Analysis of blood vessel tube formation using Rab and Rac fusion proteins in Zebrafish

by

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TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Abstract

Proper development of complex organ systems depends on the formation of vascular networks to transport fluids and exchange gases and metabolites. Understanding the processes that guide vascular network and endothelial tube formation is imperative because these mechanisms are also likely used to maintain homeostasis in the vessel wall. Most of what we understand about the molecular mechanisms that guide vascular tube formation is from knock down studies performed *in vitro*. These studies have shown that vesicles fuse to form a primary vessel lumen. Here, we focus on two small GTPases, Rab5c, which was identified in screen as being required for endothelial tubulogenesis in zebrafish, and Rac1, which is required for endothelial lumen formation *in vitro*. We have created zebrafish transgenic lines that label these proteins with the enhanced green fluorescent protein (EGFP) to understand their role in vascular formation in early embryonic development *in vivo*. EGFP fusion with Rab5c and Rac1 along with co-injection with *Tol2* transposase has allowed us to establish transgenic zebrafish to follow and observe protein the dynamics of Rab5c and Rac1 localization during the forming lumen. By crossing these EGFP lines to an *flk1a:mcherry* lines, which expresses the red fluorescent protein, mcherry, in vascular endothelial cells, we determined where the proteins localize during the forming vasculature. Confocal microscopy was used to create time-lapse movies when the

embryos were at 30 hours post fertilization (hpf). These images show that the Egfp-rab5c protein likely localizes to forming lumen and putative cellular junctions in the intersegmental vessels. The *UAS:egfp-rac1* transgenic line shows consistent GFP localization to the putative luminal or apical membranes. Overall, this project defines critical cellular components that regulate vesicle transport and adherens junction remodeling during tube formation. This knowledge can then lead to discovery of new targets for novel angiogenic therapies to ultimately alleviate cancer and vascular diseases.

Background

Proper development of complex organ systems depends on vascular networks to transport fluids and exchange gases and metabolites. Tube formation of blood vessels is a critical step to establish circulation and involves several signaling and cellular processes. Trafficking of vesicles and endocytosis controls blood vessel homeostasis and development, and improper regulation of these processes contributes to cardiovascular disease. The molecules involved in angiogenesis and vasculogenesis serve to stabilize and destabilize the vessel wall. Hemorrhagic stroke and edema are two examples of how defects in endothelial cell junction arrangement lead to human pathologies (Stainier, Fouquet et al. 1996).

Vasculogenesis and Angiogenesis

The vertebrate vascular system is made of a branched web of arteries, veins, and capillaries. This network extends into every body tissue and is specific to its local physiological function. The

development of the vascular system is carried out through two different processes, vasculogenesis followed by angiogenesis (Flamme, Frolich et al. 1997, Risau 1997). The creation of three-dimensional tube structures by endothelial cells during angiogenesis and vasculogenesis is a crucial for functional blood vessels to form, which can use mechanisms including wrapping, cord hollowing, cavitation, budding, and cell hollowing for vascular lumen formation (Lubarsky and Krasnow 2003).

Vasculogenesis involves differentiation and migration of endothelial precursor cells called angioblasts to form new blood vessels de novo or blood vessel formation from individual endothelial cells coming together. Vasculogenesis represents formation of multicellular endothelial tubes and utilizes a chord hollowing mechanism for assembly (Jin, Beis et al. 2005). Dorsal aorta formation and yolk sac circulation are direct results of vasculogenesis (Lubarsky and Krasnow 2003).

Angiogenesis is the sprouting of endothelial cells to form new blood vessels from pre-existing vessels. This is required for the healing of wounds and organ development. Angiogenesis utilizes budding, single cell hollowing and cord hollowing mechanisms for endothelial tube formation (Lubarsky and Krasnow 2003, Kamei, Saunders et al. 2006, Wang et al. 2010). Angiogenesis is also important in growth of tumors and metastasis. The development of potential angiogenesis-related targets for drug treatment could be significant in alleviating vascular diseases and cancer (Tobia, De Sena et al. 2011).

Zebrafish as a model organism

Zebrafish is a useful vertebrate animal model for developmental and genetic studies. Their visual transparency, large-scale progeny production, short generation time and external development are some of their major advantages (Isogai, Lawson et al. 2003). Many adult fish can be kept within a laboratory and a great number of offspring can be gathered from one mating. Most developmental or clinically relevant process can be studied though careful assay development (Dooley and Zon 2000). Because of this, investigating the function of a gene through genetic approaches is easily achievable. These approaches include chemical mutagenesis, TALEN and CRISPR nuclease-based mutagenesis, retroviral insertional mutagenesis, morpholino knockdown, and target-selected mutagenesis (Haffter, Granato et al. 1996, Amsterdam, Burgess et al. 1999, Nasevicius and Ekker 2000, Wienholds, Schulte-Merker et al. 2002, Amsterdam, Nissen et al. 2004, Meng, Noyes et al. 2008, Asakawa and Kawakami 2009). Many zebrafish mutant phenotypes recovered from genetic screens are similar to human disease states, giving us a powerful approach for studying pathophysiology (Dooley and Zon 2000). These advantages and the rapid development of a primary cardiovascular system make zebrafish ideal for high-resolution analysis of vasculogenesis and angiogenesis.

Gene Editing in Zebrafish

Targeted engineering is an essential approach for understanding gene function *in vivo*. Advances in genome editing/engineering using directed DNA damage and repair techniques including Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and

Clustered Regularly Interspaced Palindromic Repeats (CRISPRs) have allowed null alleles to be generated as well as integration of reporter genes at targeted loci that replicate endogenous gene expression (Hoang, Whelan et al. 2004, Doyon, McCammon et al. 2008, Meng, Noyes et al. 2008, Foley, Maeder et al. 2009, Foley, Yeh et al. 2009, Sander, Cade et al. 2011, Sander, Yeh et al. 2011, Zhu, Smith et al. 2011, Auer and Del Bene 2014). In zebrafish, a multitude of studies have used these new tools to perform targeted mutagenesis by inserting small sequence stretches using single stranded oligonucleotides, open reading frames and big fragments, and whole plasmid vectors (Miller, Holmes et al. 2007, Siekmann and Lawson 2007, Maeder, Thibodeau-Beganny et al. 2008, Boch, Scholze et al. 2009, Moscou and Bogdanove 2009, Christian, Cermak et al. 2010, Cifuentes, Xue et al. 2010, Tesson, Usal et al. 2011).

ZFNs function to cause DNA double-strand breaks as dimers. The zinc fingers are bound to non-specific FokI nuclease domain, with each zinc finger binding about three base pairs of DNA. TALENs have the ability to bind DNA via a transcription activator-like (TAL) effector repeat domain that comes from *Xanthomonas*, a bacterial plant pathogen. Every TAL effector binds one base pair of DNA with its selectivity a result of repeat variable di-residues (RVDs) which are the amino acids inside of the repeat (Boch, Scholze et al. 2009, Moscou and Bogdanove 2009, Christian, Cermak et al. 2010). Similar to ZFNs, TAL effector repeats are fused with the FokI nuclease domain in order to make TALENs that can cleave DNA. Any sequence can be targeted with TALENs though modular assembly of multiple repeats. Double stranded breaks made by TALENs and ZFNs can utilize non-homologous end joining (NHEJ) for repair. This course of action creates mutations with short insertions or deletions referred to as indels that can change the reading frame, ultimately causing an early termination of translation. ZFNs have been created to target DNA mutations inside of zebrafish somatic cells that can cause

heritable loss-of-function mutations, however, TALENs have a larger targeting range (Siekmann and Lawson 2007, Meng, Noyes et al. 2008, Foley, Maeder et al. 2009, Zhu, Smith et al. 2011, Moore, Reyon et al. 2012). TALENs and RNA guided nucleases (RGNs) have recently been used for more sophisticated genome modifications by using methods of homologous recombination (HR) and non-homologous end joining (NHEJ) mediated knock-in in model organisms (Auer and Del Bene 2014).

The CRISPR system is a bacterial defense mechanism against invasion of foreign nucleic acids derived from exogenous plasmids or bacteriophages (Barrangou, Fremaux et al. 2007, Marraffini and Sontheimer 2008, Hale, Zhao et al. 2009, Garneau, Dupuis et al. 2010, Bhaya, Davison et al. 2011). It has 2 parts: an array of small CRISPR RNAs (crRNAs), auxiliary *trans*-activating crRNA (tracrRNA) and a nuclease associated with the CRISPR locus (Cas). Unique spacers control target recognition and are the result of additional nucleic acids from foreign DNA to the CRISPR locus. Cas9 cleaves the foreign DNA, leading to inactivation of the invading pathogen (Sashital, Wiedenheft et al. 2012, Wiedenheft, Sternberg et al. 2012). Due to the ease of engineering, the CRISPR/Cas9 system has been used extensively to inactivate genes in zebrafish by utilizing the ability to direct double strand breaks to specific genomic sites.

Transgenesis: Gal4 UAS and *Tol2* transposition

The Gal4-UAS system is useful in examining gene function *in vivo*. Gal4 is a yeast transcriptional activator with a DNA binding domain found inside the N-terminus that binds the yeast-specific, upstream activating sequence (UAS) (Keegan, Gill et al. 1986). Gal4 expression

in targeted zebrafish tissues stimulates tissue specific gene expression when that gene is linked to UAS (Asakawa and Kawakami 2008). Gal4-VP16 contains a transcriptional activation domain originally found in herpes virus VP16 protein linked to the same DNA-binding domain as Gal4 (Sadowski, Ma et al. 1988). Gal4VP16 has more transcriptional activity when compared to full length Gal4, and can be used for even higher UAS transgene expression (Koster and Fraser 2001).

To induce expression of a desired gene, a transposon-donor plasmid can be created within a *Tol2* transposon construct, which has the UAS or *cis*-sequences necessary for transposition along with a fragment of DNA. The transposase mRNA is co-injected with the plasmid into fertilized eggs. Figure 1 shows the two constructs examined in this project, as well as expression of the *UAS:rac1* construct at 30 hpf. During transposition, the *Tol2* sequence is removed and inserted into the genome using a cut and paste mechanism (Kawakami, Shima et al. 2000). This form of transgenesis leads to high efficiency insertions in the germ line (Urasaki, Morvan et al. 2006). *Tol2*-mediated transgenesis is a widely used method for creating transgenic zebrafish because insertions mediated by transposons are more effectively transferred to the offspring compared to DNA microinjection. This allows for creation of a vast number of insertions in F1 fish (Davison, Akitake et al. 2007, Scott, Mason et al. 2007, Asakawa and Kawakami 2008).

Mosaic expression of GFP expression in injected embryos is observed, suggesting that Gal4-VP16 can induce *UAS:egfp* reporter expression on the same construct, as well as control the activation of different transgenes (Sagasti, Guido et al. 2005, Scott, Mason et al. 2007, Asakawa and Kawakami 2009). Because the *Tol2* transposase inserts the construct hundreds of

times throughout the genome, there may be differences in GFP expression driven by the Gal4-VP16 system in different fish, as well as in germline transmission.

Tube formation in Zebrafish

Results from previous studies in vitro using human endothelial cells demonstrate that endothelial tubes can assemble from intracellular vacuoles or large vesicles (Bayless and Davis, 2002). This involves the fusion of intracellular vesicles, resulting in single cell hollowing and the formation of the future apical surface from the vesicle membrane. In zebrafish, vesicles are also observed during lumen formation of the intersegmental vessels (ISVs) early on in embryonic angiogenesis (Kamei, Saunders et al. 2006, Wang, Kaiser et al. 2010).

In the first stage of embryonic angiogenesis in zebrafish, endothelial cells that have migrated from the dorsal aorta come together to form intersegmental vessels at around 20 hours post fertilization (hpf) (Stainier, Fouquet et al. 1996, Lawson and Weinstein 2002). ISVs initiate tube formation around 28 hpf and show circulation around 36 hpf (Isogai, Lawson et al. 2003). 3-4 cells contribute to each ISV, and those cells divide during migration between somites (Siekmann and Lawson 2007). ISVs assemble from intracellular vacuoles that form, fuse, disappear, and reform during the ISVs tube formation (Kamei, Saunders et al. 2006, Blum, Belting et al. 2008). During ISV tube formation, the dynamic junctional patterns show that the endothelial cells line up over extended regions with neighboring cells, forming tubes with at least two cells surrounding the lumen (Isogai, Horiguchi et al. 2001, Childs, Chen et al. 2002, Blum, Belting et al. 2008). Visualizing circulatory patterns in zebrafish embryos has also become

possible by microangiography (Weinstein, 1999). Microangiography is the labeling and subsequent detection of properly formed vessels by injection of a fluorescent dye into the posterior cardinal vein (Schmitt, Holland et al. 2012).

Junctions

Epithelial cells have two surfaces: an apical or luminal surface, which faces lumen, and basolateral or abluminal surface, which interacts with the extracellular matrix. Epithelia have two forms of polarity, apical/basolateral and planar polarity. Septate junctions in invertebrates and adherent and tight junctions in vertebrates separate the apical and basolateral surfaces to keep proteins and outer leaflet lipids from mixing (Martin-Belmonte and Mostov 2008). Tight junctions are made of different proteins and form a selective barrier between the luminal and abluminal sides of a tube and function in maintenance throughout the basolateral and apical membranes. Remodeling and expansion of these junctions is coordinated with the expansion of the apical membrane (Martin-Belmonte and Mostov 2008). Cell to cell adhesion is supported by adherens junctions, which are made up of transmembrane cadherins that have hetero- and homophilic binding through extracellular domains. Cadherin intracellular domains are associated with alpha catenin, a critical protein involved in for cell polarity and linking junctions to the actin cytoskeleton (Iden, Rehder et al. 2006, Wang and Margolis 2007, Miyoshi and Takai 2008, Niessen and Gottardi 2008).

VE-Cadherin

Vascular endothelial cadherin, VE-cadherin, is an adherens junction protein found in endothelial cells that is necessary for tubulogenesis (Yang, Graham et al. 1999). In mice, knockout models of VE-cadherin have shown that it functions in maintaining vascular integrity through vascular endothelial growth factor (VEGF) signaling (Carmeliet, Lampugnani et al. 1999, Carmeliet and Collen 2000). Other studies involving endothelial cells from human umbilical have concluded that VE-cadherin is necessary for cell polarity and formation of endothelial tubes. The *VE-Cadherin* gene is also called *Cdh5*. Knockdown of Cdh5 in zebrafish prevents development of a functioning circulatory system (Nicoli, Ribatti et al. 2007). As member of the cadherin family, Cdh5 functions in endothelial cell adhesions and cell-to-cell adhesion molecules (Heimark, Degner et al. 1990, Wang, Kaiser et al. 2010). During angiogenesis and vasculogenesis, Cdh5 can be used to monitor endothelial cell development because it marks blood vessels both before and after circulation is established (Larson, Wadman et al. 2004). Control of endothelial cell behaviors including cell migration, proliferation, and changes in cell shape are dependent on Cdh5 because of its ability to sense, resist, and transmit mechanical forces (Tzima, Irani-Tehrani et al. 2005, Conway, Breckenridge et al. 2013). In zebrafish, knockdown of Cdh5 causes hyper sprouting shown in multiple endothelial cell processes, demonstrating that it is required to facilitate angiogenic signals and stabilize existing vessels (Abraham, Yeo et al. 2009, Montero-Balaguer, Swirsding et al. 2009, Sauteur, Krudewig et al. 2014).

Rab5c

Development and cellular homeostasis are regulated by vesicle trafficking and endocytosis, and improper regulation of these processes are associated with several disease states. The Rab family of small GTPases is important in regulating the transport of endosome sub-types, as well as other organelles involved in trafficking (Clark, Winter et al. 2011). The largest family of small GTPases that are monomeric is the Rab family. Figure 1 shows depicts the different roles of Rab proteins in various vesicle trafficking. Rab proteins make up the biggest group in the superfamily of Ras GTPases (Urbe, Huber et al. 1993). As known orthologs of proteins SEC4 and YPT1 found in yeast, Rab proteins modulate several cellular processes such as cytoskeleton organization and receptor-mediated signaling by controlling protein internalization, trafficking, and recycling through endocytic compartments (Seachrist and Ferguson 2003, Nishimura and Sasaki 2009, Clark, Winter et al. 2011). The SEC/YPT/RAB proteins are conserved across multiple organisms, and 65 Rab genes with distinct membrane localization and functions have been identified in vertebrates (Clark, Winter et al. 2011). Rab proteins regulate vesicle transport through their on/off regulatory function that is dependent on nucleotide exchange and hydrolysis rates. This switch is specific to where the Rab proteins are localized in membrane compartments and the stage in the vesicular transport pathway (Zerial and McBride 2001, Alone, Tiwari et al. 2005). Rab proteins induce transport once they are activated and bound to soluble effectors. Rab effectors have a great amount of structural heterogeneity, correlating with how highly specialized they are for transport between membrane bound organelles (Zerial and McBride 2001, Alone, Tiwari et al. 2005).

Cells must control cell adhesion during translocation in order to migrate, which can be accomplished through endocytosis and recycling of adhesion molecules (Lauffenburger and Horwitz 1996, Le, Yap et al. 1999). Rab5 is a key component in intracellular trafficking regulation during early endocytosis and endosome remodeling, as wells as in the regulation of FGF and Wnt-signaling pathways (Zerial and McBride 2001). Activation of Rab5 stimulates endocytosis and endosome motility which causes increases cell migration and disassembly of adherens junctions (Murphy, Saffrich et al. 1996, Spaargaren and Bos 1999, Palacios, Tushir et al. 2005, Ulrich, Krieg et al. 2005). In general, Rab5 and early endosomes are involved in regulating multiple receptor-mediated signaling pathways (Clark, Winter et al. 2011).

Rab5 has been shown to be associated with vesicles and membranes inside the cytoplasm, specifically early endosomes (Barbieri, Hoffenberg et al. 1998, Simonsen, Lippe et al. 1998, Christoforidis, McBride et al. 1999, Rubino, Miaczynska et al. 2000, Zerial and McBride 2001). (Chavrier, Vingron et al. 1990). Early on in the endocytic pathway, Rab5 is necessary for fusion of donor and acceptor membranes of the early endosome (Gorvel, Chavrier et al. 1991, Bucci, Parton et al. 1992). The Rab5 effector EEA1, a highly coiled protein with two ZFNs and two Rab5-binding domains, controls tethering and docking of early endosomes and is thought to bridge the two Rab5 bearing membranes (Barbieri, Hoffenberg et al. 1998, Simonsen, Lippe et al. 1998, Christoforidis, McBride et al. 1999, Rubino, Miaczynska et al. 2000, Zerial and McBride 2001). Rab5 is also able to induce cell migration and lamellipodia formation through mechanisms different from other Rho GTPases because of its role in actin reorganization (Spaargaren and Bos 1999).

Vertebrates generally have three paralogs of Rab5, but zebrafish have two Rab5a genes. Rab5a transcripts are ubiquitously expressed in zebrafish embryos, but Rab5b expression is restricted to the yolk syncytial layer and telencephalon while Rab5c is highly expressed in endothelial cells (Thisse, Heyer et al. 2004, Clark, Winter et al. 2011).

Rac 1

The Rho family of GTPases are crucial for controlling the functions of the endothelial barrier (Wojciak-Stothard and Ridley 2002, Wennerberg, Rossman et al. 2005). Rho GTPases can change back and forth from a GTP bound, active state to a GDP bound, inactive state to function as a switch in multiple signaling pathways (Lei, Lu et al. 2000, Bryan and D'Amore 2007). After stimulation, these proteins have increased GTP binding which leads to greater membrane localization (van Nieuw Amerongen, Koolwijk et al. 2003). Small GTPases can bind effectors in order to forward upstream signals in their active state (Beckers, van Hinsbergh et al. 2010, Spindler, Schlegel et al. 2010).

There are 23 known Rho GTPases that belong to the Ras super family of GTPases, which contains 36 known proteins (Wennerberg, Rossman et al. 2005, Bustelo, Sauzeau et al. 2007). Characteristic of the Ras superfamily, Rho GTPases have the ability to control and regulate the function and structure of the actin, microtubule and intermediate filament cytoskeletons (Kaibuchi, Kuroda et al. 1999, Bishop and Hall 2000, Hall and Nobes 2000, Schwartz and Shattil 2000, Ridley 2001, Takai, Sasaki et al. 2001). Additional roles of the Rho GTPases include modulation of important steps in the morphogenesis of endothelial cells (ECs), such as gene

expression, polarity, and migration (Kaibuchi, Kuroda et al. 1999, Hall and Nobes 2000, Ridley 2001, Settleman 2001, Takai, Sasaki et al. 2001, Wojciak-Stothard, Potempa et al. 2001). Phagocytosis, endocytosis, and micropinocytosis are vesicle trafficking mechanisms that are also regulated by Rho GTPases (Greenberg 1995, Swanson and Watts 1995, Garrett, Chen et al. 2000, Ridley 2001).

GTPases are necessary for angiogenesis *in vivo* and capillary formation *in vitro* through their role in VEGF signaling (Connolly, Simpson et al. 2002, Hoang, Whelan et al. 2004). Endothelial cell migration modulated by VEGF relies on Rho protein coordination for the processes of actin polymerization and depolymerization (Bryan and D'Amore 2007, Spindler, Schlegel et al. 2010). Davis and Bayless showed that when Rho, Rac, and Cdc42 were blocked with toxin B, endothelial lumen formation in fibrin gels and collagen was blocked, supporting their functions in this processes (Davis, Bayless et al. 2002). Rac1 is inhibited by both lethal toxin and toxin B, showing that it is a key player in endothelial barrier maintenance (Waschke, Baumgartner et al. 2004, Baumer, Burger et al. 2008). Endothelial cells containing Rac1 utilize direct association, gene expression, phosphorylation, and substrate availability to control how eNOS functions (Sawada, Li et al. 2010).

Rac1 maintains the integrity of adheren and tight junctions through mobilization of the cortical actin network, phosphorylation modulation, and redistribution of intracellular junction proteins (Wojciak-Stothard, Tsang et al. 2006, Birukova, Zagranichnaya et al. 2007, Seebach, Donnert et al. 2007, Tan, Palmby et al. 2008, Baumer, Spindler et al. 2009, Gavard, Hou et al. 2009, Knezevic, Predescu et al. 2009, Monaghan-Benson and Burridge 2009, Peng, Wang et al.

2010). When constitutively active Rac1 is overexpressed, adherens junctions are stabilized in contrast to the dissociation of the adheren junctions that happens when dominant negative Rac1 was overexpressed (Kuroda, Fukata et al. 1997). Vacuole and lumen formation in ECs that were overexpressing DN Rac1 could be initiated but would eventually collapse, showing that Rac1 is necessary for the latter stages of vessel development (Bryan and D'Amore 2007). Increasing Rac1 expression in ECs throughout adenoviruses leads to increased formation of lumens and vacuoles, however, branching and sprouting are not increased (Bayless and Davis 2002). Barrier enhancement is dependent on activation of Rac1 to cause reorganization of the cytoskeleton (Birukova, Zagranichnaya et al. 2007, Baumer, Drenckhahn et al. 2008, Birukova, Zagranichnaya et al. 2008, Baumer, Spindler et al. 2009). The processes of cortactin, an actin-binding protein that accumulates at cell borders, is dependent on Rac1 (Weed, Du et al. 1998, Jacobson, Dudek et al. 2006, Waschke, Burger et al. 2006, Baumer, Drenckhahn et al. 2008).

Cdc42 and Rac1 both increase the stability of microtubules through mediating stathmin phosphorylation by Pak-1. Rac1 is also responsible for formation of lamellipodia and filopodia (Daub, Gevaert et al. 2001). Along with regulation of Fc receptor-mediated phagocytosis and macropinocytosis, Rac1 and Cdc42 also aid in the formation of capillary lumens in fibrin matrices (Caron and Hall 1998, Cardelli 2001, Ridley 2001, Bayless and Davis 2002). Rac1 and Cdc42 both localize at cell-cell contacts in ECs that have stabilized junctions.

Thesis Organization

My thesis consists of four chapters. Chapter 1 is a general introduction and provides background for the experimental approaches and findings in Chapters 2 and 3. Chapter 2 describes the characterization of *rab5c* mutants and follows Rab5c localization using a fluorescent reporter gene, *egfp-rab5c*, during blood vessel formation. Chapter 3 describes the production and isolation of an *egfp-rac1* transgenic zebrafish to follow actin dynamics during endothelial tube formation. Chapter 4 contains the general conclusions of the findings in Chapters 2 and 3. The *flk1a:egfp-rab5c* and *rab5c*-/- transgenic lines described in Chapter 2 were generated by Ying Wang. The *rab5c*-/+, *cdh5*-/+ fish were identified by Melanie Torrie. I characterized the *flk1a: egfp-rab5c* transgenic and *rab5c*-/- mutant lines in Chapter 2 and created and characterized the *UAS: egfp-rac1* transgenic line described in Chapter 3.

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Figures and Tables

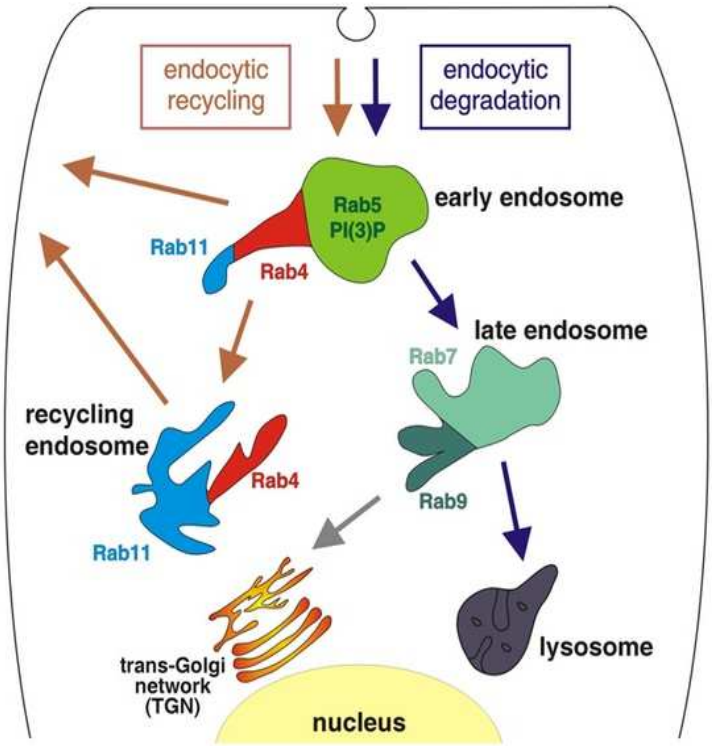

Figure 1. Depiction of how different Rab proteins are involved with interactions and movement of other organelles (Wandinger-Ness and Zerial 2014)

CHAPTER 2

ROLE OF RAB5C IN ENDOTHELIAL TUBE FORMATION

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Abstract

Proper development of complex organ systems depends on the formation of vascular networks to transport fluids and exchange gases and metabolites. Understanding formation of the vascular system is imperative because the mechanisms that guide its development are likely used to maintain homeostasis in the vessel wall. Most of what we understand about the molecular mechanisms that guide vascular tube formation is from *in vitro* gene knock down studies. On a cell biological level, *in vitro* studies have shown that vesicles fuse to form a primary vessel lumen. Rab5c is a small GTPases that is expressed in endothelial cells and required for endothelial tubulogenesis based on knockdown studies in zebrafish. Zebrafish transgenic and mutant lines for Rab5c were generated to understand further the role in Rab5c vascular formation in early embryonic development *in vivo*. These lines include an EGFP fusion with Rab5c as well as a Rab5c knock out model. By crossing the Egfp-*rab5c* line to an *flk1a:mcherry* background to identify endothelial cells with a red signal, we were able to show that the Egfp-Rab5c likely localizes to forming lumens or junctions in the intersegmental vessels by using confocal microscopy. Preliminary analysis of the Rab5c knockout mutants suggest there are

defects in endothelial migration does this correlate with the genotype. This project begins to define the role of Rab5c in critical cellular mechanisms that regulate vesicle transport and adherens junction remodeling during tube formation. This knowledge may lead to discovery of new targets for novel angiogenic therapies to ultimately alleviate cancer and vascular diseases.

Introduction

Rab proteins modulate several cellular processes such as cytoskeleton organization, cellular polarity, and receptor-mediated signaling by controlling protein internalization, sorting, trafficking, and recycling through endocytic compartments (Seachrist and Ferguson 2003, Nishimura and Sasaki 2009, Clark, Winter et al. 2011) . Rab proteins induce vesicle transport once they are activated and bound to soluble effectors. Rab5 is a key component in intracellular trafficking regulation during early endocytosis and endosome remodeling, as well as in the regulation of FGF and Wnt-signaling pathways (Zerial and McBride 2001). Activation of Rab5 stimulates endocytosis and endosome motility which causes increases cell migration and disassembly of adherens junctions (Murphy, Saffrich et al. 1996, Spaargaren and Bos 1999, Palacios, Tushir et al. 2005, Ulrich, Krieg et al. 2005). The role of rab5c in and the contribution of the early endosomal compartment to endothelial tube formation is lacking. Based on previous studies, Rab5c may affect junctional remodeling during endothelial lumen formation.

Endothelial tubes can assemble from intracellular vacuoles or large vesicles (Bayless and Davis, 2002). This involves the fusion of intracellular vesicles, resulting in single cell hollowing and formation of the future apical surface from the vesicle membrane. In zebrafish, vesicles are

also observed during lumen formation of the intersegmental vessels (ISVs) early on in embryonic angiogenesis (Kamei et al., 2006). In the first stage of embryonic angiogenesis in zebrafish, endothelial cells that have migrated from the dorsal aorta come together to form intersegmental vessels at around 20 hours post fertilization (hpf) (Stainier, Fouquet et al. 1996, Lawson and Weinstein 2002). 3-4 cells contribute to each ISV, and those cells divide during migration between somites (Siekmann and Lawson 2007). During ISV tube formation, the dynamic junctional patterns show that the endothelial cells line up over extended regions with neighboring cells, forming tubes with at least two cells surrounding the lumen (Isogai, Horiguchi et al. 2001, Childs, Chen et al. 2002).

Cadherin intracellular domains are associated with alpha catenin, critical proteins involved in cell polarity and linking junctions to the actin cytoskeleton (Iden, Rehder et al. 2006, Wang and Margolis 2007, Miyoshi and Takai 2008, Niessen and Gottardi 2008). In contrast, tight junctions are composed of multiple cellular proteins and form a selective barrier between the luminal and abluminal sides of an endothelial tube and function in maintenance of polarity. Remodeling and expansion of these junctions is coordinated with the expansion of the apical membrane (Martin-Belmonte and Mostov 2008). Cell to cell adhesion is supported by adherens junctions, which are made up of transmembrane cadherins that have hetero- and homophilic binding through extracellular domains.

I have putatively identified that Egfp-Rab5c localizes to the lumen and junctions during endothelial tube formation in zebrafish in *Tg(flk1a: egfp-rab5c)*. I propose that it plays a role in junctional remodeling by moving adherens junctional components from the cell surface. I tested

this hypothesis by examining a Rab5c knockout line that contains a putative null mutation. Some of the *rab5c* homozygous mutants showed mis-migration and a failure to form lumens, but this was not consistent with all mutants. In contrast, all *rab5c* mutants displayed a reduced dorsal aorta caliber.

Materials and Methods

Generation of the Tg(flk1a: egfp-rab5c) line

This transgenic fish line was generated by Ying Wang. The DNA construct in a Tol2 transposon is shown in Figure 1.

Generation of rab5c mutants

The *rab5c* knock out fish line was generated using TALEN-mediated mutagenesis by Ying Wang and outcrossed to *Tg(fli1a-egfp)*^{y1} line. The TALEN details are as follows for *rab5c* gene targeting: TAL1 starts at 176 bp and TAL2 starts at 221 bp from the beginning of the cDNA. TAL1 contains 15 RVDs: HD HD NG HD NI HD NI HD NI NN NI HD NI HD NG,. TAL2 also contains 15 RVDs: HD NG HD NI NI NI HD NG NG NG NI HD NG NN NG. The spacer length between the two TALs is 16 bp, corresponding to 191-206 bp from the beginning of the cDNA and is located in exon 2; CCTCACACAGACACT CTGCTTGGACGATACGACAGTAAAGTTTGAG. The following PCR primers were used to produce a PCR product of 231 bp: r5c-L: 5’ TAGCTCCGCAATAAGGAAGC, r5c-R: 5’ TCG TAG ACG ACG ATG GCT.

Genotyping and fin clips

The F3 generation of the Rab5c knockout fish line was fin clipped by placing fish 100 mL fish water in Tricane. A small piece of fin was cut with a razor, placed in a PCR tube with 100 uL NaOH, and heated to 95^{o}C for 30 minutes. 10 ul of Tris HCL pH 7.5 was added. PCR was carried out with 0.4 ul each primer (r5c-L and r5c-R), 7.5 ul 2X Gotaq mastermix (Promega), 1 ul DNA, 5.7 ul water for a total reaction volume of 15 ul. The following PCR conditions were used: 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds with a final extension at 72°C for 2 minutes. Samples were run on a 10% Acrylamide gel in Tris Borate EDTA (TBE) buffer and categorized as being heterozygous and homozygous for the *rab5c* deletion. The gel in figure 2 shows the difference in the bands of the identified fish, where the homozygous fish contain a 11 bp deletion.

Crosses of rab5c mutants

Rab5c homozygous mutant zebrafish from were grown to maturity crossed to the Cdh5 mutant line to test requirements for adherens junctions. These embryos were incrossed and selected for the Cdh5 phenotype of no circulation, anesthetized in tricane, and mounted in 1% low melt agarose on a coverslip and imaged with a 40X water emersion objective and on a Zeiss LSM 700 confocal microscope.

Microscopy

Embryos *Tg(flk1a: egfp-rab5c)* were anesthetized in tricane and screened for GFP on a Zeiss DIC Axioscope at 10x magnification. Positive embryos for Egfp-Rab5c and mcherry were imaged as above.

Microangiography

Microangiography was carried out as described (Kalen et al., 2009). At 48 and 72 hpf, embryos were injected with Tetramethylrhodamine Dextan (TMRD) in the sinus venous of the heart and imaged on a Zeiss DIC Axioscope at 5x and 10x magnification. Genotypes were identified using PCR as outlined above.

Results

Rab5c localization

To follow Rab5c mediated vesicle trafficking during endothelial tube formation, a transgenic line was created by co-injecting *Tol2-flk1a-egfp-rab5c-polyA* construct with *Tol2* transposase mRNA (Figure 2). *flk1a* is vascular specific gene, and its promoter and upstream region can be used to express fluorescent proteins specifically in the endothelial cells (Lawson and Weinstein 2002). The *Tg(flk1a: egfp-rab5c)* transgenic line was crossed to the *Tg(flk1a: mcherry)* line to visualize the vesicle movement in the developing vasculature. Using confocal microscopy, small patches of green fluorescence likely representing Egfp-rab5c protein could be seen moving back in forth and appeared to localize to the forming lumens of the endothelial tubes (Figure 3 and 4). A narrow band of egfp-*rab5c* localization was often observed, suggesting localization to junctional regions between endothelial cells.

Rab5c mutants do not show a fully penetrant phenotype

To examine the requirement for *rab5c* during development, mutants in *rab5c* were examined. TALEN mediated mutagenesis was used to create small insertions and deletions in the *rab5c* coding sequence of exon 2. *rab5c* mutant zebrafish contain an 11 bp deletion in the in the *rab5c* gene, which creates a putative null allele. Heterozygous *rab5c* fish were crossed to one another, and the progeny were examined for phenotypic changes. At 48 hpf, the homozygous mutants were associated with mismigration of the intersegmental vessels (3/9 embryos) (Figure 5 and 6). In contrast, heterozygous and wild type embryos from the same cross did not display defects in migration (Table 1). In addition, the intersegmental vessels that are not fully lumenized compared to the heterozygous and wild type fish as assessed by microangiography (Table 1). During microangiography, the dye may not transfer to every vessel, as shown by gaps in the vasculature in the wild type fish. Together, these results suggest that migration and lumen formation is compromised in some *rab5c* homozygous mutant embryos.

At 72 hpf, the homozygous mutant *rab5c* embryos have similar lumen defects in the intersegmental vessels as at 48 hpf, (Figure 6 and 7). After genotyping, these defects were not observed in all homozygous fish, suggesting that the observed phenotype is not fully penetrant (4/9 embryos) (Table 1). In contrast, all *rab5c* homozygous embryos displayed a reduced dorsal aorta caliber (Figures 6 and 7, Table 1).

Discussion

Using time lapse confocal microscopy, Egfp-Rab5c fluorescence in the *Tg(flk1a:egfp-rab5c)* line was observed moving back and forth inside the forming lumen in structures consistent with junctional morphology. The fluorescence observed is consistent with endosomal trafficking during junctional remodeling during endothelial tube formation. Further experiments will be to determine the co-localization of these vesicles with cellular junctions in both transgenic lines that allow observation of cell junctions and by immunolabeling of junctional proteins.

The mismigration observed in *rab5c* mutants was consistent with the *rab5c* homozygous phenotype, but it was not fully penetrant. By knocking out *rab5c*, the endosome may not be able to properly internalize cell surface receptors, such as VEGF and Semiphorins. VEGF gives guidance cues to endothelial cells in a pathway that leads to cellular migration and other processes such as tube formation. Endosomal trafficking regulated by Rab5c may play a role in the regulation of VEGF signaling which may explain why Rab5c knockdown leads to mismigration (Eichmann and Simons 2012).

Extensive remodeling of junctions occurs during tube formation, which is potentially mediated by the endosome. Another possibility is that Rab5c is interacting with VE-cadherin to affect lumen formation. VE-cadherin functions in maintaining vascular integrity through VEGF signaling (Carmeliet, Lampugnani et al. 1999, Carmeliet and Collen 2000). In the absence of Rab5c protein, the remodeling VE-cadherin-based junctions may be compromised. Alternatively,

Rab5c and VE-cadherin could play a role in VEGF signaling, also leading to mismigration of endothelial cells.

Incomplete penetrance of the phenotype could be a result of genetic compensation from another gene involved in lumen formation that allows for properly formed lumens without Rab5c. Although some embryos had lumenal defects, all of the embryos still had some lumens that were fully formed.

Acknowledgements

Both Rab5c transgenic lines were created by Ying Wang. I imaged and characterized both the Egfp-Rab5c transgenic line and *rab5c* mutants. Melanie Torrie identified the *rab5c* mutants outcrossed to *cdh5*.

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Figures and Tables

Figure 1. The *Tol2-flk1a: egfp-rab5c* injecteion construct. The *Tol2-flk1a:egfp-rab5c-polyA* construct injected into embryos with *Tol2* transposase mRNA, which produces widespread insertion of injected DNA.

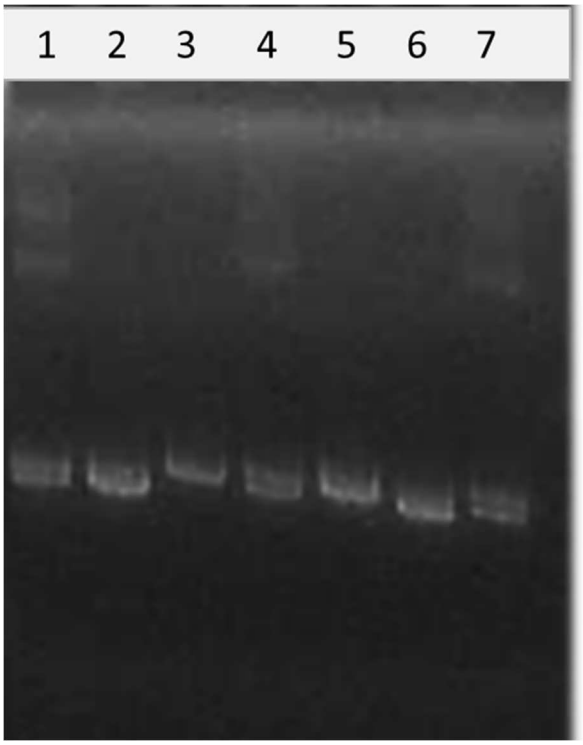

Figure 2: Genotyping of *rab5c* mutant zebrafish on 10% acrylamide gel. Lanes 3 and 5 are wild type, lanes 1,4 ,7 are heterzygous and contain two bands, and lanes 2 and 6 are homozygous with a 11 bp deletion.

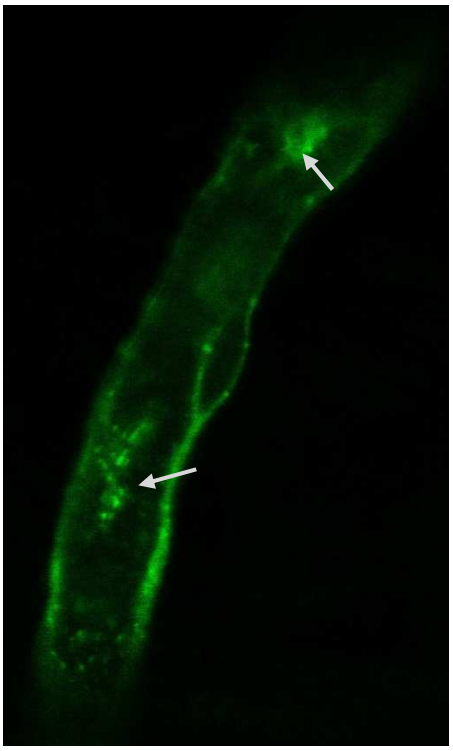

Figure 3: *Tg(flk1a:egfp-rab5c)* expression inside fully formed intersegmental vessel. Egfp-Rab5c positive vesicles inside of forming vessel were identified in *Tg(flk1a:egfp-rab5c)* transgenic line at 30 hpf. Compressed z-series at 40x magnification.

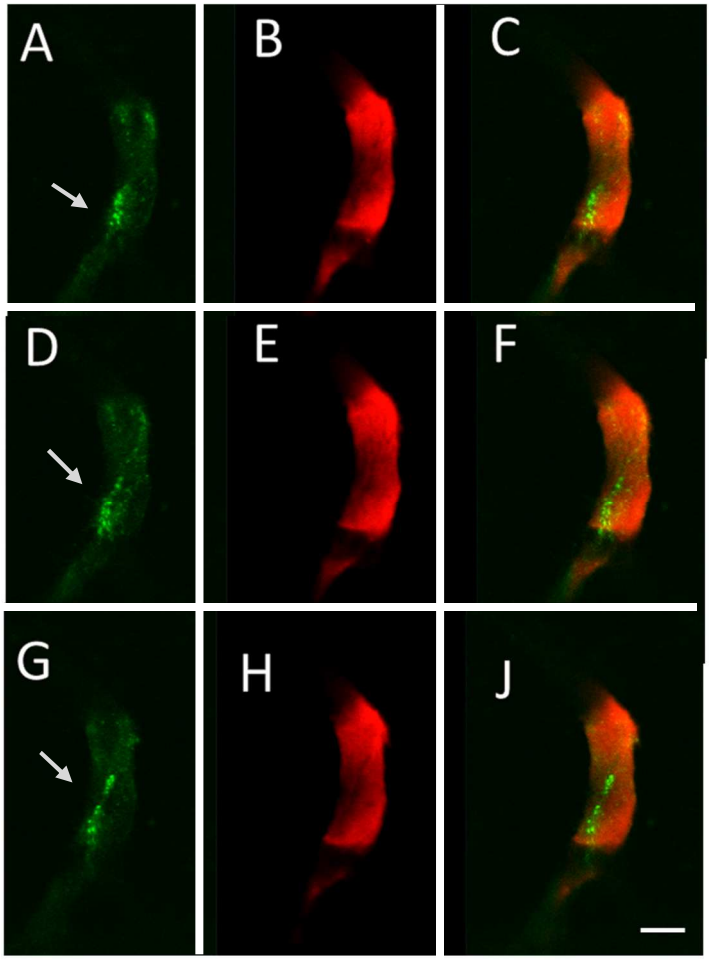

Figure 4: *flk1a:egfp-rab5c* expression inside forming vessel. At 30 hpf at 40 x magnification on confocal microscope, elongated lines of Egfp-Rab5c consistent with localization to cellular junctions. A, B, C likely represent green fluorescence from Egfp-Rab5c vesicles, *flk1a:mcherry*, and merged channels, respectively. D, E, F, and G, H, J are the same vessel in A, B, and C at 2 and 4 minutes after the first panel, respectively. Scale bar represents 5 microns.

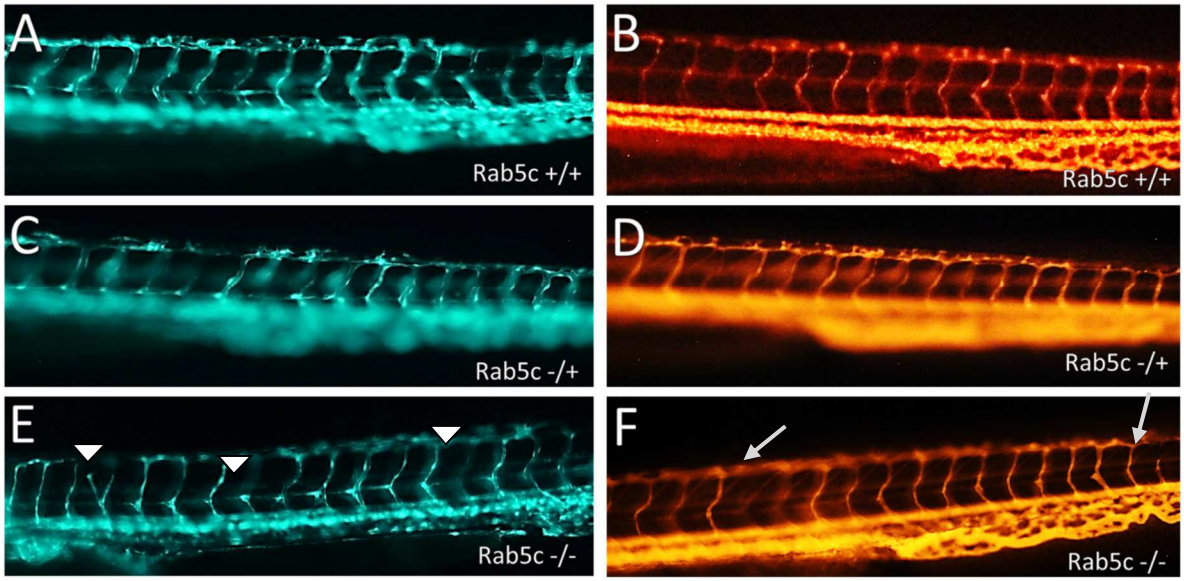

Figure 5: *Tg(fli**1a:egfp)*** **and TMRD microangiography in embryos derived from a cross of *rab5c* heterozygous zebrafish display changes in lumen formation**. At 48 hpf, defects in migration (arrowheads) and lumen formation (arrows) are detected . A and B are wild type, C and D are heterozygous, E and F are homozygous *rab5c* mutants. A, C, E, show *fli1a:egfp* expression, B, D, F, show injected TMRD dye in vascular system (10X magnification).

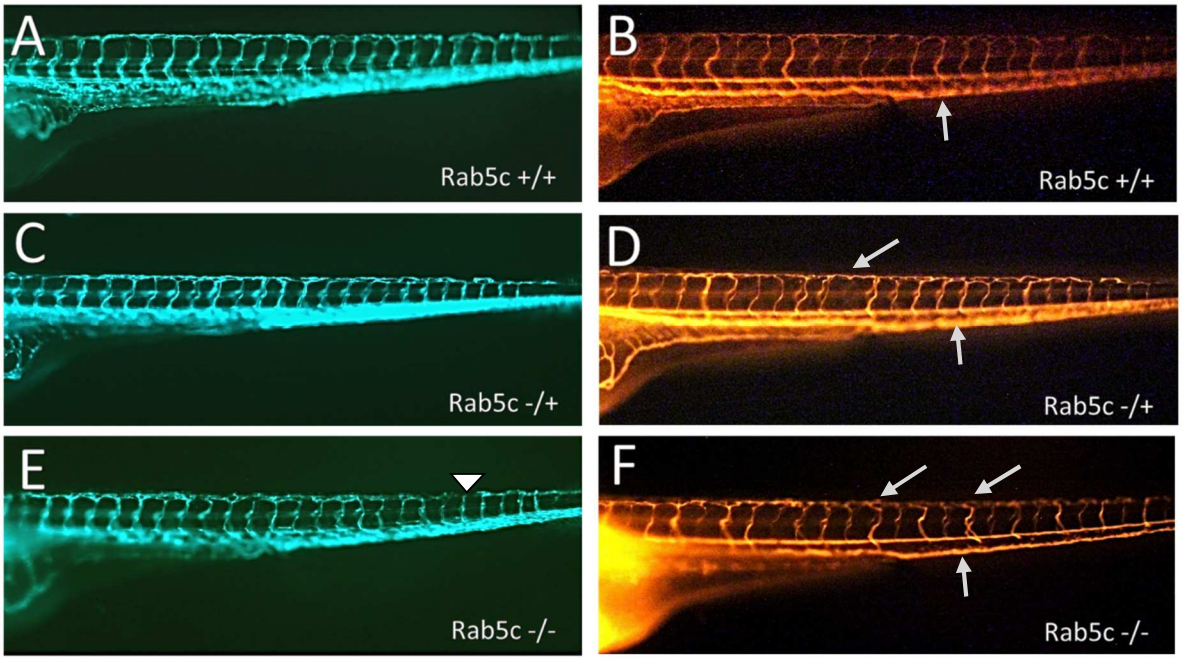

Figure 6: ***Tg(fli1a:egfp)*** **and TMRD microangiography in embryos derived from a cross of *rab5c* heterozygous zebrafish display defects in endothelial migration, lumen formation, and reduced dorsal aorta caliber**. At 72 hpf, defects in migration (arrowheads) and lumen formation (arrows) are detected. A and B are wild type, C and D are heterozygous, E and F are homozygous. A, C, E, show *Tg(fli1a:egfp)* expression, B, D, F, show injected TMRD dye in vascular system (5X magnification).

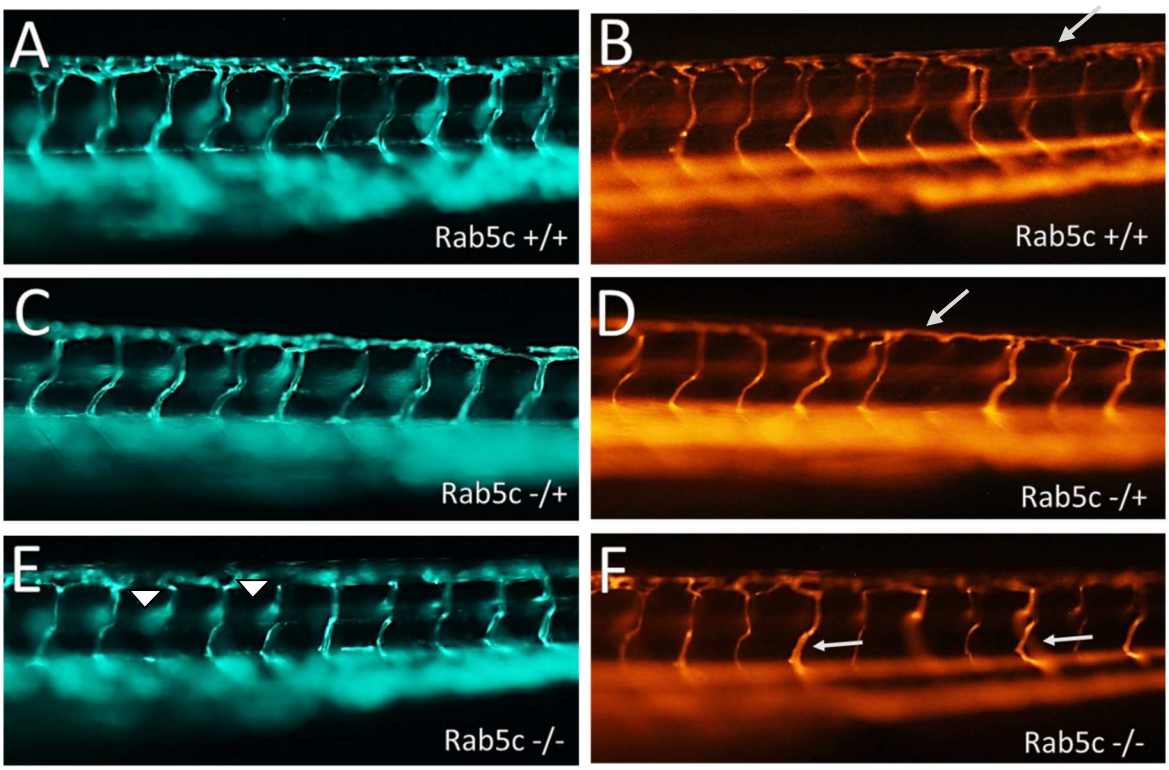

Figure 7: ***Tg(fli1a:egfp)*** **and TMRD microangiography in embryos derived from a cross of *rab5c* heterozygous zebrafish display defects in endothelial migration, lumen formation, and reduced dorsal aorta caliber**. At 72 hpf, defects in migration (arrowheads) and lumen formation (arrows) are detected. A and B are wild type, C and D are heterozygous, E and F are homozygous. A, C, E, show *Tg(fli1a:egfp)* expression, B, D, F, show injected TMRD dye in vascular system (10X magnification).

Table 1: **Summary of Rab5c heterozygous incross differences at 48 and 72 hpf.** 40 embryos total were examined and genotyped as wild type, heterozygous, or homozygous for the *rab5c* deletion.

Genotype	**Number with genotype**	**Differences at 48 hpf**	**Differences at 72 hpf**
Wild Type	12	1 showed smaller lumen	No differences
Heterozygous	19	2 showed dye transfer only on one side	All look wild type
Homozygous	9	3 show mismigration Few changes in microangiography	4 show luminal defects None were wild type Reduced DA in all

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF A ZEBRAFISH EGFP-RAC1 TRANSGENIC LINE

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Abstract

Proper development of complex organ systems depends on the formation of vascular networks to transport fluids and exchange gases and metabolites. Understanding of vascular development is imperative because these mechanisms are likely used to maintain homeostasis in the vessel wall. Most of what we understand about the molecular mechanisms that guide vascular tube formation is from knock down studies carried out *in vitro*. These studies have shown that vesicles fuse to form a primary vessel lumen. Rac1 is a small GTPase that is necessary for capillary lumen formation in human endothelial cells. Here, a zebrafish transgenic line was created to follow the localization of Rac1 during vascular tubulogenesis in early embryonic development *in vivo*. For this, an EGFP fusion protein with Rac1 was generated and expressed using the Gal4 UAS system. A stable transgenic line was isolated by cloning and injecting a *Tol2* transposon carrying the *UAS:egfp-rac1* DNA construct along with *Tol2* mRNA. This allowed visualization of the forming lumen during endothelial tubulogenesis. By crossing the *Tg(UAS:egfp-rac1)* line to a *Tg(flk1a:mcherry)* background with a *Tg(flk1a:Gal4-VP16)* driver, mcherry was observed

throughout the endothelial cells and *egfp-rac1* was observed enriched in apical membranes. Confocal microscopy was used to create time-lapse movies when the embryos were 30 hours post fertilization. Overall, this project establishes a transgenic line that allows critical cellular components that regulate vesicle transport to be followed in real time and *in vivo* during endothelial tube formation. This knowledge can then lead to discovery of new targets for novel angiogenic therapies to ultimately alleviate cancer and vascular diseases.

Introduction

Rho GTPases work in regulating the functions of the endothelial barrier and vesicle trafficking (Greenberg 1995, Swanson and Watts 1995, Garrett, Chen et al. 2000, Ridley 2001, Wojciak-Stothard and Ridley 2002, Wennerberg, Rossman et al. 2005). GTPases are necessary in VEGF signaling where they are involved in angiogenesis *in vivo* and capillary formation *in vitro* (Connolly, Simpson et al. 2002, Hoang, Whelan et al. 2004). Endothelial cell migration modulated by VEGF relies on Rho protein coordination for the processes of actin polymerization and depolymerization (Bryan and D'Amore 2007, Spindler, Schlegel et al. 2010). Rac1 is a GTPase that maintains the integrity of adherens and tight junctions through mobilization of the cortical actin network, phosphorylation modulation, and redistribution of intracellular junction proteins (Wojciak-Stothard, Tsang et al. 2006, Birukova, Zagranichnaya et al. 2007, Seebach, Donnert et al. 2007, Tan, Palmby et al. 2008, Baumer, Spindler et al. 2009, Gavard, Hou et al. 2009, Knezevic, Predescu et al. 2009, Monaghan-Benson and Burridge 2009, Peng, Wang et al. 2010). Barrier enhancement is dependent on activation of Rac1 to cause reorganization of the

cytoskeleton (Birukova, Zagranichnaya et al. 2007, Baumer, Drenckhahn et al. 2008, Birukova, Zagranichnaya et al. 2008, Baumer, Spindler et al. 2009).

Actin filaments can assemble into different structures to assist in many critical cellular processes. Actin polymerization allows for formation of protrusions in the plasma membrane during cell migration and morphogenesis. Stress fibers are important in morphogenesis and cellular adhesions and are made up of actomyosin. Stress fibers are plentiful in endothelial cells in animal tissues. Stress fibers that are attached to focal adhesions aid in the process of mechano-transduction (Kaksonen, Toret et al. 2006, Tojkander, Gateva et al. 2012). Focal adhesions are a link between extracellular matrices and actin cytoskeleton. They consist of tight adhesions that have some signal transduction to control cellular growth (Burridge and Chrzanowska-Wodnicka 1996).

I have made a transgenic zebrafish line that expresses Egfp-Rac1 in the endothelial cells to follow actin dynamics during endothelial tube formation *in vivo*. Other studies have shown that Rac1 is necessary for proper tube formation, but its specific capacity is unknown. We hypothesize that Rac1 is localizing to apical membranes, which would be consistent with its known role in actin rearrangement. We examined this hypothesis by creating and characterizing the *Tg(UAS: egfp-rac1)* transgenic line which tags Rac1 with green fluorescent protein to follow it's movement and localization *in vivo*.

Materials and Methods

Generation of the Tg(UAS:egfp-rac1) line

The following primers were used to amplify *egfp* and *rac1* from the respective template to create the *pTol2-UAS:egfp-rac1polyA* construct from the *pTol2-UAS-rac1-Egfp-PA* template (a kind gift from Ryan Cheng): EGFP F-ATGCATCGATACCATGGTGAGCAAGGGCGAGGAG, EGFP R-GCATCCCGGGAGGAGCAGGAGCAGGAGCAGGAGCAGGAGCCTTGTACAGCTCGTCCATGC, Rac1 F- ATCGCCCGGGATGCAGGCCATAAAGTGTGT, Rac1 R-GACTGAATTCTTACAGAAGGAGACATCTTCTCC. The following primers were used to amplify the zebrafish beta actin poly adenylation sequence (polyA or pA) from the T2sboncgb2 template (a kind gift from Dr. Maura McGrail): polyA tail F-ATCGGAATTCACGGACTGTTACCACTTCAC, polyA tail R-ATCGACTAGTTTTATTTAGCAGTAGATAGC, RFP F-ATCGATCGATACCATGGCCTCCTCCGAGGACGT, RFP R-CGATCCCGGGAGGAGCAGGAGCAGGAGCAGGAGCAGGAGCGGCGCCGGTGGAGTGGCGGC. Fragments were amplified with KOD (Life Sciences) and purified prior to digestion with the following enzymes prior to ligation with T4 ligase (Promega): Cla1, Spe1, Xma1, and EcoR1.

The *pTol2-UAS:egfp-rac1polyA* construct was purified with the Qiagen Miniprep kit, and again with the Promega Miniprep kit. The construct was injected at a concentration of 25 pg with 100

pg of *Tol2* transposase mRNA into *Tg(flk1a:gal4VP16polyA)* (provided by Ying Wang) embryos with *Tg(UAS:rfp-polyA)* in the background (provided by Darius Balciunas).

Microscopy

At 30 hours post fertilization, embryos were anesthetized in Tricane and screened for GFP expression in the vascular system using a Zeiss DIC Axioscope at 10x magnification. Embryos positive for EGFP were mounted in 1% low melt agarose on a coverslip and imaged with a 40X water emersion objective lens on a Zeiss LSM 700 confocal microscope. The embryos expressing GFP raised to adulthood. At maturity, these F0 fish were outcrossed to *Tg(flk1a:mcherry)* line fish (Wang et al., 2010) and screened in the same manner. This time point was chosen because it is when the lumens are forming.

Results

To examine actin dynamics during lumen formation, the *Tg(UAS:egfp-rac1)* transgenic fish line was created using *Tol2*-mediated transgenesis (Figure 1). By injecting this construct with *Tol2* transposase into driver *Tg(flk1a:gal4VP16polyA)* embryos with *Tg(UAS:rfp-polyA)* in the background, expression of Egfp-Rac1 and RFP was detected in the endothelial of the vascular system. Injection usually led to mosaic expression of the construct, meaning that each successfully injected fish could show different patterns of Egfp-Rac1 expression in the vasculature. Two founder zebrafish that showed germline transmission, with the germlines displaying highly mosaic transmission of Egfp-Rac1 fluorescence. These fish were crossed to

Tg(flk1a:mcherry) and screened for vascular expression of GFP. Table 1 shows the total number of embryos screened from each injected founder when outcrossed to *Tg(flk1a:mcherry).* Transmission of *Tol2-UAS:egfp-rac1* as observed by fluorescence was detected in 1% and 4.1% of the offspring.

The embryos showing the most vascular Egfp-Rac1 expression were imaged at 30 hpf to observe dynamics during lumen formation (Figures 2 and 3). Embryos displayed Egfp-Rac1 localization to what appears to be actin filaments and potential focal adhesions. Through further examination of the *Tg(UAS:egfp-rac1)* embryos, we were able to see specific actin movements and dynamics during lumen formation and to actin rich filopodia. Localization of Egfp-Rac1 seemed to show similar localization in the forming and fully formed lumen to the lumenal membranes, consistent with localization of Actin and Moesin to these sites (Figures 4, 5 and 6) (Wang et al., 2010). Localization of Egfp-Rac1 also appeared to identify vesicles, also consistent with previous findings in human endothelial cells (Bayless and Davis 2002). Together, the *Tg(UAS:egfp-rac1)* transgenic line will allow the examination of actin dynamics in real time and *in vivo*.

Discussion

The GTPase Rho regulates focal adhesions assembly (Bryan and D'Amore 2007). Rac1 is in the same family of GTPases as Rho and regulates actin dynamics (Jacobson, Dudek et al. 2006), which may explain why it appears to localize to the apical membrane, which interact with focal adhesions. Rho GTPases regulate intracellular regulation of cellular responses to external

signaling cues, resulting in actin rearrangements (Poukkula, Kremneva et al. 2011). Actin localization has long been recognized as being important in tube formation, however the role of downstream cell signaling remains elucidated. The *Tg(UAS:egfp-rac1)* transgenic line will allow us to address the precise roles of actin and Rac1 in this process. This tool will also allow us to examine the dynamics of actin associated with junctions and actin-mediated vesicle transport.

Since remodeling and expansion of junctions is coordinated with the expansion of the apical membrane, it would be logical that Rac1 localizes to the apical membrane because of its role in maintaining the integrity of adherens and tight junctions (Martin-Belmonte and Mostov 2008). Junctional remodeling is also controlled by vesicles, which Rac1 may be localizing to through its involvement with the actin cytoskeleton. It is well established that endothelial tube formation requires actin polymerization. This powerful *in vivo* tool, *Tg(UAS:egfp-rac1),* will allow detailed analysis of actin-based vesicle transport to follow in real time, complementing the seminal work of in vitro from Kayla Bayless and George Davis (Bayless and Davis 2002).

We can additionally observe how mutants affect vascular development in great detail with the *Tg(UAS:egfp-rac1)* line. By crossing *Tg(UAS:egfp-rac1)* to the *cdh5* mutant line, we can characterize the contribution of VE-cadherin to actin dynamics. VE-cadherin is linked to the actin cytoskeleton via alpha-catenin. By placing Cdh5 in this background, we could examine what causes failure of lumen formation and how actin dynamics are changed.

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Figures and Tables

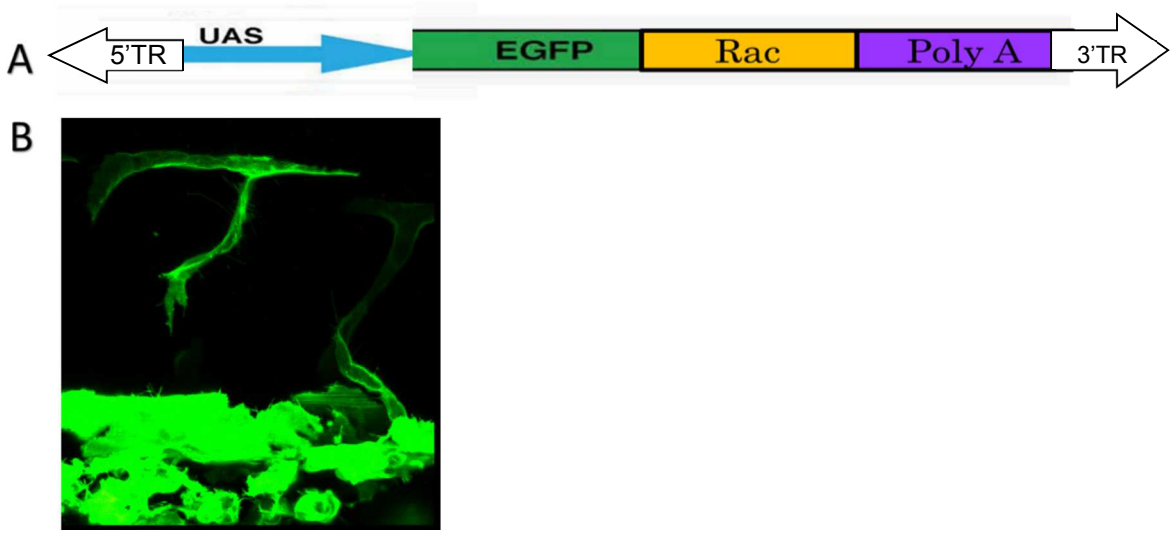

Figure 1: *Tol2-UAS:egfp-rac1* DNA construct and transient expression of Egfp-Rac1 in endothelial cell following injection with Tol2 mRNA. A. The *Tol2-UAS:egfp-rac1* DNA construct. B. Injection of the *Tol2-UAS:egfp-rac1* DNA construct into *Tg(flk1a:gal4VP16-polyA)* with *Tg(UAS: rfp-polyA)* transgenic embryos with *Tol2* transposase mRNA produces widespread insertion and expression of injected DNA. Forming vessels show Egfp-Rac1 expression at 30 hpf. Compressed z-series at 40x magnification,

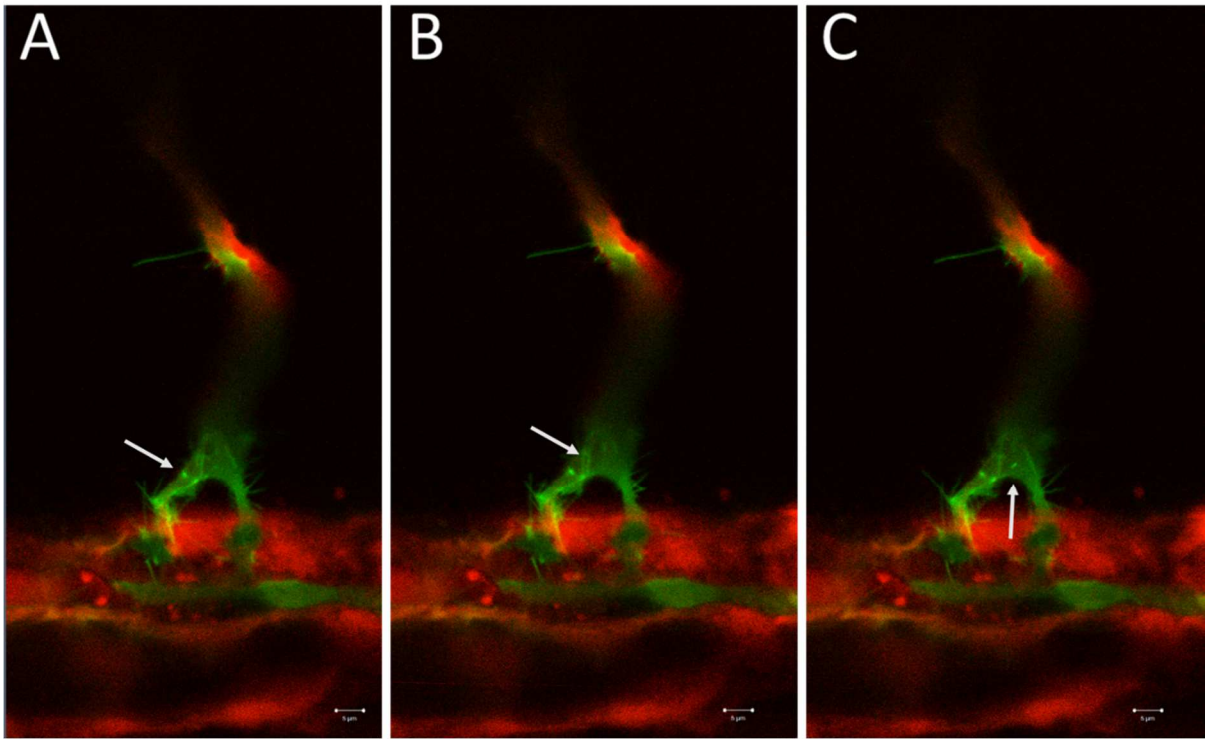

Figure 2: Egfp-Rac1 expression in endothelial cells highlights putative actin dynamics during endothelial tube formation. At 30 hpf at 40 x magnification on confocal microscope, elongated lines of Egfp-Rac1 are consistent with localization to actin and focal adhesions. A, B, C represent green fluorescence from Egfp-Rac1 and uniform red fluorescence from mCherry from *Tg(flk1a:mcherry)* in merged channels. B, and C are 2 and 4 minutes after the first panel, respectively. Scale bar represents 5 microns.

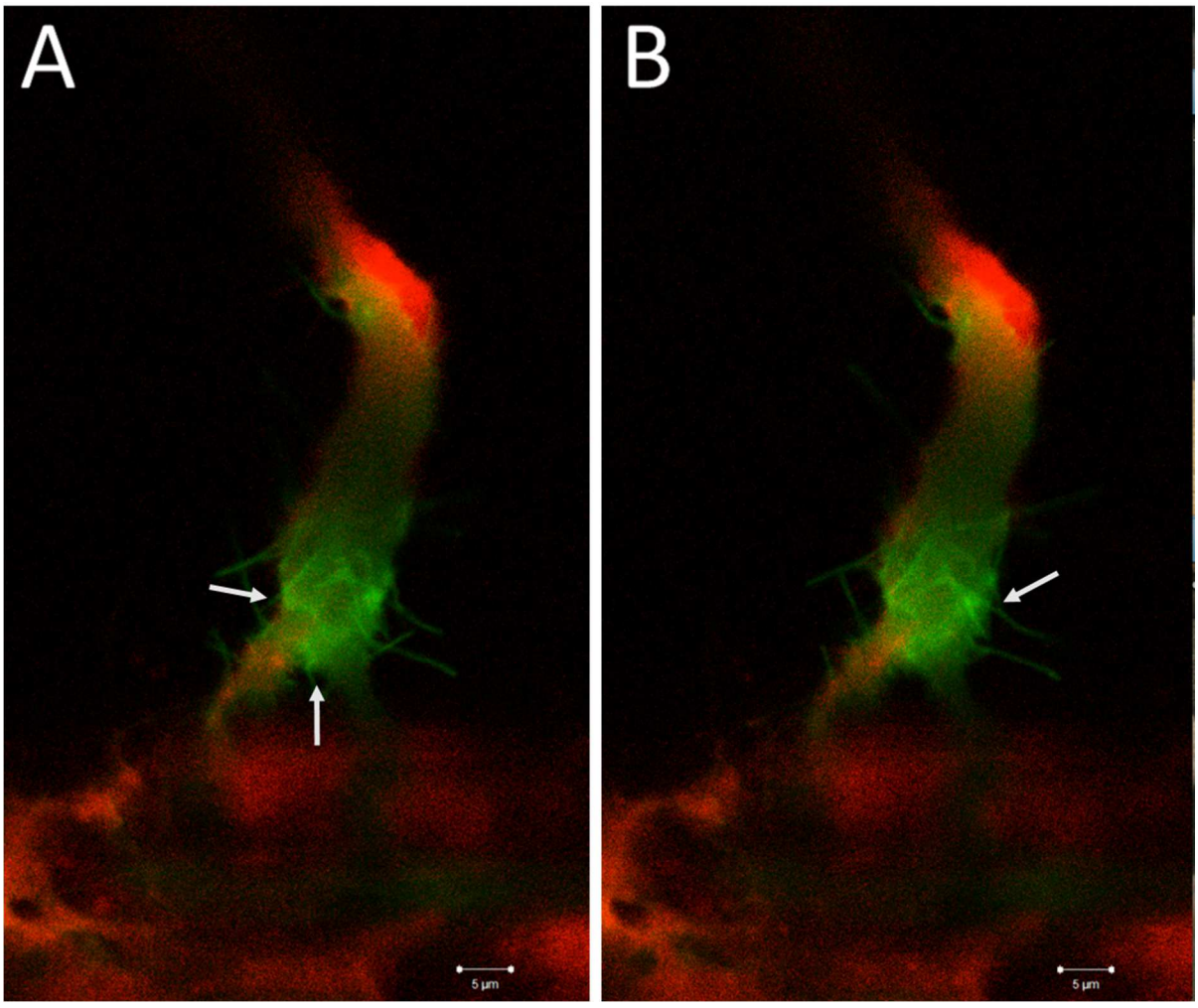

Figure 3: Egfp-Rac1 expression in endothelial cells highlights putative actin dynamics during tube formation. At 30 hpf at 40X magnification on confocal microscope, elongated lines of Egfp-Rac1 are consistent with localization to actin or focal adhesions. A and B represent green fluorescence from Egfp-Rac1 and uniform red fluorescence from mCherry from *Tg(flk1a:mcherry)* in merged channels. B is 2 after the first panel. Scale bar represents 5 microns.

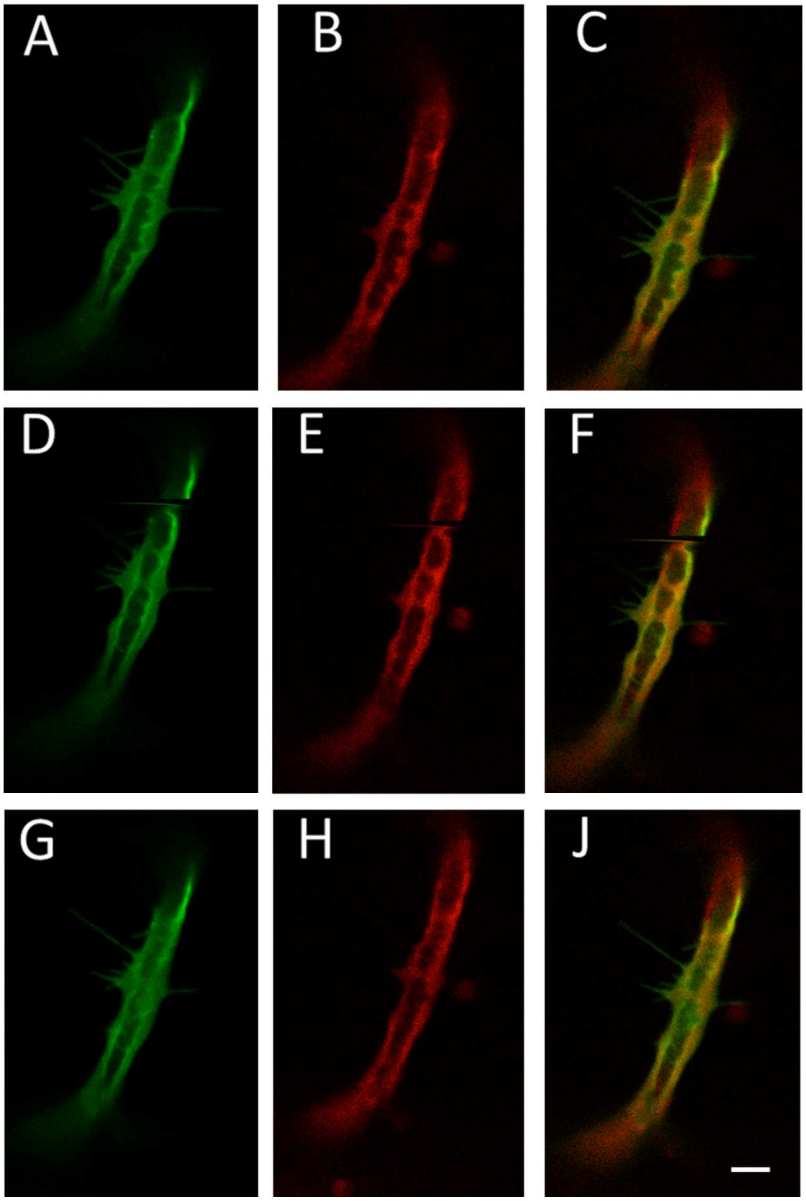

Figure 4: **Egfp-Rac1 expression in endothelial cells highlights putative apical-dynamics in a forming lumen during endothelial tube formation.** At 30 hpf at 40A magnification on confocal microscope. A, B, C represent green fluorescence from Egfp-Rac1 and uniform red fluorescence from mCherry from *Tg(flk1a:mcherry)* in merged channels. D, E, F, and G, H, J are the same vessel in A, B, and C 2 and 4 minutes after the first panel, respectively. Scale bar represents 5 microns.

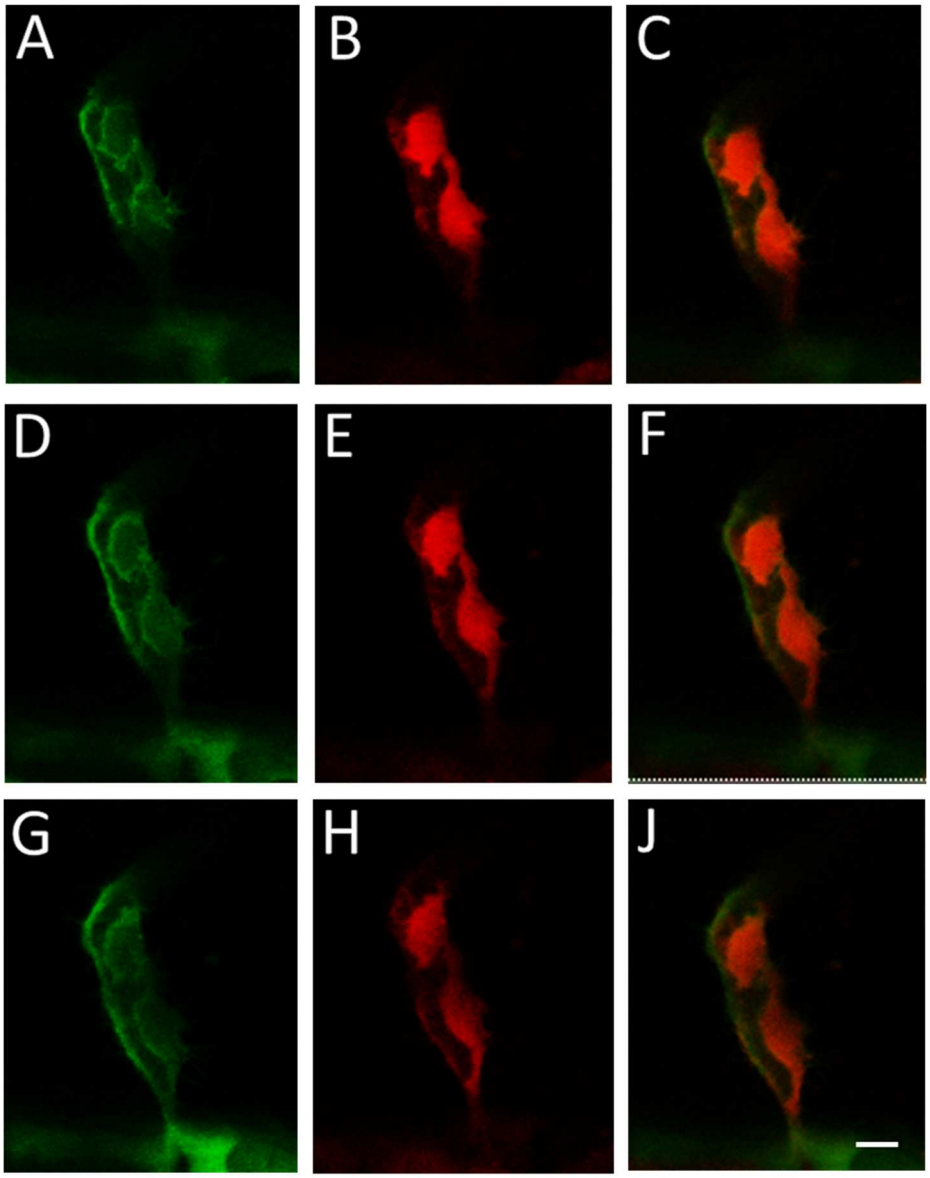

Figure 5: **Egfp-Rac1 expression in endothelial cells highlights putative apical-dynamics in the forming lumen during tube formation.** At 30 hpf at 40 x magnification on confocal microscope. A, B, C represent green fluorescence from Egfp-Rac1 and uniform red fluorescence from mCherry from *Tg(flk1a:mcherry)* in merged channels.. D, E, F, and G, H, J are the same vessel in A, B, and C 2 and 4 minutes after the first panel, respectively. Scale bar represents 5 microns.

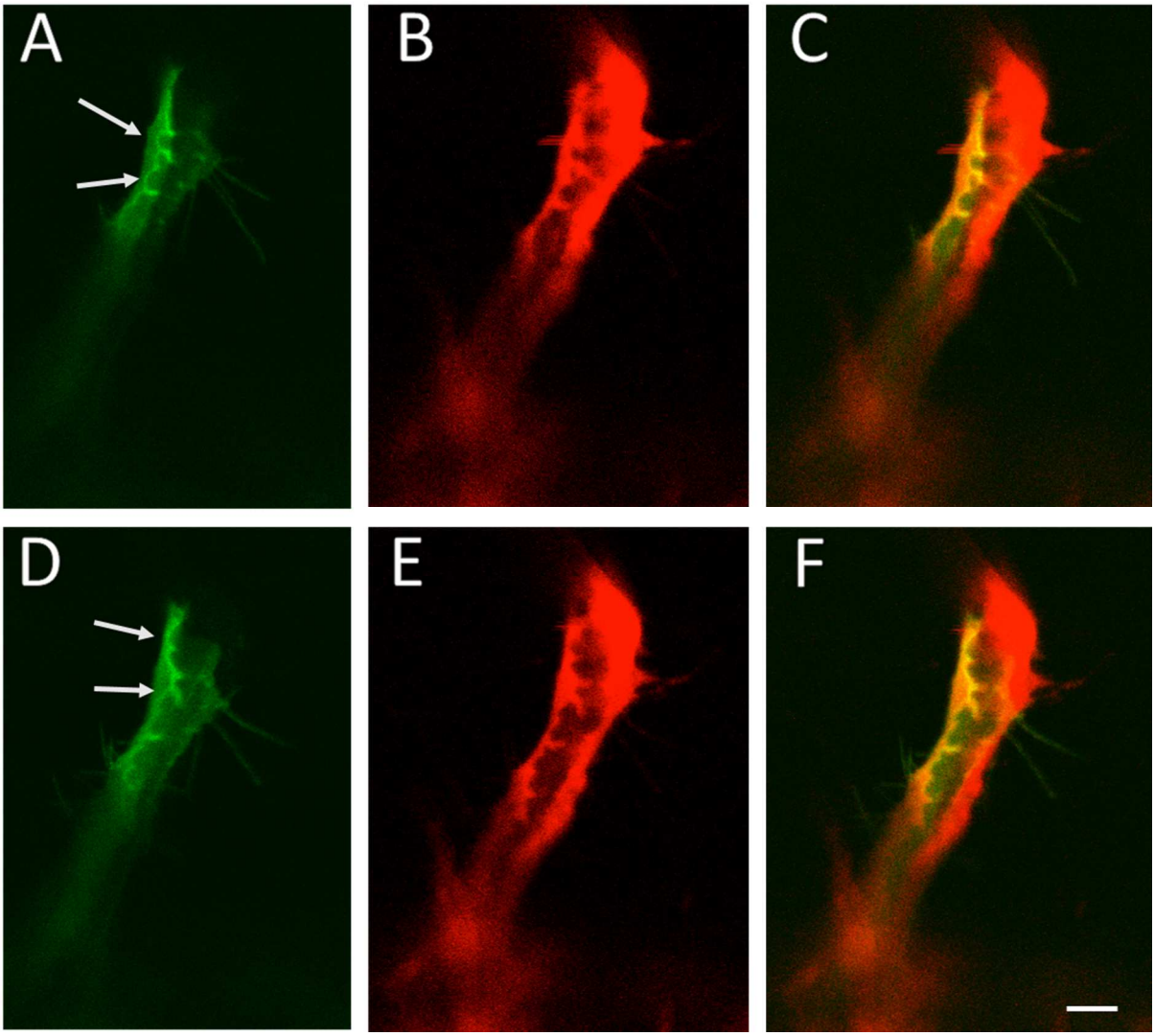

Figure 6: **Egfp-Rac1 expression in endothelial cells highlights putative apical-dynamics in the early forming lumen during tube formation.** At 30 hpf at 40X magnification on confocal microscope. A, B, C represent green fluorescence from Egfp-Rac1 and uniform red fluorescence from mCherry from *Tg(flk1a:mcherry)* in merged channels. D, E, F are the same vessel in A, B, and C 2 after the first panel. Scale bar represents 5 microns.

Table 1. *Tg(UAS:egfp-rac1)* F1 fish screened for germline transmission of GFP.

Screening of Rac 1 founder fish		
Founder	# of embryos	# with GFP
1	~50	0
2	~50	0
3	~200	0
4	~100	0
5	~20	0
6	~170	7 (4.1%)
7	~50	0
8	~400	4 (1%)
9	~100	0

CHAPTER 4

GENERAL CONCLUSIONS

The mismigration observed in the *rab5c* homozygous mutants may be due to improper cell surface signaling and binding. VEGF gives guidance cues to endothelial cells in a pathway that leads to cellular migration and tube formation. Endosomal trafficking, mediated by Rab5c, may play a role in the regulation of VEGF. This could explain why rab5c knockdown leads to mismigration. Rab5c vesicles could also be seen moving back and forth inside the forming lumen in the *Tg(flk1a:egfp-rab5c)* line, which would be consistent with endosomal trafficking. Extensive remodeling of junctions needs to occur during endothelial tube formation, which is likely mediated by the endosome. Although there are some potential lumenal defects in the homozygous *rab5c* mutant, this phenotype was not fully penetrant. This could be a result of genetic compensation and redundancy in the zebrafish genome.

Rho GTPases regulate intracellular regulation of cellular responses to external signaling cues, resulting in actin rearrangements (Poukkula, Kremneva et al. 2011). Actin localization has long been recognized as being important in tube formation, however, the role of downstream cell signaling remains elucidated. The *Tg(UAS:egfp-rac1)* transgenic line will allow us to address the precise roles of actin and Rac1 in this process. This tool will also allow us to examine the dynamics of actin associated with junctions. Since remodeling and expansion of junctions is coordinated with the expansion of the apical membrane, it would be logical that Rac1 localizes to the apical membrane because of its role in maintaining the integrity of adherens and tight

junctions. Junctional remodeling is also controlled by vesicle transport, where Egfp-Rac1 may be localizing.

My work with Rab5c and Rac1 can lead to further defining the roles of these GTPases, as well as highlight novel processes in endothelial tube formation. This work can also lead to better understanding of vascular tube morphogenesis. This is an important process in multiple disease states, and these *in vivo* models can better define the cell biology and genetic requirements of endothelial tubulogenesis.

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