 30 to 48hpf (n=2 embryos; Fig. 3.5 A- C). Using time- lapse imaging I showed that the pattern of interstitial spaces within the plexus changes over time. Interestingly in *rasa1a* morphants, venous endothelial cells are likely unable to break junctions to enable them to form interconnecting vessels once they are formed, thus In the absence of interconnecting vessels, there are no interstitial spaces in between the vessels and the caudal plexus does not undergo vascular maturation and remodeling (n=2 embryos; Fig. 3.5 D- F). Notably, the main cellular role of *rras* and Rap subfamilies of GTPases is regulating integrin- dependent cell- ECM (extracellular matrix) adhesions. Therefore defective cellular adhesion in *rasa1a*-deficient embryos is a plausible cellular defect that causes the phenotype.

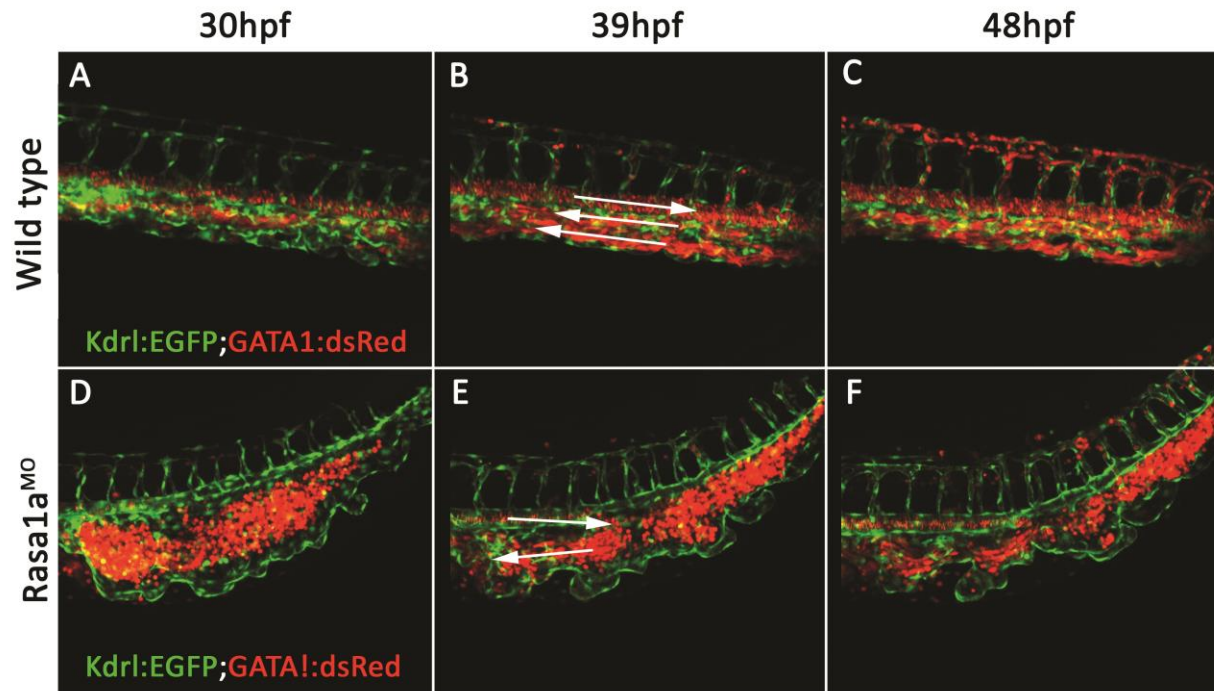


**Figure 3.5 *rasa1a* controls remodeling and maturation of the caudal vein plexus**

Time lapse imaging of the CVP between 30 and 48hpf in *Tg (fli1a: neGFP)<sup>y7</sup> (kdrl: mCherry)<sup>ci5</sup>* transgenic fish (images converted to greyscale) shows the pattern of interstitial spaces between interconnecting vessels changes over time in uninjected controls (A-C). In *rasa1a* morphants the CVP does not undergo remodeling and maturation (D-F). Red shows the artery, blue shows the vein. Interstitial spaces are marked in yellow to show the change of the pattern in wild type fish. In *rasa1a* morphants there is no visible interstitial space.

### **3.2.4 *rasa1a* knockdown causes arteriovenous shunts and disrupts blood circulation**

Arteriovenous malformations and shunts are very common defects in *rasa1a* morphants. At around 26hpf, wild type embryos circulate blood to the most caudal end of the embryo. The majority of blood flows through the aorta and the ventral vein, while some blood cells also pass through the small capillary-like interconnecting vessels (Fig. 3.6 A- C). Using time-lapse imaging from 30 to 48hpf in *Tg(Kdr1:eGFP<sup>ci5</sup>;GATA1:dsRed)* transgenic fish, I showed that as a result of improper connection between the dorsal aorta and the posterior cardinal vein in *rasa1a* morphants, arterial blood flow makes a shunt and returns into the cardinal vein, anterior to the enlarged vascular cavity that forms (n=3 UIC, n=3 *rasa1a* MO; Fig. 3.6 D- F). The improper blood circulation to the caudal part results in the formation of blood pools.



**Figure 3.6 *rasa1a* knockdown causes arteriovenous shunts that disrupt blood circulation**

Time lapse imaging of the CVP in *Tg(Kdr1:eGFP<sup>ci5</sup>;GATA1:dsRed)* transgenic fish (green: endothelial cytoplasm; red: blood). Between 30-48hpf in uninjected controls the majority of blood flows through the aorta and the ventral vein, while some blood cells also passes through the small capillary-like interconnecting vessels (A-C), while in *rasa1a* morphants arterial blood flow makes a shunt and returns into the cardinal vein, anterior to the enlarged vascular deformity (D-F).

### **3.3 *rasa1a* molecular function**

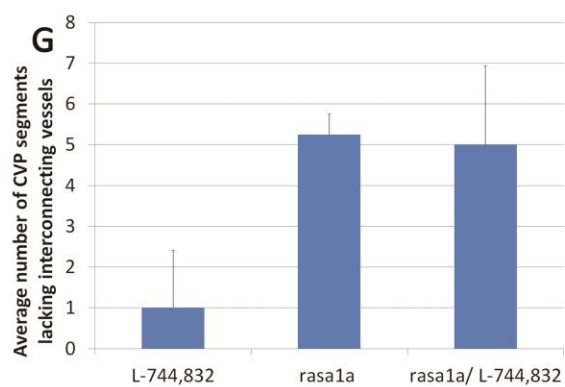
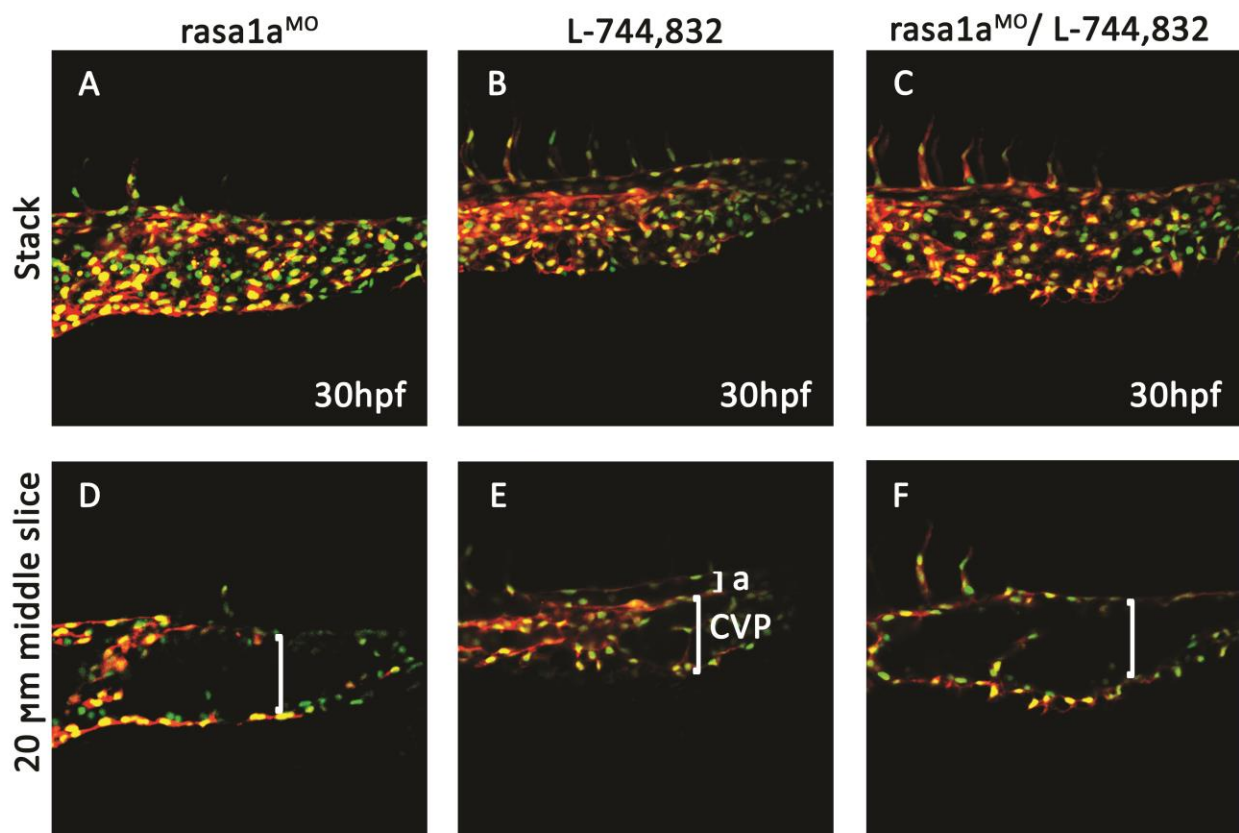
#### **3.3.1 *rasa1* does not appear to interact with classic Ras proteins**

I used the logic that post- transcriptional prenylation is required for most members of Ras subfamily to assist the association with the membrane. Most classic Ras subfamily members are farnesylated (addition of 15-carbon Farnesyl); however, Rras and some Rap and Ral proteins require geranylgeranylation (addition of 20-carbon geranylgeranyl). Geranylgeranylation is not common among Ras oncoproteins; however, KRas and NRas become geranylgeranylated in the absence of farnesyl (Whyte et al., 1997). Inhibition of farnesyltransferase (FTase) using farnesyltransferase inhibitor (FTI) results in improper functioning of the Ras and therefore pharmacologically inhibiting Ras using farnesyltransferase inhibitors is a good test of the role of classic Ras proteins in CVP formation downstream of *rasa1*.

In order to determine whether farnesyl- modified RAS proteins are downstream effectors of *rasa1a*, I conducted a rescue experiment in *rasa1a* morphants using small molecule L-744,832, a potent RAS farnesyl transferase inhibitor. L-744,832 induces apoptosis in human cancer cells and causes tumor regression in a variety of transgenic mouse tumor models (Dai et al., 2005; Barrington et al., 1998). This inhibitor has also been used in zebrafish previously (Choi et al., 2011). If Rasa1a inactivates classic farnesyl-modified RAS proteins through enhancing their GTPase activity, I would expect to see a rescue of the *rasa1a* knockdown phenotype by inhibiting farnesylation. I scored embryos at 30 hpf by counting the average number of caudal plexus segments lacking interconnecting vessels using confocal images. Double-injected embryos had

an average of 5 (out of 6 segments, n=9; Fig. 3.7C, F) segments without a CVP as compared to 5.25 (out of 6 segments, n=4) in *rasa1a* morphants (N=2 experiments, P value > 0.05 as determined by ordinary one-way ANOVA with Tukey's multiple comparison test; Fig. 3.7 A, D) at 30hpf. For these experiments I both injected (5mM-3nl) and soaked (100  $\mu$ M-18 somite to 30hpf) embryos with the drug, and the effect was the same.

These data suggest that *rasa1a* does not act through farnesyl-modified RAS proteins. However some farnesylated proteins like Kras and Nras can become geranylgeranylated in the presence of FTIs, and thus we cannot rule out this possibility. Taken together, these results suggest that the activity of GTPases modified by farnesylation is dispensable for CVP formation. Thus *rasa1a* might primarily modulate Ras-family proteins that are geranylgeranylated *in vivo*.



H	<i>rasa1a</i> <sup>e515</sup> MO	L-744,832	<i>rasa1a</i> / L-744,832
n	4	10	9
mean	5.25	1	5
SD	0.5	1.41	1.93

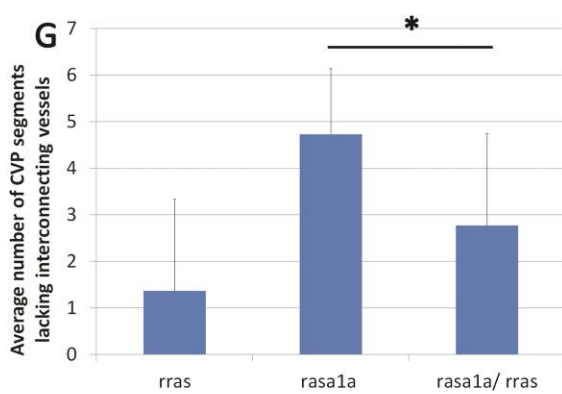
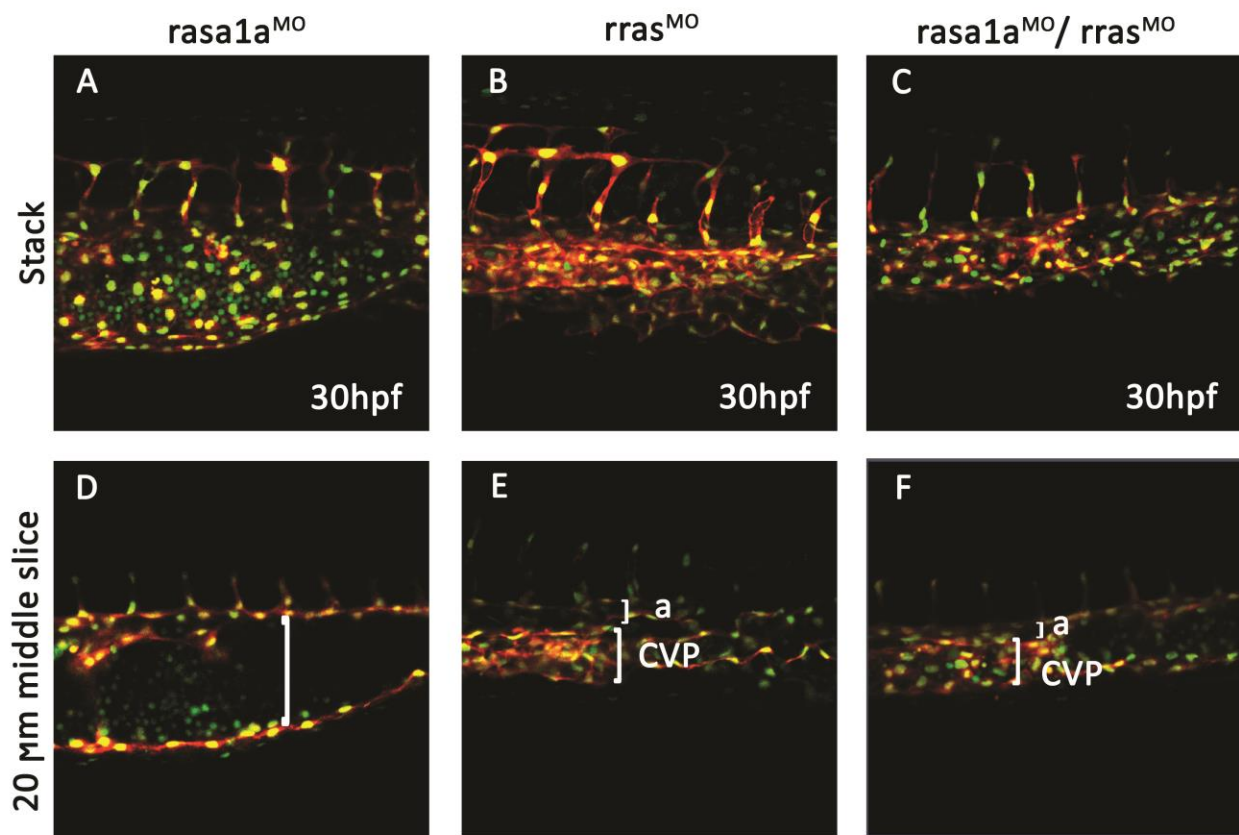


**Figure 3.7 Inhibition of Ras Farnesylation does not rescue *rasa1a* phenotype**

*rasa1a* morphants (A) vs. L-744,832 treated embryos (B) vs. double injected (*rasa1a*<sup>MO</sup> + L-744,832) embryos (C) using *Tg (fli1a: neGFP)<sup>y7</sup> (kdrl: mCherry)<sup>ci5</sup>* transgenic fish. (D-F) single slices used to count the CVP segments lacking interconnecting vessels. a: artery; CVP: caudal vein plexus. Brackets in D and F show the interstitial space lacking interconnecting vessels. (G-H) The average number of caudal plexus segments lacking interconnecting vessels is 5 in double injected embryos as compared to 5.25 in *rasa1a* morphants at 30hpf. P value > 0.05 as determined by ordinary one-way ANOVA with Tukey's multiple comparison test. Two-tailed errors.

### **3.3.2 *rras* Knockdown partially rescues the *Rasa1a* knockdown phenotype**

My results with inhibition of farnesylation seemed to indicate that geranylgeranylated GTPases might be more important than classic Ras proteins in caudal vein plexus formation. Therefore I focused on genetic interactions with geranylgeranyl-modified Ras proteins such as Rras. In order to investigate the possible molecular interaction between *rasa1* and *rras*, I conducted a rescue experiment in *rasa1a* morphants, using an *rras* splice morpholino targeting exon4/intron4 boundary (*rras*<sup>*e4i4*</sup>). *rras* morpholino mis-splicing was verified by Ryan Sobering, a former graduate student in the laboratory (Sobering, 2012). Similar to what Ryan had reported, I did not observe any vascular specific phenotype after *rras* knockdown at 30hpf. At this stage, the morphology of the morphants is quite normal with a slightly shorter anteroposterior axis, however the number of somites was the same, indicating the same developmental stage (Fig. 3.9 B, E). The caudal vein plexus also forms normally between 30 to 48hpf. Interestingly, inhibition of *rras* in *rasa1a*-deficient embryos partially rescues the enlarged CVP phenotype as scored by the number of CVP segments that lacks interconnecting vessels (N=2 experiments, P value < 0.05 as determined by ordinary one-way ANOVA with Tukey's multiple comparison test). The average number of caudal plexus segments showing the enlarged single vessel phenotype with no interconnecting vessels was 2.76 (out of 6 segments, n=15) in double-injected embryos (Fig. 3.9 C, F), comparing to 4.75 (out of 6 segments, n=10) in *rasa1a* morphants (Fig. 3.9A, D) at 30hpf (C-D). Arteriovenous shunts were still present, however they were formed more posteriorly as compared to *rasa1a*-deficient embryos. These findings suggest that *rasa1a* may interact with *rras* to enhance its intrinsic GTPase activity. Thus *rasa1a* knockdown leads to excessive *rras* activity in vasculature which consequently results in overactivation of its downstream effectors.



H	rasa1a <sup>e515</sup> MO	rras <sup>e4i4</sup> MO	rasa1a / rras
n	10	15	15
mean	4.75	1.36	2.76
SD	0.79	1.96	1.97

**Figure 3.8 *rras* Knockdown partially rescues *rasa1a* knockdown phenotype**

*rasa1a* morphants (A) vs. *rras* morphants (B) vs. double injected (*rasa1a*<sup>MO</sup> + *rras*<sup>MO</sup>) embryos (C) using *Tg (fli1a: neGFP)<sup>y7</sup> (kdrl: mCherry)<sup>ci5</sup>* transgenic fish. (D-F) single slices used to count the CVP segments lacking interconnecting vessels. a: artery; CVP: caudal vein plexus. (G-H) The average number of caudal plexus segments lacking interconnecting vessels is 2.76 in double injected embryos as compared to 4.75 in *rasa1a* morphants at 30hpf. P value < 0.05 as determined by ordinary one-way ANOVA with Tukey's multiple comparison test.

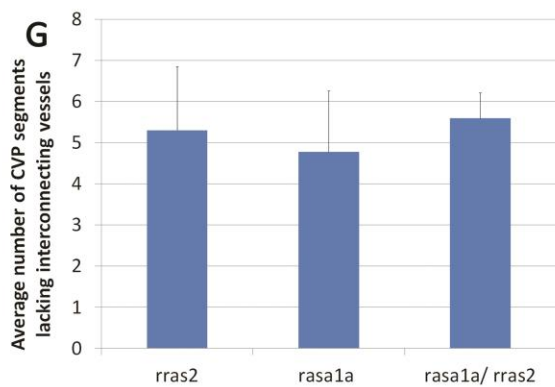
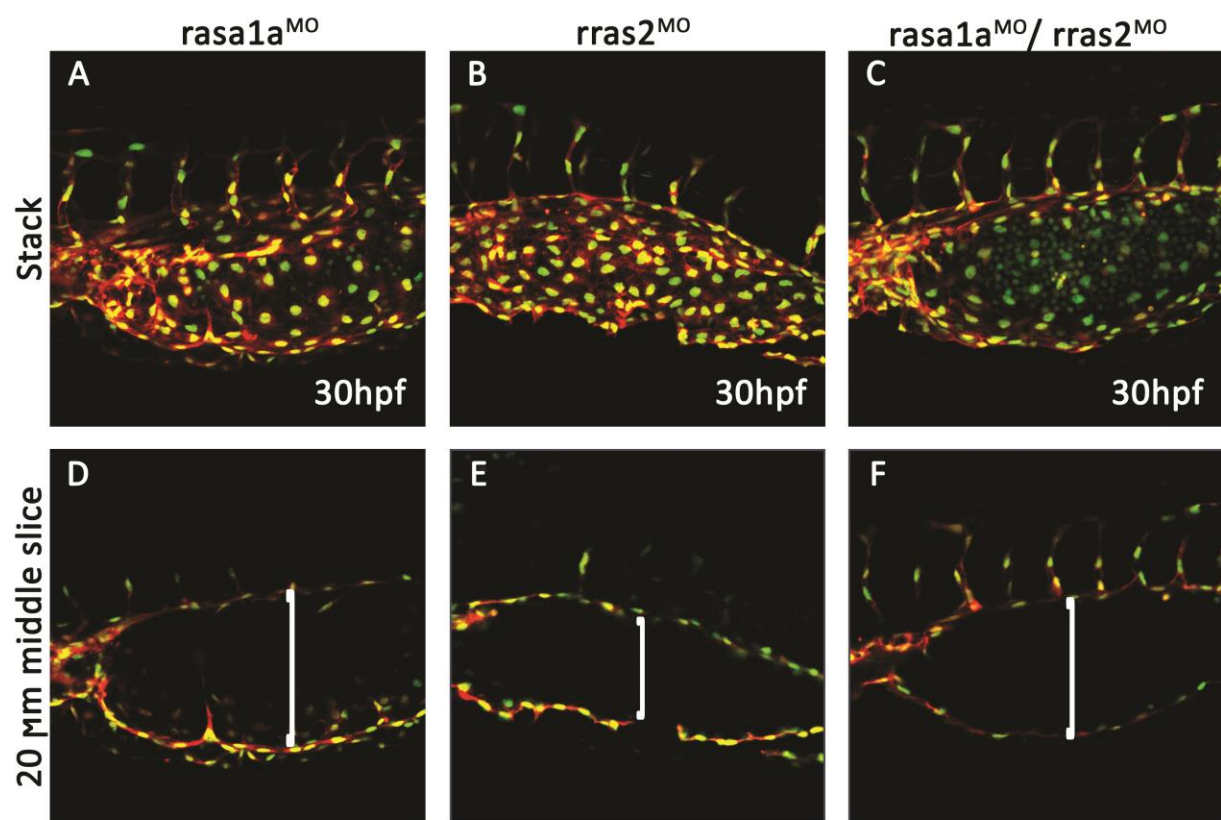
### **3.3.3 *rras2* knockdown phenocopies *rasa1a* phenotype**

*rras2* is the closest relative of *rras* and the oncogene member of the Rras (related to Ras) subfamily. Similar to Rras, Rras2 requires geranylgeranylation and activating mutations of *rras2* stimulate integrin-mediated cell migration in breast epithelial cells (Keely et al., 1999). However no known function of *rras2* has been reported in zebrafish or in the onset of vascular malformations.

In order to determine whether *rras2* signals downstream of *rasa1a* during CVP formation, I performed a rescue experiment using a splice morpholino targeting the *rras2* exon3/intron3 boundary (*rras2*<sup>e3i3</sup>). The mis-splicing of the morpholino was verified by Michela Goi, a graduate student in the lab and has been used in her research project. Interestingly, knockdown of *rras2* results in the same phenotype as *rasa1a* knockdown. *rras2*-deficient embryos displayed an enlarged single vessel instead of a plexus with no interconnecting capillary-like vessels (Fig. 3.10 B, E). Similar to *rasa1a* morphants, *rras2* knockdown embryos exhibited abnormal connections between the artery and the vein and arteriovenous shunts were observed posterior to the deformity.

Counting the number of caudal plexus segments lacking the interconnecting vessels revealed that the average number of caudal plexus segments displaying the vascular deformity was 5.5 in double-injected embryos (n=15; Fig. 3. 10 C, F) as compared to 4.73 in *rasa1a* morphants (n=15; Fig. 3.10 A, D) and 5.3 in *rras2* morphants (n=15; Fig. 3.10 B, E). Thus, knockdown of *rras2* does not rescue *rasa1a* knockdown phenotype (N=3 experiments, P value > 0.05 as determined by ordinary one-way ANOVA with Tukey's multiple comparison test). It is very interesting that *rras* and *rras2* have opposite functions in vascular context in zebrafish.

These results suggest that while *rras* appears to act downstream of *rasa1a*, *rras2* appears to act in parallel pathway.



H	<i>rasa1a</i> <sup>e5i5</sup> MO	<i>rras2</i> <sup>e3i3</sup> MO	<i>rasa1a</i> / <i>rras2</i>
n	15	15	15
mean	4.73	5.3	5.5
SD	1.41	1.54	0.61

**Figure 3.9 *rras2* knockdown phenocopies *rasa1a* phenotype**

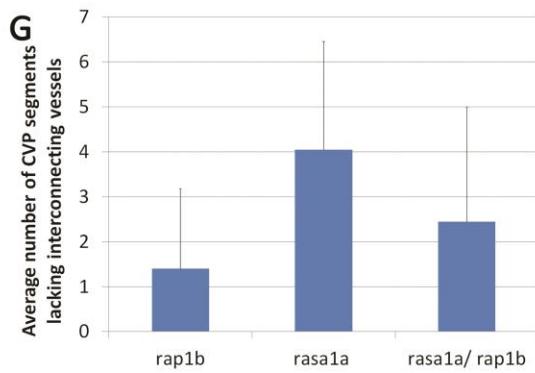
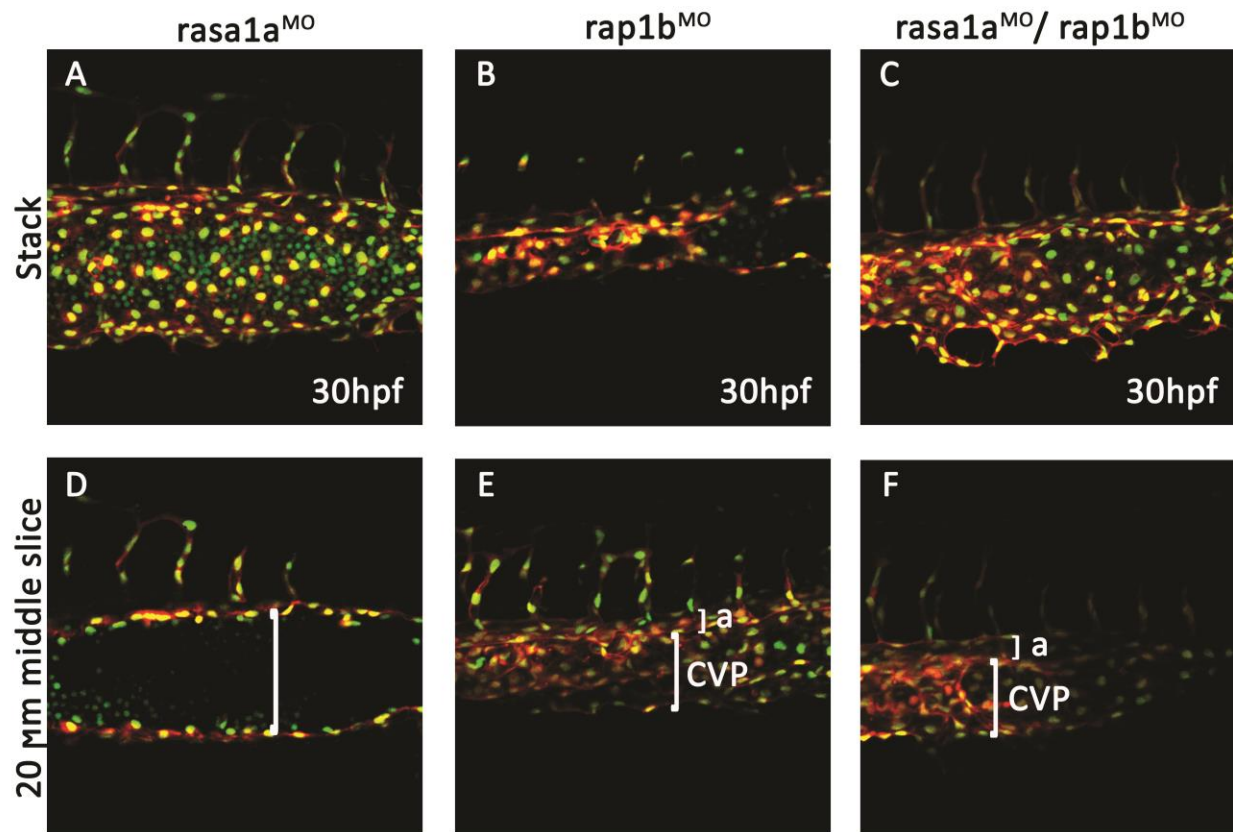
*rasa1a* morphants (A) vs. *rras2* morphants (B) vs. double injected (*rasa1a*<sup>MO</sup> + *rras2*<sup>MO</sup>) embryos (C) using *Tg (fli1a: neGFP)<sup>y7</sup> (kdrl: mCherry)<sup>ci5</sup>* transgenic fish. (D-F) single slices used to count the CVP segments lacking interconnecting vessels. a: artery; CVP: caudal vein plexus. (G-H) The average number of caudal plexus segments lacking interconnecting vessels is 5.5 in double injected embryos as compared to 4.73 in *rasa1a* morphants at 30hpf. P value > 0.05 as determined by ordinary one-way ANOVA with Tukey's multiple comparison test. Two-tailed errors.



### **3.3.4 *rap1b* Knockdown does not rescue *rasa1a* knockdown phenotype**

Like the Rras subfamily, Rap proteins also promote cell adhesion through integrin activation and require post-transcriptional geranylgeranylation. There is no evidence *in vitro* on *rasa1* interacting with members of Rap family of GTPases. However, some evidence suggests that *rap* and *rras* may act in a similar pathway and *rras* may need *rap* to induce integrin activation, indicating the possible indirect interaction of *rap* and *rasa1a*. Other evidence suggests that *rap1a* is potent inhibitor of Ras GTPase activity, perhaps by limiting the amount of *rasa1* available for interaction with *ras* (Kinbara et al., 2003).

In order to determine whether *rap1b* signals downstream of *rasa1a*, I conducted a rescue experiment using a published *rap1b* morpholino sent to us from Weinstein lab (Gore et al., 2008). I confirmed the specificity of the morpholino by producing the same published phenotype (hemorrhages in the head in 80% of the injected embryos). Knockdown of *rap1b* in *rasa1a* morphants, does not rescue the CVP deformity (N=2 experiments, P value > 0.05 as determined by ordinary one-way ANOVA with Tukey's multiple comparison test). The average number of CVP segments that are not developing interconnecting vessels reaches to 2.44 (out of 6 segments, n=9) in double injected embryos (Fig. 3.11 C, F) as compared to 4.05 (out of 6 segments, n=10) in *rasa1a* morphants (Fig. 3.11 A, C). No specific vascular phenotype was observed in the CVP region in *rap1b*-deficient embryos alone (Fig. 3.11 B, E).



H	rasa1a <sup>e515</sup> MO	rap1b MO	rasa1a / rap1b
n	10	10	9
mean	4.05	1.4	2.44
SD	2.40	1.77	2.55

**Figure 3.10 *rap1b* Knockdown does not rescue *rasa1a* knockdown phenotype**

*rasa1a* morphants (A) vs. *rap1b* morphants (B) vs. double injected (*rasa1a*<sup>MO</sup>+ *rap1b*<sup>MO</sup>) embryos (C) using *Tg (fli1a: neGFP)<sup>y7</sup>(kdrl: mCherry)<sup>ci5</sup>* transgenic fish. (D-F) single slices used to count the CVP segments lacking interconnecting vessels. a: artery; CVP: caudal vein plexus. (G-H) The average number of caudal plexus segments lacking interconnecting vessels is 2.44 in double injected embryos as compared to 4.05 in *rasa1a* morphants at 30hpf. P value > 0.05 as determined by ordinary one-way ANOVA with Tukey's multiple comparison test. Two-tailed errors.

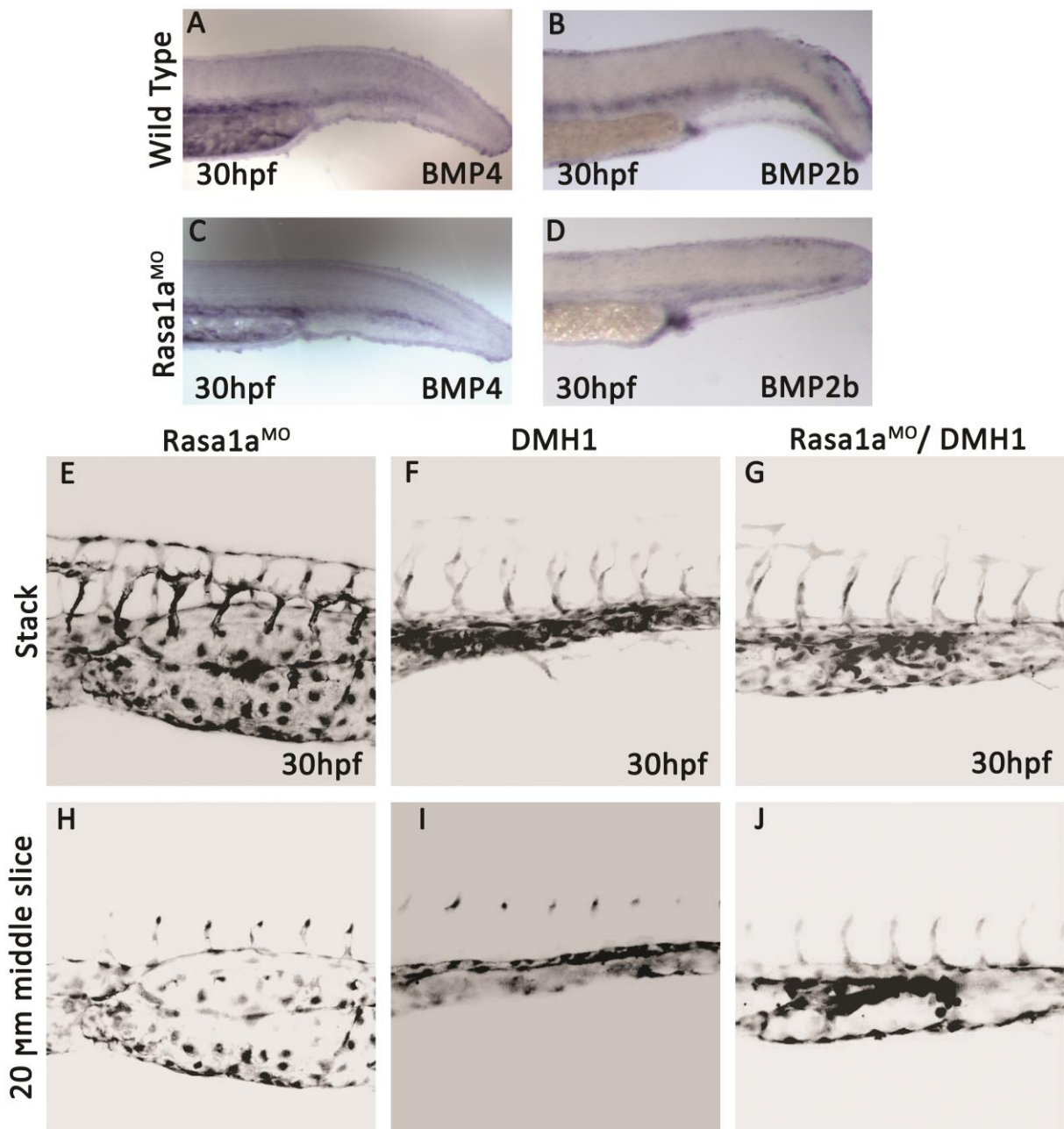
### **3.3.5 *rasa1a* knockdown does not alter the expression levels of BMP**

Since Ras GAPs and growth factors can interact at many levels, expression of growth factors could be controlled by GAP signaling. BMP signaling is an important cue in regulating the sprouting angiogenesis of the vein (Wiley et al., 2011). Inhibition of BMP ligands and receptors expression severely compromises the formation of venous sprouts, raising the possibility that *rasa1a* might interact with BMP signaling during venous angiogenesis.

In order to evaluate the impact of *rasa1a* inhibition on the expression level of BMP, I performed a whole mount *in situ* hybridization analysis comparing *bmp2b* and *bmp4* mRNA expression patterns and levels in uninjected versus *rasa1a*-deficient embryos. At 30hpf *bmp2b* is expressed in dorsal retina, otic vesicles, pharyngeal endoderm, posterior cardinal vein and the caudal vein plexus. *bmp4* ligand is also expressed in dorsal retina, pharyngeal endoderm, posterior cardinal vein and tail and tail bud epidermis at 30hpf. I did not observe any distinguishable differences in expression patterns and levels of *bmp2b* (N=3 experiments; (Fig. 3.12 B, D) and *bmp4* (N=2 experiments; Fig. 3.12 A, C) ligand in *rasa1a* morphants, suggesting that *rasa1a* might not modulate the BMP pathway.

To test the relationship of BMP signaling and *rasa1a* function I used a pharmacological approach. Dorsomorphin homologue 1 (DMH1) is a small molecule BMP inhibitor. DMH1 is a selective inhibitor of BMP type 1 receptor and has shown no inhibitory activity for MAPK phosphorylation or VEGF- induced *vegfr2* phosphorylation (Hao et al., 2010). DMH1-treated embryos have normal segmental arteries but do not develop venous ventral sprouts (Wiley et al., 2011). In order to further demonstrate that *rasa1a* does not modulate BMP pathway in venous angiogenesis, I treated wild type and *rasa1a* morphants with DMH1 (N=1 experiment, 10  $\mu$ M

DMH1, Treatment duration: 18 somite to 30hpf). Predictably, DMH1 inhibited the formation of caudal venous sprouts, leaving a single dorsal vein instead of a plexus (Fig. 3.12 F, I). I observed the same phenotype in DMH1-treated *rasa1a* morphants with a moderate vessel enlargement.

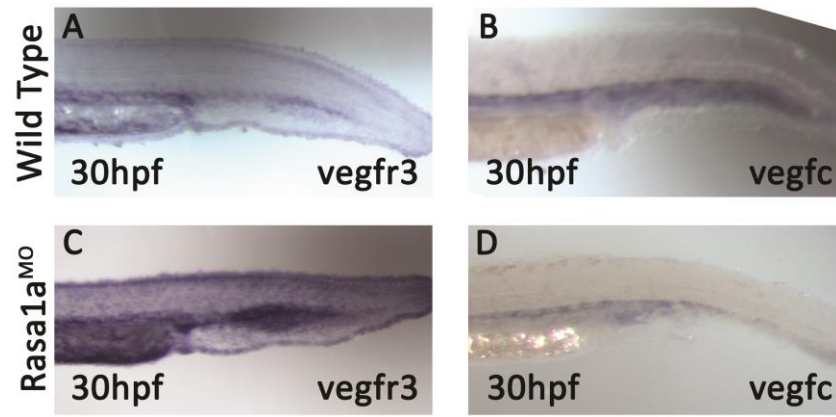


**Figure 3.11 *rasa1a* knockdown does not alter expression levels of BMP**

Wholemount in situ hybridization of *bmp2b* and *bmp4* in the tail region in uninjected controls and *rasa1a* morphants (A-D). *rasa1a* morphants (E) vs. DMH1 treated embryos (F) vs. double treated (*rasa1a*<sup>MO</sup>+ DMH1) embryos (G) using *Tg(kdrl:mCherry)<sup>ci5</sup>* transgenic fish. (H-J) single slices.

### **3.3.6 *rasa1a* knockdown alters the expression levels of *vegfc* and its receptor *vegfr3***

Similar to BMP signaling pathway, *vegfc* and its receptor *vegfr3* are required for venous angiogenesis; however, the role of *vegfc/vegfr3* signaling pathway has not been determined in the development of caudal vein plexus in zebrafish. In order to determine whether *rasa1* interacts with the *vegfc/vegfr3* pathway during caudal vein plexus angiogenesis, I looked for alterations in the expression levels of *vegfc* and *vegfr3* in wild type and *rasa1a*-deficient embryos, using whole mount *in situ* hybridization (Fig. 3.13 A-D). At 30hpf *vegfc* is expressed in the vicinity of caudal vein plexus probably in circulating erythrocytes, and its receptor *vegfr3* is also expressed the endothelial cells of the caudal vein plexus in wild type embryos. Interestingly, mRNA expression levels of both *vegfc* (N=2 experiments) and *vegfr3* (N=4 experiments) seemed to be altered at 30hpf. *rasa1a* knockdown results in decreased *vegfc* expression level in the caudal vein plexus region, while *vegfr3* expression is strongly increased. These findings indicate that *rasa1a* interacts with *vegfc/vegfr3* pathway, a known growth factor signaling pathway important for venous angiogenesis.



**Figure 3.12** *rasa1a* knockdown alters the expression levels of *vegfc* and its receptor *vegfr3*

Wholemount *in situ* hybridization of *vegfr3* (A, C) and *vegfc* (B, D) in uninjected controls and *rasa1a* morphants. While the expression level of *vegfr3* seems to increase (C), the expression level of *vegfc* seems to decrease (D) in *rasa1a* morphants as compared to uninjected controls.



## Chapter Four: General Discussion and Future Directions

### 4.1 Major Findings

#### 4.1.1 *rasa1a* knockdown causes major hemato-vascular defects

Morphological defects in *rasa1* deficient vessels: *rasa1* mutations cause arteriovenous malformations in human and mice (Revenu et al., 2013; Lapinski et al., 2012; Henkemeyer et al., 1995). In my project I sought to understand the role of *rasa1* in arteriovenous malformations using the zebrafish model. I showed that *rasa1a*-deficient embryos do not extend endothelial sprouts in the caudal vein plexus; instead a single enlarged vessel is formed in the morphants. Knockdown of *rasa1a* causes improper connection between the artery and the vein, which results in arteriovenous shunts and blood circulation defects. Kawasaki et al. found the same results in parallel to my project; however, some questions regarding the cellular and molecular defects caused by *rasa1a* knockdown have not been addressed yet. Although Kawasaki et al. showed that the enlarged vein is due to overactivation of mTORC1 and thus from an increase in cell size, effects on endothelial structure, such as cell junctions were not explored (Kawasaki et al., 2014).

Morphological defects and connection to integrin signaling: I found that *rasa1a* genetically interacts with *rras*, which is interesting because this molecule is known to regulate integrin activation. This supports my hypothesis that the formation of enlarged single vessels could be due to integrin dysregulation. To look for integrin activation one could mark focal adhesion components such as phospho- paxillin and phospho- FAK (focal adhesion kinase) with antibody staining. Paxillin and FAK are two of the key components of integrin signaling (Bellis et

al., 1997). Activation of integrins in extra cellular matrix promotes the formation of focal adhesion complexes. Confocal imaging of the CVP during development at high magnification will detect any cell junctional defects, which is integrin-induced in *rasa1a* morphants. My attempt to look at the abluminal localization of  $\beta$ -Catenin (adherens junction) by antibody staining was not informative due to the thickness of the sections, but confocal microscopy would be superior (Fig. 3.2).

Migration defects in *rasa1a* morphants: Kawasaki et al. showed that *rasa1a* signals downstream of *ephb4*. Ephrin signaling is required to direct the dorsal and ventral migration of the endothelial cells to form the dorsal aorta and posterior cardinal vein respectively (Herbert et al., 2009). Thus, it is possible that *rasa1a* inhibition causes migration defects. To test this, the individual tracing of the cells is required over the time period that the plexus undergoes active angiogenesis. It should be noted that active migration versus passive movement needs to be distinguished in future experiments (the movement of the cells due to the embryonic growth is considered as passive as the endothelial cells are carried along with other cells as the embryo grows).

Circulation defects in *rasa1* morphants: While early blood vessels such as the dorsal aorta and posterior cardinal vein pattern in the absence of flow; all angiogenic vessels develop in the presence of blood flow and pressure. Blood flow also plays a significant role in vascular remodeling (Corti et al., 2011; Lucitti et al., 2007). The caudal vein plexus is a transient niche for hematopoietic stem cells (HSC) in early stages and *rasa1a*-deficient embryos lack proper blood circulation to this region, raising the issue of whether the effects of *rasa1a* knockdown on venous

angiogenesis are secondary to circulation defects. However this is probably not the case as Choi et al. showed that angiogenic sprouts form normally in two zebrafish mutant lines without blood circulation (Choi et al., 2011). A primordial CVP is formed in these embryos at 32hpf suggesting that hemodynamic flow is not required during the active angiogenic state of the CVP. However the formation of a single lumen vessel at 48hpf suggested that the blood flow is required for maintenance of the plexus. To test the role of blood flow in *rasa1a*- deficient embryos, one could knockdown *rasa1* and simultaneously block heart function either by using the  $\text{Ca}^{2+}$  blocker BDM (Ostap, 2002), or using a morpholino against *tnnt2* that induces the silent heart phenotype (Bartman et al., 2004). These standard methods would distinguish whether patterning defects in *rasa1* morphants are intrinsic versus defects that arise as a result of defective circulation.

Genetic redundancy of *rasa1a* and *rasa1b*: Although Kawasaki et al. reported that morpholino knockdown of both *rasa1* homologs in zebrafish (*rasa1a* and *rasa1b*) leads to no worsening of the *rasa1a* phenotype, suggesting that *rasa1b* is not involved in caudal venous development, double mutants are required to assess the synergic effects and genetic interactions of both homologs.

Need for a genetic model of *rasa1* loss of function: I used two different splice blocking morpholino oligomers to knockdown *rasa1a* and found that each morpholino produced identical vascular phenotypes. I did not use the second morpholino (published with verified mis-splicing) (Kawasaki et al., 2014) for further experiments beyond the initial phenotypic screen. I was able to verify the efficiency of the first morpholino using a PCR. It is very unlikely that an identical phenotype produce by two different morpholinos be caused due to the morpholino toxicity or

off-target effects, however given the changing standards in the zebrafish field, my experiments will need to be repeated with genetic mutants (currently in preparation).

#### **4.1.2 *rasa1a* acts in venous growth in Ras-dependent mechanisms**

In my project I sought to identify the downstream effectors of *rasa1a* during venous development. While it has been established that *rasa1a* signals downstream of *ephb4* to inhibit *mTORC1* in endothelial cells in zebrafish, it was still unknown which GTPases interact with *rasa1a*. Here I showed that knock down of *rras* can partially rescue the vascular phenotype in *rasa1a* deficient zebrafish embryos, suggesting that *rasa1a* may interact with *rras* *in vivo*.

Involvement of Rho- mediated signaling pathway: I did not explore interactions with the Rho pathway, but Rasa1 SH2 domains also interact with p190 Rho Gap, suggesting the co-ordination of Ras and Rho- mediated signaling pathways. Rasa1/p190 Rho GAP complex is required for reorientation of focal adhesions and for maintaining elongate morphology, independent of Ras GTPase activity (Pamonsinlapatham et al., 2009). Thus, it is also possible that the *rasa1a* phenotype partially results from Ras- independent mechanism. To address this, one could try to rescue the *rasa1a* vascular phenotype by *rasa1a* construct which is expressed in endothelial cells with mutations in the SH2 domain. Rescue of the caudal vein plexus deformity by expression of the construct will suggest whether *rasa1a* Ras- independent mode of signaling is also important during venous development *in vivo*.

Involvement of geranylgeranylated proteins: We predict that *rasa1a* knockdown results in upregulated Ras activity; however, Inhibition of Ras Farnesylation using the farnesyl transferase inhibitor (FTI) L-744,832 results in no vascular phenotype ((Choi et al., 2011); my results),

suggesting that classic Ras proteins are not involved in this process. The caveat is that *kras* and *nras* undergo geranylgeranylation in the presence of Farnesyl transferase inhibitors *in vitro* (Whyte et al., 1997), and thus I cannot conclude that *rasa1a* does not interact with farnesyl-modified ras GTPases. It is very possible that *rasa1a* interacts with geranylgeranyl- modified ras proteins in the venous context *in vivo*, such as *rras* family members or *rap*. Inhibition of protein geranylgeranylation resulted in the formation of single-lumen caudal vein in developing zebrafish embryos and endothelial cell migration defects in human cell cultures, suggesting that geranylgeranylation is required for CVP formation (Choi et al., 2011). To further investigate whether *rasa1a* interacts with geranylgeranyl-modified Ras proteins, one could assess the CVP phenotype by blocking protein geranylgeranylation in *rasa1a* morphants. A partial rescue will also support the ras -dependent mechanism of *rasa1a in vivo*.

One difficulty I encountered during rescue experiments, was managing the toxicity of injected embryos with two morpholinos or a morpholino and a small molecule. The combination of two reagents results in a number of embryos with morphological defects and increased lethality. In developing embryos, about 15% of the morpholinos can exhibit non-specific toxicity (Ekker and Larson, 2001) and apoptosis is a key component of morpholino off-target effects (Robu et al., 2007). *tp53* activation, a transcriptional regulator with important roles in apoptosis, is mostly responsible for non-specific cell death in many morphants. Thus, additional supportive experiments, such as using *tp53* morpholino, may be required to distinguish between specific and non-specific results; however, despite the high level of toxicity, genetic rescue is the best available method for determining genetic interactions.

#### **4.1.3 *rras* interacts with *rasa1a***

A partial genetic rescue of the *rasa1a* knockdown phenotype was observed after knockdown of *rras* in zebrafish embryos using a splice blocking morpholino. This experiment demonstrated that *rasa1a* may interact with *rras* to enhance its intrinsic GTPase activity in veins. These results are consistent with the role of *rras* in integrin-mediated cell adhesions. Activating mutations of *Rras* result in highly adhesive cell lines due to integrin overactivation and promotion of integrin adhesion to laminin and collagen (Kinbara et al., 2003). Thus, the failure of the CVP endothelial cells to break the junctions might be due to the unregulated integrin expression. However, since the loss of *rras* does not individually affect venous angiogenesis, I concluded that *rras* acts upstream of integrin adhesion; however, in a redundant manner with another pathway.

A second verified *rras* (as well as *rras2* and *rap1b* morpholinos that were used for the rescue and will be discussed later) morpholino is required to replicate the rescue for more confidence in the future.

#### **4.1.4 *rras2* and *rasa1a* function in distinct pathways**

*rras* and *rras2* are not closely related (they share only 65% identity at the protein level), but they both stimulate integrin activation in breast epithelial cells *in vitro* (Keely et al., 1999). However, I found that knockdown of *rras2* in zebrafish embryos has an opposite phenotype to loss of *rras*, and has a similar phenotype to *rasa1a* knockdown suggesting that *rras2* and *rras* do not have similar functions *in vivo*. Even considering that there must be overlap of effectors

downstream of *rras* and *rras2*, my results point to the idea that *rras* and *rras2* function in distinct pathways in the same process. Ras proteins can either activate or suppress integrin activation depending on the cell context (Kinbara et al., 2003). Thus, it is possible that *rras* inhibits the suppression of *rras2* in terms of integrin regulation in endothelial cells. To address this one could conduct a genetic rescue of the *rras2* phenotype through *rras* inhibition. The alleviation of the phenotype would determine whether *rras* and *rras2* act antagonistically in the same pathway. Due to the similar phenotypes of *rasa1a* and *rras2* inhibition, I speculate that loss of *rras2* would also result in overactivation of *mTORC1*. To test this hypothesis one could conduct a rescue experiment of the *rras2* phenotype with *mTORC1* chemical inhibitor rapamycin. It is important to note that treatment of *rasa1a*-deficient embryos with rapamycin between 24 and 48hpf rescues the vascular defect and restores normal circulation (Kawasaki et al., 2014).

#### **4.1.5 *rasa1a* does not interact with *rap1b***

Since *rap1b* also regulates integrin signaling, it is possible that *Rasa1a* activates *Rap1*. However, I found that *rap1b* knockdown does not rescue of *rasa1a* loss of function. *in vitro* evidence also does not support a direct interaction between *rap1* and *rasa1*. Self et al. have reported that *rras* and *rap1* are linked in one single pathway in terms of integrin regulation in a macrophage cell line (Self et al., 2001); therefore *rasa1a* may interact with *rap1b* through *rras*. However, as we still do not know the physical interactions among these proteins *Rasa1a*, *Rras* and *Rap1* may interact directly or indirectly. Further studies are required to elucidate the *in vivo* interaction between these three Ras subfamily members. One caveat of my data is that the *rap1b* rescue experiment comprised two experiments with ~10 embryos in each group.

However, due to the huge deviation within the double morphants, further repetitions would be required for more confidence.

#### **4.1.6 *rasa1a* does not functionally interact with BMP signaling pathway**

BMP functions as a context-dependent pro-angiogenic cue. Inhibition of BMP signaling with endogenous inhibitor *noggin3* results in the formation of a CVP with aberrant sprouts that fail to make connections. In contrast BMP-overexpressing embryos contained ectopic branches that grow rapidly (Wiley et al., 2011). These data suggest a defect in endothelial cell migration, similar to that found in *rasa1* morphants. However BMP- deficient embryos show separation of the dorsal aorta and the dorsal vein with no evidence of arteriovenous shunts. Thus the arteriovenous shunts in *rasa1a* morphants suggest an additional defect, not in the BMP pathway, certainly in Eph signaling and possibly in endothelial cell junction regulation. Thus, although both BMP and Rasa1 signaling seem to regulate venous angiogenesis, they do not have identical cellular functions, possibly because *rasa1* can act downstream of multiple receptors. My *in situ* hybridization results also demonstrate that the expression levels of BMP ligands do not change following *rasa1a* inhibition suggesting that *rasa1a* does not signal upstream of BMP pathway. To further demonstrate the hypothesis that *rasa1a* does not interact with BMP pathway, one could conduct a genetic rescue experiment by overexpressing *bmp* in *rasa1a* morphants. The persistence of the arteriovenous shunts would demonstrate that *rasa1a* does act downstream of the BMP signaling pathway in this context.



#### **4.1.7 *rasa1a* may interact with *vegfc/vegfr3* signaling pathway**

The *Vegfc/Vegfr3* pathway is important in venous and lymphatic angiogenesis. Loss of *vegfc* and *vegfr3* also leads to spontaneous blood vessel abnormalities in early angiogenesis (Hogan et al., 2009). Interestingly, systemic loss of *Rasa1* as well as the conditional loss of *Rasa1* in lymphatic endothelial cells results in severe lymphatic defects in adult mice characterized by lymphatic hyperplasia and leakage that result in early lethality due to lymphatic fluid accumulation in the abdomen. *Rasa1*-deficient lymphatic endothelial cells also show evidence of constitutively activated *Ras*. Interestingly inhibition of *Vegfr3* is sufficient to rescue lymphatic hyperplasia in *rasa1a* deficient mice (Lapinski et al., 2012). My *in situ* hybridization results showed that the expression levels of both *vegfc* and *vegfr3* alter, following *rasa1a* inhibition suggesting that *rasa1a* may also interact with *vegfr3* signaling pathway during venous angiogenesis. To test this hypothesis, one could conduct a genetic rescue experiment in *rasa1a* morphants by knocking down *vegfr3* using a small molecule inhibitor of *vegfr3*, MAZ51 or by morpholinos against the *vegfr3* receptor. It would also be interesting to look at the over expression effects of *vegfc* and *vegfr3* in endothelial cells. To do this, one could over express the genes using DNA or RNA. For future experiments, it would be interesting to look at the lymphatic vessel defects in *rasa1a* morphants and conduct a genetic rescue with *vegfc/vegfr3* constructs.

#### **4.2 Summary and Conclusion**

This project began by examining the knockdown effects of *rasa1* in zebrafish vascular development *in vivo*. *RASA1* is a causative gene in the formation of capillary- arteriovenous

malformations in humans. Severe angiogenic and lymphatic defects were also observed following *Rasa1a* knockdown in mice.

*rasa1a* knockdown leads to severe vascular defects in zebrafish. Instead of a venous plexus, an enlarged single vessel forms, eliminating the capillary-like interconnecting sprouts in between. The caudal artery and the vein are not segregated properly and arteriovenous shunts were formed. A lack of blood circulation to the tail is the end result. Although there is no change in the number of venous endothelial cells, cells exhibit migration defects. In wild type vessels cell connections are able to broken and reformed; while in *rasa1a* morphants we hypothesize that they cannot. Thus the CVP does not undergo remodeling during the later stages of its development. The phenotype of *rasa1a* knockdown in zebrafish provides a strong platform for the study of cellular defects in capillary- arteriovenous malformations. Real time evaluation of vascular defects is possible in zebrafish during development.

My research is first of its kind to document the *in vivo* interaction between *rasa1a* and specific members of the *ras* subfamily. I conducted several genetic rescue experiments to show that *rasa1a* does not interact with farnesyl-modified *ras* genes; however, it likely interacts with *rras* , which is specifically involved in integrin regulation. While *rras2* had no known function in zebrafish vascular development, I showed that *rras2* knockdown results in similar type of defects as *rasa1a* knockdown, suggesting that they might function in parallel pathways in regulating angiogenesis. I was also able to show that *rasa1a* may interact with *vegfc/vegfr3* pathway, a crucial signaling pathway in venous and lymphatic angiogenesis.

The experiments presented here are uncovered a part of a pathway involve in the regulation of venous angiogenesis. It is very likely that *rasa1a* functions in several overlapping signaling pathways during vascular development. Thus, much research still needs to be conducted in order to fully characterize the molecular events leading to vascular malformations.

Finally, it will be important to verify the phenotype and the interactions of *rasa1* by creating *rasa1* mutants. Fortunately the ability to produce genetic mutants via TALEN or CRISPR technology has become available in zebrafish (Bedell et al., 2012).

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