CHARACTERIZATION OF THE CASZ1-DEPENDENT MECHANISMS UNDERLYING VERTEBRATE BLOOD VESSEL DEVELOPMENT

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ABSTRACT

Marta S. Charpentier: Characterization of the CASZ1-dependent mechanisms underlying vertebrate blood vessel development
(Under the direction of Frank L. Conlon)

The vascular system is the first organ system to form during embryonic development. Its proper establishment is required for embryonic growth and survival as well as tissue homeostasis. Blood vessels are comprised of endothelial cells which must coordinate multiple behaviors such as migration, adhesion, sprouting, and proliferation to properly assemble into cord-like structures and then undergo further remodeling and branching to form a patent vascular network. Disruptions in these processes result in a number of human disease states including tumorigenesis, stroke, and atherosclerosis necessitating a better understanding of the factors and pathways that regulate these key developmental steps. Despite the large number of identified growth factors and proteins that are critical for vascular development, the field still lacks a thorough understanding of how endothelial gene programs are regulated. CASTOR (CASZ1) is an evolutionarily conserved transcription factor expressed in the cardiovascular system. Mutations in the human *Casz1* locus have been recently genetically associated with cardiovascular disease risk factors including hypertension and high blood pressure although the mechanisms by which CASZ1 functions in vascular biology are unknown.

Here we demonstrate that CASZ1 is critical for proper vascular assembly and morphogenesis during embryonic development. Mechanistically, we have determined that

CASZ1 directly binds to and regulates the expression of *Epidermal growth factor-like domain 7* (*Egfl7*), an extracellular matrix (ECM) protein previously implicated in vessel sprouting and vascular lumen formation. We further showed that the CASZ1/*Egfl7* transcriptional pathway is required to promote the activation of the RhoA GTPase signaling pathway to directly modulate endothelial cell adhesion, proliferation, and cell shape in order to support proper cellular behavior for vessel development. Moreover, for the first time we have uncovered the molecular and cellular basis for vascular lumen formation in *Xenopus* and show that EGFL7 is required for the proper cell shape changes and resolution of cell-cell junctions necessary to facilitate the transition of cord-like structures into functional vascular tubes. Collectively, this work provides insight into how transcription factors, ECM proteins, and intracellular signaling pathways are coordinated to achieve timely and appropriate assembly of a hierarchically branched and perfusable vascular network.

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LIST OF ABBREVIATIONS

aPKC	Atypical Protein Kinase C
BMP	Bone Morphogenetic Protein
ChIP	Chromatin Immunoprecipitation
CCV	Common Cardinal Vein
Dll4	Delta-like Ligand 4
DA	Dorsal Aorta
DLPM	Dorsal Lateral Plate Mesoderm
DLAV	Dorsal Longitudinal Anastomosing Vessel
EGFL7	Epidermal Growth Factor-Like Domain 7
EC	Endothelial Cell
ECM	Extracellular Matrix
FGF	Fibroblast Growth Factor
FA	Focal Adhesion
HUVEC	Human Umbilical Vein Endothelial Cells
IP	Immunoprecipitation
ISV	Intersegmental Vessels
MEM-GFP	Membrane-Green Fluorescent Protein
miR	MicroRNA
MO	Morpholino
MOI	Multiplicity of Infection
PLA	Palatocerebral Artery

pH3	Phosphorylated Histone H3
PECAM	Platelet Endothelial Cell Adhesion Molecule
p-MLC	Phosphorylated-Myosin Light Chain
PODXL	Podocalyxn-Like Protein
PCV	Posterior Cardinal Vein
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
ROCK	Rho Kinase
Thr	Thrombin
VEGF	Vascular Endothelial Growth Factor
VE-Cadherin	Vascular Endothelial-Cadherin
VVN	Vitelline Vein Network
YSL	Yolk Syncytial Layer
ZO-1	Zonula Occludens-1

CHAPTER 1: INTRODUCTION[1]

The cardiovascular system is one of the first organ systems to arise during embryogenesis. Its proper establishment during early development is critical for meeting the demands of a rapidly growing embryo in need of nutrients, oxygen, and waste removal. Vertebrate blood vessels develop via two distinct mechanisms known as vasculogenesis and angiogenesis. During vasculogenesis, the de novo assembly of new blood vessels, a subset of mesodermal cells differentiates into endothelial cell (EC) precursors called angioblasts. Angioblasts proliferate and migrate to specified positions in the embryo where they coalesce into cord-like structures to form the primary vascular plexus. These early EC behaviors are elicited through growth factor-mediated signaling including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP) (Patel-Hett and D'Amore, 2011). Rapidly thereafter, endothelial cords transform into patent tubes and become further specified to contribute to either the venous, arterial, or lymphatic vasculature (Swift and Weinstein, 2009). The primitive plexus undergoes dramatic remodeling during angiogenesis when new vessels sprout, elongate, and expand to form a complex network of arteries and veins. Henceforth, the majority of vascular development proceeds via angiogenesis, which can also be sub-divided into two processes. In sprouting angiogenesis, new vessels branch from preexisting

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vessels whereas in intussusceptive angiogenesis, existing vessels internally split to form new vessels (Djonov et al., 2000; Eilken and Adams, 2010). In later stages of vascular remodeling, mural cells including smooth muscle cells and pericytes, are recruited to surround the EC-lined tubes and impart vessel stabilization and maturation (Potente et al., 2011).

In the adult, the vasculature is mostly quiescent, with little to no vessel growth or remodeling. However, in response to injury, reduced oxygen or hypoxic conditions, inflammation, tumor growth, or pregnancy, ECs are poised to react and form new blood vessels in a process known as neoangiogenesis. The ability of a cohort of ECs to dynamically assemble into a vascular network is remarkable, given the multitude of individual cellular behaviors that must first be coordinated, including migration, cell-cell and cell-extracellular matrix (ECM) adhesion, proliferation, ECM degradation, and sprouting. Therefore, it is not surprising that dysregulation of these behaviors can have detrimental effects on health and survival; for example, during the pathogenesis of numerous vascular diseases, including atherosclerosis and myocardial ischemia, as well as during tumor progression (Chappell and Bautch, 2010; Potente et al., 2011). Understanding the cellular and molecular mechanisms underlying EC behavior will provide a basis for the development of therapeutics for these diseases.

Over the years, substantial contributions have been made toward understanding the mechanisms underlying sprouting angiogenesis that have significantly impacted the development of therapeutics that promote or inhibit vessel growth in a wide range of diseases (Carmeliet and Jain, 2011a; Said et al., 2013). Considerably less is known about the lumen formation process, critical for establishing a patent and functional vascular system during development as well as

for efficient delivery of therapeutics to their targets during disease treatment. In this review, we will discuss the diverse mechanisms by which the lumens of various vascular beds arise. Although there is some debate as to which of these mechanisms predominate during development, the identification of several molecules and models of lumen formation has without a doubt shed light on this seemingly mysterious process, and has in recent years greatly expanded the number of groups now studying lumen morphogenesis.

Vessel Lumens Arise by Distinct Mechanisms

The ability to culture primary mammalian ECs has significantly enabled the study of cellular behaviors associated with blood vessel assembly, such as proliferation, motility, and sprouting angiogenesis. The recent advent of three-dimensional models has further enhanced the ability to visualize EC morphogenesis into vascular tubes (Davis et al., 2000; Kleinman and Martin, 2005; Nakatsu et al., 2003; Nehls and Drenckhahn, 1995). Specifically, in response to suspension in three-dimensional collagen or fibrin matrices, human umbilical vein endothelial cells (HUVECs) were shown to individually generate luminal spaces via a vacuolation mechanism known as cell hollowing. Live imaging revealed that cells use pinocytosis to internalize the plasma membrane and generate multiple vesicles that coalesce to form an intracellular lumen, defined as an ECM-free space surrounded by a single EC (Davis and Bayless, 2003). Contacts made between adjacent cells facilitate the fusion of vacuoles, resulting in a network of capillary-like tubes (Figure 1.1A). This process depends on signaling between ECs and the ECM via integrins, particularly integrin $\alpha 2\beta 1$ in collagen matrices, and integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ in fibrin matrices (Bayless et al., 2000; Davis and Camarillo, 1996). Although these observations were made in cultured cells in a manipulated environment, the generation of

an intracellular lumen via vacuolation was also observed in zebrafish intersegmental vessels (ISVs) (Kamei et al., 2006).

Recent studies, however, have indicated that the lumen is more often generated extracellularly where it lies between two adjacent ECs connected by junctions, raising questions regarding whether cell vacuolation is the primary mechanism of lumen formation (Figure 1.1B). Also known as cord hollowing, in the mouse dorsal aorta (DA) adjacent ECs are initially in contact with one another via adherens junctions (Strilic et al., 2009). The cells become polarized even before beginning to separate from each other with a number of molecules localizing specifically to the apical surface where the lumen will be generated. The establishment of apicobasal polarity is suggested to further promote cell separation by causing junctions to redistribute away from the cell-cell interface to the periphery. Furthermore, small GTPase signaling was also shown to enhance EC elongation in order to accommodate the luminal compartment in the center (Strilic et al., 2009). In addition to the mouse DA, extracellular lumens also arise in zebrafish ISVs. In contrast to the idea that intracellular vacuoles reside within the ISV ECs and fuse to form a seamless unicellular tube, labeling of multiple junction and polarity markers has in fact revealed that cells first establish apical polarity and form complex junctions that dynamically rearrange to generate a multicellular tube containing an extracellular lumen (Blum et al., 2008; Wang et al., 2010). However, ISV lumens are not generated strictly by cord hollowing as Wang et al. showed that the formation and fusion of vacuoles within ISVs also promote rapid expansion of the luminal compartment (Wang et al., 2010).

It is therefore increasingly clear that vessels arise in a heterogeneous manner and likely use a number of mechanisms to conveniently and efficiently generate lumens. Extracellular lumen formation may be the predominant mechanism by which major vessels such as the DA form, whereas small-calibre vessels like the ISVs and capillaries, which have also been demonstrated to resemble unicellular tubes in rats (Yoshida et al., 1989), may use a combination of both cord and cell hollowing. Nonetheless, analyses of the molecular determinants of extracellular and intracellular lumen formation have greatly contributed to our understanding of the cellular processes that must be coordinated in order to promote this complex step of blood vessel development (Table 1.1).

Vessels Remodel Lumens During Branching and Fusion

The processes described above reveal how the lumen forms de novo and independently of blood flow. More frequently, new vessels are formed during anastomosis, or fusion between two distinct perfused vessels that must combine luminal compartments to give rise to a patent network. The formation of the zebrafish dorsal longitudinal anastomosing vessel (DLAV) occurs via anastomosis between adjacent segmental arteries (Herwig et al., 2011). The DLAV is comprised of both multicellular and, in the majority of cases, unicellular tubes. Using a transgenic line expressing the tight junction molecule zonula occludens-1 (ZO-1) tagged to GFP, Herwig et al. found that multicellular lumens are generated by the rearrangement of junctions between three adjacent cells, resulting in the establishment of new contacts between the outer cells and the merging of local lumens into one continuous luminal compartment. Conversely in most instances, junctions between two cells are not as dynamic. Instead, a lumen is generated within a single cell by invagination of the apical membrane throughout the length of the cell resulting in a unicellular tube with an intracellular lumen. The luminal membrane of this single

cell invaginates through the point of cell-cell contact to ultimately fuse with the neighboring lumen (Herwig et al., 2011).

The presence of unicellular tubes is not unique to the DLAV and is conserved in other zebrafish vessels, such as the palatocerebral artery (PLA) in the cranial vasculature (Lenard et al., 2013). Using a similar fusion process, two distinct lumenized sprouts come into contact with each other, marked by junction markers such as ZO-1, and combine their luminal compartments by apical membrane invagination, resulting in two adjoining unicellular tubes comprised of intracellular lumens. However, later remodeling events reveal that these unicellular vessels are transient, and multicellular tubes take precedence within the PLA. The two cells adjacent to the unicellular tube are initially not in contact with each other but move and redistribute their junctions to form a new contact, thereby forcing the cell comprising the unicellular tube to split to one side of the tube (Figure 1.2). Therefore, an extracellular lumen surrounded by multiple ECs is generated, analogous to the mouse DA lumen assembled via cord hollowing. In both the DLAV and PLA, apical membrane invagination and the subsequent generation of the lumen is dependent on blood flow, likely because of the force generated by increased blood pressure. However, polarity and junctional rearrangements still take place in the absence of flow (Herwig et al., 2011; Lenard et al., 2013).

The presence of vacuole-like structures was not addressed in the above zebrafish studies and hence it is debatable whether vacuole coalescence and apical membrane invagination truly represent distinct mechanisms of lumen formation. However, because in both cases the lumen is completely surrounded by a single cell and devoid of any junctions throughout the unicellular

region, vacuole coalescence and apical membrane invagination can likely be annotated as sub-categories of intracellular lumen formation (Figure 1.1A). It is also unknown what molecular forces govern lumen formation during vessel fusion. Because several distinct cellular behaviors are necessary for successful anastomosis, including migration, sprouting, and adhesion, it may be difficult to tease apart the specific molecular mechanism underlying apical membrane invagination and subsequent cell splitting. It is likely that multiple mechanisms are involved, and that different combinations of interacting factors and intersecting signaling pathways at each step of vascular morphogenesis may elicit more precise cellular responses.

The Establishment of Polarity Initiates Lumen Formation

Distinct Surface Markers Define the Apical and Basal Membranes

Despite the distinction between intracellular, extracellular, and transcellular lumens, a common theme reconciling these differences is that in all cases, cells must first establish polarity to define the apical/luminal ("inside") and basal/abluminal ("outside") vessel surface (Lizama and Zovein, 2013). This specialized distinction between the inside and outside of the tube is not unique to ECs. Multiple other tubular organs such as the kidney and intestine also display apicobasal polarity, and many of the molecular players currently being investigated in vessel lumen formation unsurprisingly have reported roles in epithelial tubulogenesis as well (Rodriguez-Fraticelli et al., 2011).

A number of markers have been established that have preferential localization to either the apical or basal side of EC-lined tubes. Notably, cell-surface transmembrane proteins such as the CD34 sialomucins, consisting of CD34 and podocalyxin-like proteins 1 and 2 (PODXL/PODXL2), and the Ezrin-Radixin-Moesin (ERM) protein moesin are enriched at the

apical membrane (Lampugnani et al., 2010; Nielsen and McNagny, 2008; Strilic et al., 2009; Wang et al., 2010). However, the function of these proteins extends beyond expression at the apical surface. The negatively charged sialic acids coating the extracellular domain of the CD34 sialomucins are required for the electrostatic repulsion needed to separate the adjoining ECs from each other to accommodate the central luminal compartment (Strilic et al., 2010a). Furthermore, in PODXL-null mice, moesin no longer localizes to the apical membrane of the early DA, and only 40% of lumens fully develop (Strilic et al., 2009). ERM proteins modulate cytoskeletal dynamics and signal transduction by anchoring the actin cytoskeleton to various transmembrane proteins (Fehon et al., 2010; Speck et al., 2003). Accordingly, moesin depletion was associated with significantly diminished F-actin enrichment at the apical surface of the developing mouse DA and zebrafish ISVs, ultimately resulting in reduced or failed lumen formation (Strilic et al., 2009; Wang et al., 2010). In addition, the partition-defective (Par) polarity complex (Par3/Par6/atypical protein kinase C [aPKC]) has also been implicated in apical polarity in epithelial and ECs, and disruption of the Par complex is strongly associated with failed lumen formation (Joberty et al., 2000). Depletion of Par3 and Par6 in HUVECs embedded in collagen matrices results in failed intracellular lumen formation, whereas chemical inhibition of PKC in the mouse DA prevents moesin phosphorylation and subsequent lumen generation (Koh et al., 2008; Strilic et al., 2009).

On the basal surface of the vessel, ECs contact various constituents of the ECM that facilitate interactions with mural cells as well as influence whether vessels will become stable and quiescent or "activated" to sprout (Hynes, 2007). Basement membrane markers include fibronectin, laminin, collagen IV, and integrins. A recent study suggests that this specialized

separation of apical and basal membranes in vertebrate blood vessels is a consequence of EC evolution. Unlike vertebrate vessels, the lumen of the aorta in the invertebrate cephalochordate amphioxus, which lacks ECs, is directly lined with the laminin-containing basement membrane implying that ECs serve to separate basal surfaces from the luminal compartment (Kucera et al., 2009; Strilic et al., 2010b).

VE-Cadherin Promotes Proper Localization of Polarity Markers

What is the basis for initiating polarity in the first place? In the case of extracellular lumen formation, ECs first coalesce into cords where they adhere to each other via adherens junctions. Numerous markers, such as β-catenin and N-cadherin, constitute adherens junctions in multiple cell types. Vascular endothelial (VE)-cadherin (also known as Cdh5), however, specifically marks junctions in ECs (Lampugnani, 2012). Far from simply labeling cell-cell junctions, VE-cadherin plays a critical role in vascular morphogenesis. VE-cadherin deficiency in mice results in early embryonic lethality due to severe vascular defects, including failed establishment of yolk sac vasculature, disorganized and disconnected embryonic vessels, and notably, minimal or absent lumens (Carmeliet et al., 1999; Gory-Faure et al., 1999). A functional vascular network also fails to form in VE-cadherin-depleted zebrafish embryos in which vessel lumens are small (or absent) and fail to connect to form a complete circulatory loop (Montero-Balaguer et al., 2009). In the adult, VE-cadherin is essential for maintaining vascular homeostasis and endothelial barrier integrity (Dejana et al., 2008; Dejana and Vestweber, 2013).

Subsequent studies in cultured ECs as well as in vivo have determined that the molecular basis for aberrant lumen morphogenesis in VE-cadherin-deficient animals is associated with impaired establishment of cell polarity. In VE-cadherin null mouse DAs, apical markers such as

CD34, PODXL, and moesin fail to localize to the cell-cell contact, and consequently lumens do not form (Strilic et al., 2009). These results were confirmed in detailed analyses of VE-cadherin-depleted zebrafish where it was noted that moesin was no longer enriched at the luminal surface of the ISVs (Wang et al., 2010). In addition, the distribution of ZO-1-containing junctions was disorganized in VE-cadherin-depleted zebrafish, indicating a role for VE-cadherin in maintaining adherens junctions as well as tight junctions. Tubular HUVEC networks in collagen matrices also display specific localization of apical and basal markers (Lampugnani et al., 2010). However, in the absence of VE-cadherin, PODXL and collagen IV are aberrantly localized with little distinction between apical and basal membranes. Consequently, there are multiple small lumens instead of one central compartment. In addition, members of the Par complex are also absent from cell-cell contacts in VE-cadherin-depleted cells (Lampugnani et al., 2010). Moreover, VE-cadherin has been shown to directly associate with Par3 and Par6 in cultured ECs, strongly suggesting that the interplay between junctional and polarity molecules promotes lumen formation (Iden et al., 2006) (Figure 1.3). Interestingly, VE-cadherin is not required for polarity during PLA anastomosis. Instead, it is needed to stabilize the initial contact made between fusing sprouts, implying diverse roles for this junctional marker during various steps of vascular assembly (Lenard et al., 2013).

Transcriptional Regulation of Lumen Formation

CASTOR (CASZ1)

Transcriptional regulation of blood vessel development has been extensively studied, and numerous transcription factors have been identified that regulate endothelial gene expression (De Val, 2011; Park et al., 2013). However, because many of these factors fall in common families, such as the Ets and forkhead factors, deletion or mutation of individual genes results in little to

no vascular phenotypes, likely due to functional redundancy or cooperative regulation (Barton et al., 1998; De Val, 2011; De Val and Black, 2009; Lelievre et al., 2001). Recent work has provided evidence for a novel transcription factor, CASTOR (CASZ1), in vascular biology (Charpentier et al., 2013a). CASZ1 is an evolutionarily conserved para-zinc finger factor expressed in multiple tissues, including the cardiovascular system, across vertebrate species (Charpentier et al., 2013a; Christine and Conlon, 2008; Zaidel-Bar et al., 2007). Consistently, CASZ1 is required for cardiomyocyte differentiation and subsequent heart formation in *Xenopus* embryos (Christine and Conlon, 2008). Independent of its cardiac function, CASZ1 was recently shown to be required for blood vessel assembly and lumen formation (Charpentier et al., 2013a; Charpentier et al., 2013b). In CASZ1-depleted *Xenopus* embryos, ECs are properly specified but rather than assembling into a dense vascular network, vessels fail to branch and form an incomplete network of thickened cords that predominately run laterally to each other. Furthermore, vessels such as the posterior cardinal veins fail to form lumens. Recent genome-wide association studies have uncovered a genetic link between the human *Casz1* locus and hypertension, implying a role for CASZ1 in cardiovascular disease in addition to development (Takeuchi et al., 2010).

CASZ1 Regulates Expression of ECM Factors During Vascular Development

The molecular mechanisms by which CASZ1 functions in the heart and vasculature remain to be elucidated. Thus far, efforts to identify direct transcriptional targets of CASZ1 have been restricted to the *Xenopus* cardiovascular system. Targets were isolated using a cloning chromatin immunoprecipitation method from dissected cardiovascular-enriched regions (Charpentier et al., 2013a; Charpentier et al., 2013b). One gene that was confirmed to be directly

bound and activated by CASZ1 was *Epidermal Growth Factor-Like Domain 7* (*Egfl7*; also known as *VE-statin*). EGFL7 is a 30 kD protein secreted exclusively by ECs during embryonic development (Fitch et al., 2004; Parker et al., 2004). In the adult vasculature, *Egfl7* levels are significantly reduced, but expression is upregulated in response to neoangiogenesis during injury repair, inflammation, and tumor growth (Fan et al., 2013; Nichol and Stuhlmann, 2012; Nikolic et al., 2010). Parker et al. were the first to demonstrate a role for EGFL7 in vessel lumen formation. In EGFL7-depleted zebrafish, ECs of several primary vessels fail to separate, resulting in disorganized or absent lumens (Parker et al., 2004). Using *in vitro* assays, these authors also showed that primary ECs retain stronger adhesion to substrates such as fibronectin and collagen I compared to EGFL7, thus implying that EGFL7 may provide a permissive environment for cell motility and vascular assembly. These results corroborate the *Xenopus* studies in which depletion of EGFL7 phenocopies depletion of CASZ1, resulting in reduced vessel branching and failed lumen formation (Charpentier et al., 2013a). Furthermore, analyses of transgenic mice expressing *Egfl7* under the Tie2 promoter revealed abnormal EC aggregates, collapsed or uneven lumens, and knot-like structures within the veins, further implicating EGFL7 in vascular patterning and morphogenesis (Nichol et al., 2010). However, the recent discovery of the microRNA miR-126 within the *Egfl7* locus has caused some controversy over the specific function of EGFL7 because many of the earlier phenotypes attributed to deletion of EGFL7 were in fact due to the loss of miR-126 (Kuhnert et al., 2008; Schmidt et al., 2007). miR-126 is required for vascular integrity maintenance and VEGF-mediated angiogenesis, functions similar to, but not identical to, those described for EGFL7 (Fish et al., 2008; Wang et al., 2008). Hence, although EGFL7 acts initially to open vessel lumens early during vascular development, miR-126 may serve to fine-tune later remodeling events and keep patent vascular networks stable.

Interactions between ECs and their microenvironment are clearly significant for eliciting the proper behaviors associated with vascular assembly. However, the specific function of many ECM constituents, including EGFL7, remains unclear. Identification of interaction partners of EGFL7 and potential downstream signaling pathways are only beginning to be illuminated. Through interactions with the lysyl oxidase (LOX) enzymes, EGFL7 has been shown to negatively affect vascular elastogenesis, a process in which elastic fibers are deposited into the ECM and must be regulated to maintain proper blood vessel stretch and resiliency (Lelievre et al., 2008). EGFL7 has also been shown to antagonize Notch signaling in HUVECs, whole mouse embryos, and cultured neurospheres via its interaction with Notch receptors, particularly NOTCH4 (Nichol et al., 2010; Schmidt et al., 2009). Recently, EGFL7 was shown to interact with integrin αvβ3 to promote proper vessel structure and angiogenic sprouting (Nikolic et al., 2013). However, it is still unclear how these interactions contribute to the role of EGFL7 during lumen formation.

The importance of EGFL7 in vascular assembly and morphogenesis is highlighted by current clinical trials in which antibodies against EGFL7 are being tested as tumor growth-suppressing therapies in the treatment of colorectal cancer and non-small cell lung cancer (Parsatuzumab; http://www.gene.com/medical-professionals/pipeline). Understanding the mode of function and affected downstream pathways of such drug targets is beneficial for the potential discovery of new targets and may reveal a link to other pathways for which therapies have been developed, thereby providing potentially efficacious treatment options through combinatorial dosages of anti-EGFL7 and other known growth-suppressing agents.

Small GTPases Modulate the Cytoskeleton to Facilitate Lumen Formation

In addition to cell-cell contact and the establishment of polarity, lumen morphogenesis requires cells to make dynamic contacts with the underlying ECM as well as rearrange their internal actin cytoskeleton to adopt a suitable shape conducive to expansion of the luminal compartment. Directly involved in regulating cell motility and cytoskeletal organization, the Rho family of small GTPases has been at the forefront of cell biology for decades. The number of studies on their downstream effectors, roles in diverse biological processes such as gene expression, proliferation, and membrane trafficking, and implications for human health is continually growing (Burridge and Wennerberg, 2004; Karlsson et al., 2009; Parsons et al., 2010). The most well-known GTPases, RhoA, Rac1, and Cdc42, have been extensively characterized in fibroblasts, where they have critical roles in organizing the actin cytoskeleton during cell migration and mitosis (Maddox and Burridge, 2003; Nobes and Hall, 1995a, b; Olson et al., 1995). It is therefore unsurprising that ECs also depend on GTPase activity for a multitude of cellular behaviors. However, the functions of the Rho GTPases in vascular development and disease are only just beginning to be uncovered, including their roles in lumen formation.

Rac1 and Cdc42 Positively Regulate Lumen Formation

Rac1 and Cdc42 are classically known to promote lamellipodial and filopodial protrusions, respectively, at the leading edge of migrating cells including fibroblasts, ECs, and smooth muscle cells (Nobes and Hall, 1995b; Ridley, 2006; Ridley et al., 1992). Early cell culture studies first showed that Rac1 and Cdc42 have distinct functions in ECs, such as stabilizing cell-cell junctions to regulate vascular permeability and mediating downstream

signaling in response to shear stress (Beckers et al., 2010; Broman et al., 2006; Hoang et al., 2011a, b; Spindler et al., 2010; Tzima et al., 2002; Wojciak-Stothard et al., 2001). Although global Rac1 inactivation in mice results in defects far preceding vascular development, endothelial-specific ablation of Rac1 results in embryonic lethality at slightly later stages with severe defects in angiogenic sprouting and remodeling of secondary vessels (Sugihara et al., 1998; Tan et al., 2008). Genetic inactivation of Rac1 in cultured ECs reinforced the requirement for Rac1 in adhesion, migration, and tube assembly (Tan et al., 2008). Similarly, global ablation of Cdc42 also results in early developmental abnormalities prior to vessel development (Chen et al., 2000), however, mouse embryonic stem cells devoid of Cdc42 fail to assemble into vascular networks during embryoid body differentiation due to impaired directional migration (Qi et al., 2011). Very recently, an endothelial-specific Cdc42-null mouse has been generated and displays severe defects in overall vasculogenesis likely due to increased EC apoptosis (Jin et al., 2013).

Rac1 and Cdc42 have been strongly implicated in promoting formation of epithelial tubes and therefore, have recently been the targets of study during vascular tubulogenesis (Jaffe et al., 2008; Liu et al., 2007; Myllymaki et al., 2011). Indeed, inhibiting Rac1 or Cdc42 activity or expression in HUVECs suspended in collagen matrices prevents cell vacuolation and subsequent lumen formation (Bayless and Davis, 2002; Koh et al., 2008). Furthermore, signaling downstream of Rac1 and Cdc42 that is associated with polarity (Par complex) and cytoskeletal dynamics (Pak2/Pak4) is also required for intracellular lumen formation in collagen matrices (Koh et al., 2008; Koh et al., 2009; Sacharidou et al., 2010). In vivo, Cdc42 localizes to intracellular vacuolar structures in zebrafish ISVs; however, studies to examine whether depleting Cdc42 in these vessels hinders lumen formation have yet to be performed (Kamei et

al., 2006). In a mouse skin model of angiogenesis, retroviral delivery of VEGF and a dominant negative form of Cdc42 decreased the size and number of perfusable vessels whereas the constitutively active form promoted formation of lumens, thereby supporting a positive role for Cdc42 in lumen generation (Hoang et al., 2011b). Rac1 has also been linked to in vivo lumen formation through its interaction with cerebral cavernous malformation-1 (CCM1) protein. In CCM1-depleted zebrafish embryos, although lumens of major vessels form normally, the lumens of the smaller ISVs fail to form, most likely because intracellular vacuoles are not generated (Liu et al., 2011a). In addition, depleting CCM1 in HUVECs inhibits Rac1 activity, implying that GTPase signaling downstream of CCM1 is necessary for lumen morphogenesis in microvessels. Taken together, these studies support a function for Rac1 and Cdc42 in lumen generation, additionally potentiating the hypothesis that intracellular lumen formation may indeed be a mechanism by which small-calibre vessels open and expand.

RhoA Activity Must Be Tightly Controlled During Lumen Formation

The major physiological outputs of RhoA-mediated signaling in various cell types include the formation of focal adhesions between cells and their underlying substrates as well as formation of stress fibers that promote actomyosin contraction (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992). The role for RhoA in lumen morphogenesis has been controversial. Early studies showed that disrupted RhoA function had no effect on intracellular vacuole formation in collagen matrices (Bayless and Davis, 2002; Koh et al., 2008). However, later studies demonstrated that disruption of microtubules using chemical agents, an event known to activate RhoA, results in the collapse of lumens in three-dimensional assays. This is prevented when RhoA activity is inhibited, implying that increased RhoA activity in fact antagonizes the stability of tubular networks (Bayless and Davis, 2004). Furthermore, Xu et al. recently showed

that negative regulation of RhoA signaling via interaction between Ras-interacting protein 1 (RASIP1) and the GTPase activating protein (GAP) Arhgap29 is required for lumen formation (Xu et al., 2011). In the absence of either interacting protein, RhoA activity increases but Rac1 and Cdc42 levels are decreased, in agreement with studies highlighting the requirement for the former two GTPases in lumen formation. Consequently, cultured and mouse embryo ECs are unable to maintain adhesion to the underlying ECM and display polarity defects with improper localization of apical and basal markers. These data strongly suggest that de-regulation of RhoA activity prevents proper cell behaviors associated with lumen formation.

However, results from a number of studies conflict with the hypothesis that RhoA suppresses lumen formation and instead, propose a positive role for RhoA during this process. Introduction of VEGF and a dominant negative form of RhoA in mouse skin resulted in fewer new blood vessels, and in the vessels that did form, most did not contain lumens (Hoang et al., 2004). Further evidence highlighting a requirement for RhoA signaling was demonstrated in the mouse DA, where normal lumen formation proceeds when F-actin and non-muscle myosin II first localize to the apical surface between the two opposing ECs (Strilic et al., 2009). In the presence of a Rho kinase (ROCK) inhibitor, F-actin correctly positions at the cell-cell contact; however, myosin II fails to colocalize with F-actin, thus resulting in defective lumen formation. These results indicate that RhoA downstream signaling through ROCK is required for the proper EC shape changes that facilitate opening of the luminal compartment.

More recently, a study demonstrated that RhoA lies downstream of a transcriptional cascade responsible for promoting proper endothelial cell adhesion, shape, and proliferation to

facilitate vessel assembly (Charpentier et al., 2013a; Charpentier et al., 2013b). The transcription factor CASTOR (CASZ1), previously implicated in cardiac development (Christine and Conlon, 2008) and genetically linked to hypertension and high blood pressure (Takeuchi et al., 2010), and its direct target *Epidermal Growth Factor Like-Domain 7* (*Egfl7*) are required for vessel branching and lumen formation in *Xenopus* embryos (Charpentier et al., 2013a). EGFL7 is an ECM-associated protein exclusively secreted by ECs during embryonic development that has been demonstrated to play a role in vessel morphogenesis (Fitch et al., 2004; Parker et al., 2004). In CASZ1 and EGFL7-depleted HUVECs, RhoA expression levels, and consequently activity, are significantly diminished, resulting in reduced stress fiber and focal adhesion formation associated with contractility defects and loss of adhesion between cells and their underlying substrate. These defects can be rescued by reintroduction of EGFL7 strongly suggesting that the CASZ1/*Egfl7* transcriptional hierarchy is required for proper expression of RhoA and the cellular outputs associated with its activation (Figure 1.4) (Charpentier et al., 2013a). Therefore, these results would favor a model by which RhoA is necessary for eliciting the proper EC behaviors (i.e. cell shape and adhesion) for vascular morphogenesis to proceed. Whether reduced RhoA levels explain the impaired lumen formation in CASZ1 and EGFL7-depleted *Xenopus* embryos remains to be established. However, because these small GTPases likely have roles in numerous processes during vascular development, this is a situation where small animal models can be particularly valuable. Pharmacological inhibition or the use of caged morpholinos to inhibit GTPase activity in specific tissues during a tightly controlled temporal window can be useful in teasing apart these diverse roles (Deiters et al., 2010; Langdon et al., 2012; Morckel et al., 2012). Nonetheless, it is evident that too much or too little GTPase signaling, particularly RhoA, can be detrimental to vascular morphogenesis.

Endothelial Cells Enclose Around a Central Lumen via a Novel Mechanism

The molecular players and pathways discussed above have been deciphered based on two primary mechanisms of lumen morphogenesis: intracellular lumen formation via vacuole coalescence or membrane invagination and the formation of extracellular lumens via cell-cell separation. Nonetheless, characterization of these mechanisms has been confined to limited, albeit informative, vascular contexts, such as cultured ECs suspended in ECM matrices, the mouse DA, and zebrafish ISVs. Recent evidence has elucidated a new mechanism by which lumens form. Helker et al. demonstrated that the zebrafish common cardinal veins (CCVs), which bifurcate from the major posterior cardinal vein, develop a central compartment via a novel lumen ensheathment mechanism (Helker et al., 2013). In this model, specified angioblasts destined to become ECs of the CCV are first positioned in a monolayer on top of the yolk syncytial layer (YSL). These cells then detach from the YSL and migrate to the epidermal side, joining together and shaping a new lumen-containing tube located between the YSL and the epidermis, prior to the commencement of circulation. The cells continue to migrate as a sheet to extend the tube and connect it to the heart inflow tract. Interestingly, although VE-cadherin was shown to be required for detachment and migration of ECs during this process, establishment of apicobasal polarity does not appear to be a prerequisite for lumen formation, based on the absence of the apical marker PODXL2. Because the ECs are not initially in contact with each other during the detachment and ensheathment steps, perhaps defining the apical surface between adjacent cells is superfluous. However, it would be interesting to determine whether CCVs form properly in moesin-depleted fish or other polarity mutants.

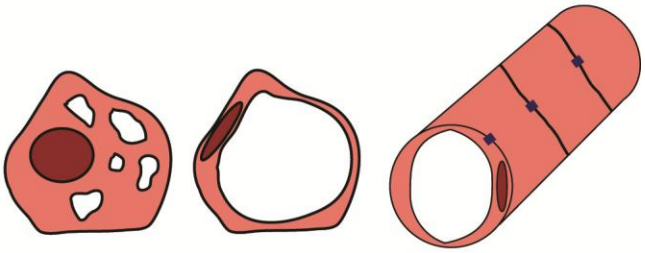
Conclusion and Implications

With the amount of heterogeneity that occurs in different vascular beds throughout embryonic and neonatal development, it would not be surprising to identify additional mechanisms of lumen formation. The assembly of the vasculature through distinct processes such as de novo EC coalescence into cords, angiogenesis, anastomosis, or in response to mechanical forces such as blood flow, may directly influence how the lumen is generated, expanded, and maintained. With advances in imaging technology and more detailed understanding of the spatial and temporal requirements of critical molecules during vascular morphogenesis, the discovery of novel lumen formation mechanisms is very likely (Table 1.1). The need for effective cancer therapies continues to sustain drug development industries, and trials testing the efficacy of anti-angiogenic therapies in controlling tumor vasculature growth are ongoing (Meadows and Hurwitz, 2012). Although the majority of current treatments are aimed at inhibiting vessel growth and expansion in order to starve tumors of oxygen and induce shrinkage, a seemingly contradictory idea know as "vascular normalization" has emerged (Carmeliet and Jain, 2011b). The tumor vasculature is highly abnormal; vessels are tortuous, leaky, uneven, and disorganized. Inducing tumor vessels and ECs to become more "normal" by stabilizing junctions and recruiting mural cells to provide cohesive coverage of EC-lined tubes may lead to improved perfusion of drugs and soluble factors directly to the tumor site as well as reduce the chance of metastasis. Obviously, these outcomes are concomitant with the presence of an impermeable, stable lumen. Thus, a greater understanding of lumen morphogenesis, particularly the molecular players involved, may contribute to new approaches for normalizing tumor vessels.

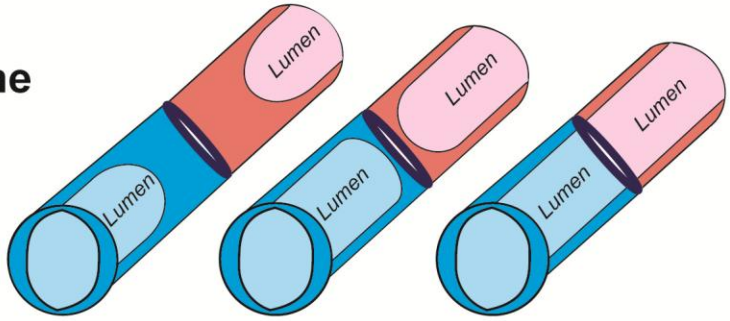
Figure 1.1. Mechanisms of blood vessel lumen formation. (A) Intracellular lumens refer to hollow spaces enclosed by a single endothelial cell and devoid of junctions. Intracellular lumen formation can be sub-divided into two mechanisms. **(i)** During vacuole coalescence (top panel), individual endothelial cells generate vacuole-like structures through membrane internalization. The vacuoles fuse to form a hollow compartment devoid of ECM. Therefore, when vacuolated cells assemble to form a network, contacts between adjacent cells are made (purple rectangles) however no junctions are found interrupting the cell-lumen boundary resulting in a unicellular tube. **(ii)** In apical membrane invagination (middle panel), once two distinct vessels (blue vs. pink) have established contact (purple oval), the luminal compartment is expanded by invagination of the membrane (light blue/pink). The membrane continues to extend through the point of contact resulting in one continuous lumen. Each cell now makes up a unicellular tube containing an intracellular lumen within the patent vessel. **(B)** During extracellular lumen generation (bottom panel), endothelial cells first form adherens junctions between their contact surfaces to generate cord-like structures. These junctions become redistributed from the center of the contact region to the periphery in order to allow a luminal compartment to take shape between the cells. Thus, multicellular tubes are generated in which the lumen is surrounded by two or more endothelial cells that remain in contact with each other as well as with adjacent cells.

A) Intracellular Lumen Formation

i) Vacuole Coalescence



ii) Apical Membrane Invagination



B) Extracellular Lumen Formation

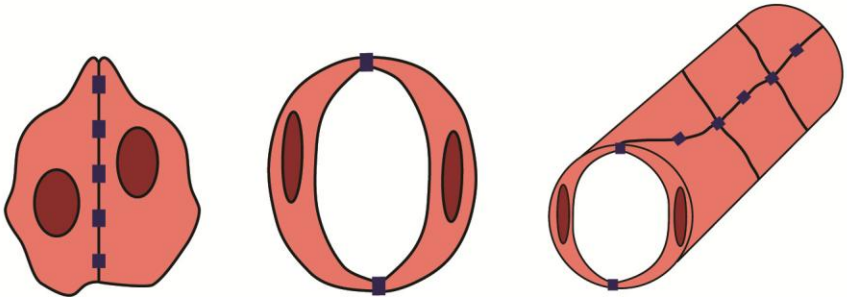


Figure 1.2. Lumen formation during vessel fusion. Unicellular tubes remodel into multicellular tubes by cell splitting. **(A)** Two unicellular tubes (blue and pink) are flanked by multicellular tubes (green/yellow and red/brown) with junctions represented by purple ovals. **(B)** Two cells flanking the blue unicellular tube (yellow and pink) rearrange their junctions and approach each other (black arrows) while the blue cell is forced to split to one side of the vessel (white arrow). **(C)** The yellow and pink cells establish a new contact (gray oval) and the blue cell has narrowed to one side of the vessel incorporating into a multicellular tube. **(D)** The second unicellular tube (pink) is now flanked by the blue and red cells which rearrange their junctions and approach each other (black arrows) forcing the pink cell to the opposite side (white arrow). **(E)** The blue and red cells establish a new contact (gray oval) and the whole vessel is now comprised of multicellular tubes.

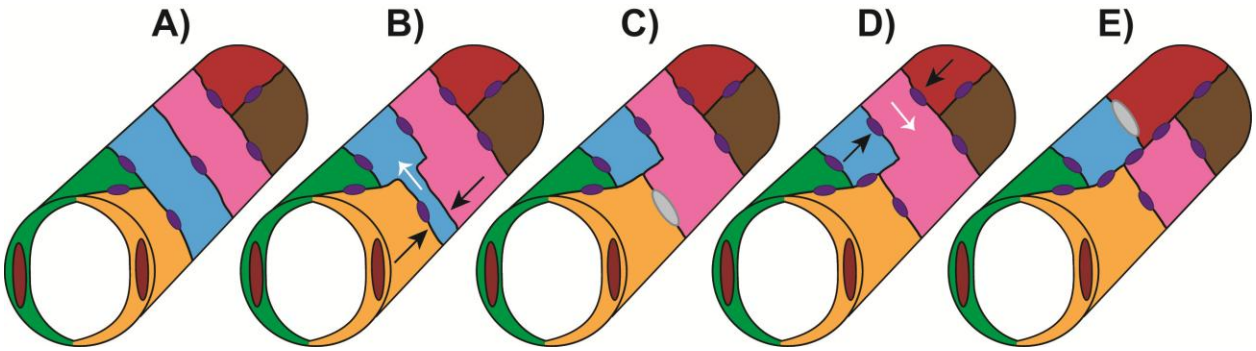


Figure 1.3. Role of VE-Cadherin in establishment of polarity. In the presence of VE-Cadherin (left panel), endothelial cells establish proper polarity with a specified apical surface, as marked by proteins such as the CD34 sialomucins, the ERM protein moesin, and members of the Par polarity complex, and a basal surface marked by ECM constituents and integrins. However, when VE-Cadherin is absent (right panel), apical and basal markers are disorganized and fail to localize to the appropriate surface resulting in smaller or absent vessel lumens.

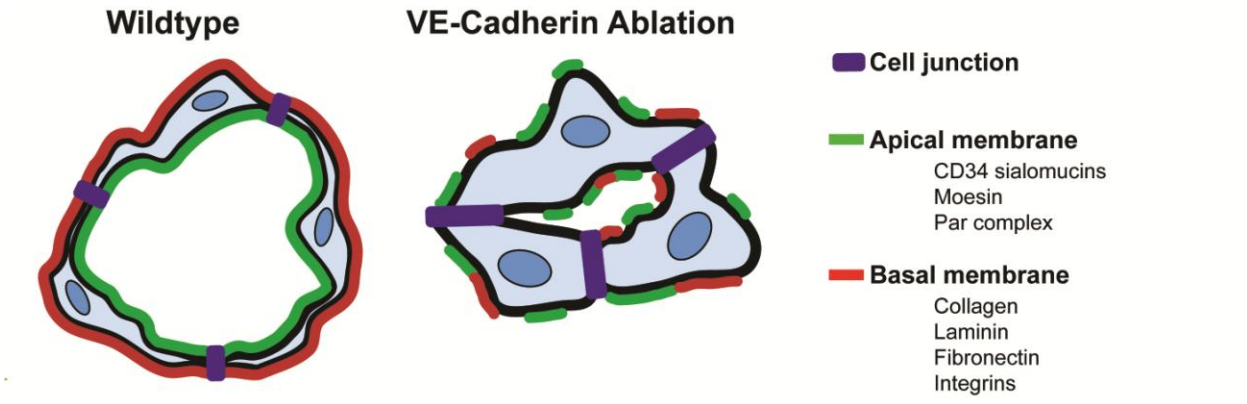


Figure 1.4. RhoA lies downstream of the CASZ1/Egfl7 transcriptional network. (A) The zinc-finger transcription factor CASZ1 binds to and activates expression of *Egfl7* in endothelial cells (top panel). EGFL7 is then secreted to the ECM and by a yet unknown mechanism, promotes RhoA expression and subsequent GTPase activity (star) leading to proper EC shape and adhesion to the substrate (as indicated by blue lines). **(B)** In the absence of CASZ1 (bottom panel), transcriptional regulation of *Egfl7* is dysregulated and *RhoA* expression is diminished resulting in thin, elongated cells that no longer maintain adhesion to the substrate and eventually round up and detach.

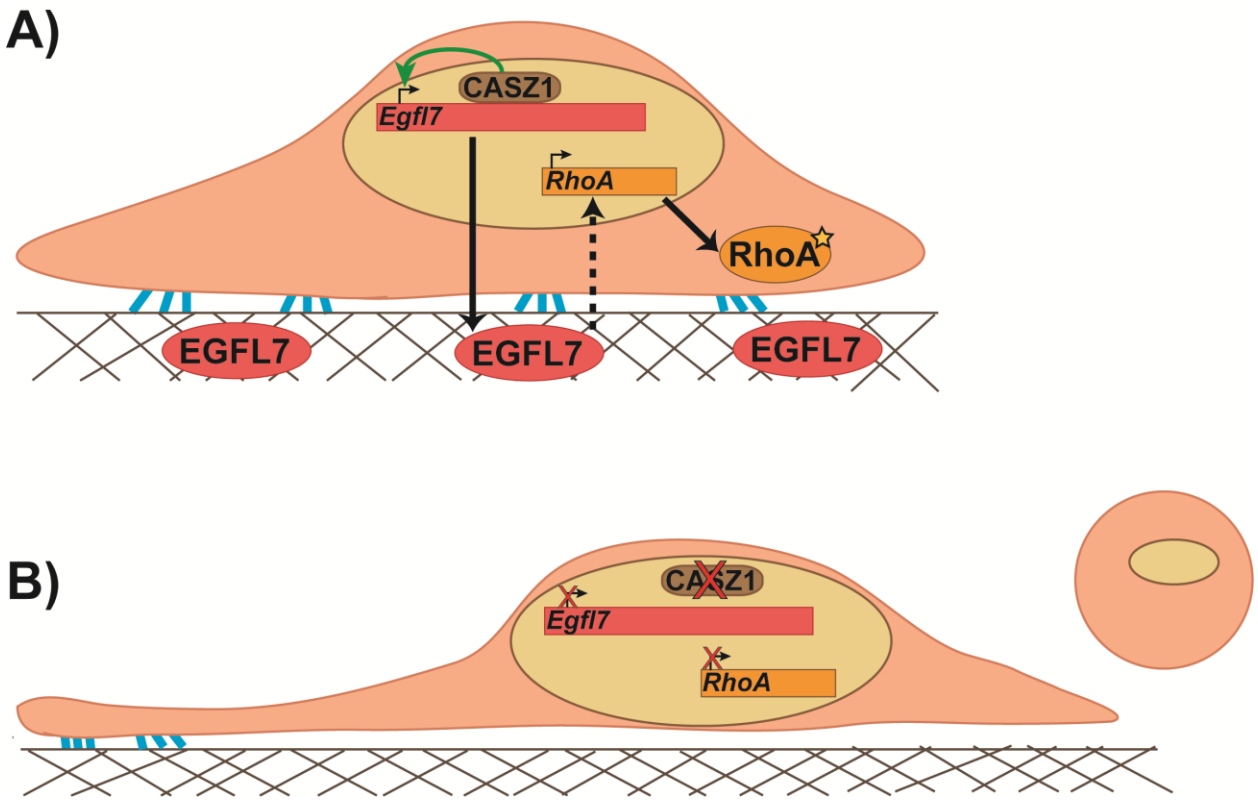


Table 1.1. Molecular determinants that play diverse roles during lumen morphogenesis.

Molecule	Function/Localization during Lumen Formation	Model System	Reference
VE-Cadherin	Localizes to adherens junctions in ECs; Establishes apicobasal polarity	Mouse, zebrafish, cultured ECs	(Carmeliet et al., 1999; Gory-Faure et al., 1999; Lampugnani et al., 2010; Montero-Balaguer et al., 2009; Strilic et al., 2009; Wang et al., 2010)
Par complex (Par3-Par6-aPKC)	Promotes endothelial polarization; Associates with tight and adherens junctions	Mouse, cultured ECs	(Iden et al., 2006; Koh et al., 2008; Strilic et al., 2009; Zovein et al., 2010)
Moesin	Localizes to apical membrane; Recruits F-actin to apical surface	Mouse, zebrafish	(Strilic et al., 2009; Wang et al., 2010)
CD34/PODXL	Localize to apical membrane; sialic acids promote cell-cell separation	Mouse, cultured ECs	(Lampugnani et al., 2010; Strilic et al., 2010a; Strilic et al., 2009)
Rac1/Cdc42	Localize to vacuolar structures; Mediate downstream signaling during intracellular lumen formation	Mouse, zebrafish, cultured ECs	(Bayless and Davis, 2002; Hoang et al., 2011a, b; Kamei et al., 2006; Koh et al., 2008; Koh et al., 2009; Liu et al., 2011a)
RhoA	Positive and negative roles; Modules proper adhesion to ECM and ROCK-mediated shape changes	Mouse, cultured ECs	(Bayless and Davis, 2004; Charpentier et al., 2013a; Hoang et al., 2004; Strilic et al., 2009; Xu et al., 2011)
Rasip1	Regulates RhoA activity with Arhgap29 to promote lumen formation	Mouse, cultured ECs	(Xu et al., 2011)
Casz1	Binds and maintains *Egfl7* expresion in ECs	*Xenopus*, cultured ECs	(Charpentier et al., 2013a)
Egfl7	Secreted into ECM and required for initial establishment of lumens but specific function still unknown	*Xenopus*, zebrafish, cultured ECs	(Charpentier et al., 2013a; Parker et al., 2004)

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CHAPTER 2: CASZ1 PROMOTES VASCULAR ASSEMBLY AND MORPHOGENESIS THROUGH THE DIRECT REGULATION OF AN EGFL7/RHOA-MEDIATED PATHWAY[2]

Introduction

Endothelial cells (ECs) are building blocks for the formation of a functional vascular system during embryonic development. Early stages of blood vessel development occur via vasculogenesis whereby mesodermal cells differentiate into EC progenitors that subsequently proliferate, migrate, and assemble into vascular cords. Cords then undergo tubulogenesis, or lumen formation, continuing to mature by angiogenesis where vessels sprout, branch, and remodel. Vessels then become surrounded and stabilized by pericytes and smooth muscle cells that provide structural support (De Val, 2011; Patan, 2000). The critical nature of these events is emphasized by observations that disruption of these processes is associated with human disease states including cancer, stroke, and atherosclerosis, therefore necessitating a better understanding of transcriptional networks that regulate these key developmental steps (Carmeliet, 2003; Carmeliet and Jain, 2011a; Potente et al., 2011).

While numerous transcription factors have been discovered to regulate endothelial gene expression, relatively few factors are necessary for development. Deletions or mutations of a number of single genes, especially members of Ets and forkhead families, result in moderate to

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no vascular phenotypes likely due to functional redundancy (Barton et al., 1998; De Val, 2011; De Val and Black, 2009; Lelievre et al., 2001). Previous studies have implicated the zinc finger transcription factor CASZ1 in cardiovascular development with depletion of CASZ1 in *Xenopus* embryos resulting in failure of a subset of progenitor cells to differentiate into cardiomyocytes (Christine and Conlon, 2008). Recent genome-wide association studies have shown a genetic link between human *Casz1* and high blood pressure and hypertension, suggesting a possible role for CASZ1 in EC biology (Levy et al., 2009; Takeuchi et al., 2010). However, no studies have addressed expression, function, or transcriptional targets of CASZ1 in vascular tissue.

Despite an essential role for the vasculature in development and disease, our knowledge of molecular mechanisms controlling these events remains incomplete (De Val and Black, 2009). One protein recently shown to play a role in EC morphogenesis is *Epidermal Growth Factor-Like Domain 7* (*Egfl7*), an extracellular matrix (ECM) protein expressed exclusively in EC progenitors and vessels during embryonic and neonatal development. EGFL7 is also expressed in highly vascularized adult organs and is upregulated upon injury (Campagnolo et al., 2005; Fitch et al., 2004; Parker et al., 2004). Depletion studies in zebrafish have defined a role for EGFL7 in tubulogenesis likely through modulation of cell-matrix interactions (Nikolic et al., 2010; Parker et al., 2004). In addition, overexpression of *Egfl7* in mouse results in abnormal patterning of embryonic and postnatal vasculature (Nichol et al., 2010). However, the specific function of EGFL7 has been complicated by recent discovery of microRNA miR-126, the only EC-specific microRNA, within intron 7 of the *Egfl7* locus. While *Egfl7* and miR-126 are co-expressed, the function of miR-126 has indicated a distinct role from its host gene in maintaining vessel

integrity and modulating angiogenesis through repression of targets *Spred1* and *PIK3R2* (Fish et al., 2008; Kuhnert et al., 2008; Wang et al., 2008).

In this study, we demonstrate CASZ1 is required for angiogenic sprouting and lumen morphogenesis. CASZ1 regulates EC contractility, adhesion, and sprouting promoting assembly and tubulogenesis of blood vessels. Furthermore, we demonstrate CASZ1 activates *Egfl7*/miR-126 in mammals and *Xenopus* by directly binding to an intronic element but does not regulate miR-126 in *Xenopus*. Moreover, defects associated with CASZ1-depletion can be phenocopied by EGFL7-depletion, and importantly, rescued by restoration of *Egfl7* levels. We further show CASZ1 transcriptional control of EGFL7 modulates EC behavior through RhoA. Collectively, our studies point to a network whereby CASZ1 regulates an EGFL7/RhoA-mediated pathway to promote vessel assembly and morphogenesis.

Materials and Methods

Xenopus Embryo Collection and Morpholino Design

Preparation and collection of *Xenopus* embryos was performed as described (Showell et al., 2006). Briefly, *X. laevis* eggs were collected after priming females with 10 U of human chorionic gonadotropin (hCG; Sigma) the night before. Eggs were fertilized with the testis of a male and treated with 2% cysteine hydrochloride to remove their jelly coat. Embryos were then injected at the one-cell stage with morpholino, cultured between 16-23° in Modified Barth's Solution (MBS), and staged according to criteria set out in the Normal Table of *Xenopus laevis* (Nieuwkoop and Faber, 1967). Morpholino sequences are as follows:

Casz1 MO (80ng)-[5'TTTATACAATTCAGTCCTACCTGGC] Targets splice donor on Exon 8.

[5' ATCTGCAAGGGAGAATCATTTGCTG] Targets splice acceptor on Exon 9.

Egfl7 MO (40ng)-[5'ACAGCACTAAAGAGAGAGACACAAG] Targets splice acceptor on Exon 6.

miR-126 MO (80ng)-[5'TGACACAGCGCGTACCAAAAGTAAT] Targets primary miR-126 sequence preventing proper processing.

Control MO-[5'CCTCTTACCTCAGTTACAATTTATA] Targets human beta-globin gene.

In Situ Hybridization

Whole mount *in situ* analysis was carried out as described (Harland, 1991) using probes of *Casz1* (Christine and Conlon, 2008), *Msr* (Devic, 1996), *Ami* (Inui and Asashima, 2006), *Erg* (cloned from st. 39 *X. laevis* cDNA), *Egfl7* (cloned from st. 33-38 *X. tropicalis* cDNA), and *EphrinB2* (cloned from st. 39 *X. laevis* cDNA). miR-126 locked nucleic acid probe from Exiqon (38523-05) and hybridization was performed according to manufacturer's instructions at 51°C.

Mouse Immunohistochemistry

After sacrificing time-pregnant dams (E14.5), embryos were isolated in phosphate-buffered saline (PBS), fixed overnight in freshly dissolved paraformaldehyde (4% w/v in PBS), processed through a graded series of sucrose, embedded in OCT, and serial sectioned on a cryostat at 10 µM. Immunohistochemistry was carried out with rabbit anti-Casz1 (Santa Cruz Biotechnology #SC-135453) and rat anti-CD31 (BD Pharmingen #553370) as described (Goetz et al., 2006). Prior to incubation with primary antibodies, slides were pretreated with Sodium Citrate Buffer (pH6.0) for twenty minutes in a steamer (Sunbeam) followed by preincubation with 1% fetal bovine serum (FBS) in PBS. Secondary antibodies (Molecular Probes) used included donkey

anti-rabbit Alexa 488 and donkey anti-rat Alexa 546. Nuclei were visualized using DAPI. Fluorescence was visualized using a Zeiss 700 confocal laser scanning microscope.

Chromatin Immunoprecipitation (ChIP)

Four hundred cardiovascular-enriched regions were dissected from st. 29 *X. tropicalis* embryos and processed for ChIP as reported (Weinmann and Farnham, 2002) (Taranova et al., 2006) with following exceptions: (1)Sonication was carried out with Branson Digital Sonifier at 20% amplitude (2.5 cycles, 30s [1s on/0.5s off]) to yield 4kb DNA fragments. (2)Affinity-purified rabbit anti-CASZ1 polyclonal antibody was added and incubated overnight at 4°C. (3)DNA was digested with *NlaIII* (NEB) and ligated into *SphI*-digested (NEB) pUC19 using DNA ligation kit (Stratagene). Ligated DNA was transformed into NEB10β electrocompetent cells. Transformants were selected by blue/white screening, cultured, and isolated plasmids were sequenced. CASZ1 target DNA sequences were assessed by BLAT analysis using UCSC *X. tropicalis* Genome Browser (http://genome.ucsc.edu, August 2005 assembly). DNA scaffold location coordinates were imported into the annotated Joint Genome Institute. v4.1 database (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). Validation of targets by ChIP PCR was performed as above with following exceptions: (1)Thirty st. 32 *X. tropicalis* embryos were collected and processed as described. (2)Nuclear samples were sonicated at 20% amplitude (5 cycles, 30s [1s on/0.5s off]) to generate ~300bp DNA fragments.

Cloning and Sequencing of *Egfl7* Intronic Regions

The ISB-1 *X. tropicalis* BAC library obtained from Children's Hospital Oakland Research Institute (CHORI) was screened for the *Egfl7* locus using a probe corresponding to *Egfl7* intron 5

that was amplified from *X. tropicalis* genomic DNA by polymerase chain reaction (PCR). Three BAC clones (A20, C10, F23) were identified and *Egfl7* introns 2-4 were amplified from BAC clone A20 by PCR. The introns were subcloned and sequenced to confirm that they truly were *X. tropicalis Egfl7* intronic regions.

In vivo Transcriptional Assays

Egfl7 intronic regions (introns 2,3,4,5) were subcloned into pGL3-Promoter firefly luciferase vector (Promega). *X. laevis* embryos were injected at 1-cell stage with 300 pg reporter plasmid and 10 pg Renilla reporter plasmid in presence or absence of CASZ1 mRNA. Injected embryos were cultured until st. 11.5. Ten injected embryos were lysed in 50 µl Passive Lysis Buffer (Promega) in triplicate. 20 µl of cleared lysates were assayed using Dual-Luciferase Reporter Assay System (Promega).

Cell Culture

Pooled population of HUVEC (Lonza) were maintained in Complete EBM-2 (Lonza) containing 10% fetal bovine serum, 100 U/mL penicillin and streptomycin and used between passages 1-6. hCASZ1 was immunoprecipitated from HUVECs using polyclonal rabbit anti-human CASZ1 (LifeSpan Biosciences) and probed by western blot with same antibody.

Short Hairpin RNA (shRNA)

shRNA viral constructs specific to human *Casz1* and *Egfl7* were obtained from Open Biosystems TRC1 shRNA library. *Casz1, Egfl7,* and control scrambled sequence (Addgene) shRNA lentiviral particles were prepared by UNC Lentiviral Core Facility. 40-50% confluent HUVECs

were infected with $1x10^6$ IU lentivirus combined with 10 µg/mL polybrene (Sigma) for 7.5 hr. Infected cells were placed under 1.5 µg/mL puromycin selection for 3 days and processed for further analysis.

SYBR Green Quantitative RT-PCR

For assessing miR-126 knockdown by morpholino, RNA was isolated from stage 29 embryos using TRIzol (Invitrogen) and purified by phenol:chloroform extraction and ethanol precipitation. miR-126-specific cDNA was synthesized using 100 ng of RNA as described previously (Shi and Chiang, 2005). RNA was isolated from HUVECs using RNeasy mini kit (Qiagen) and cDNA synthesis with 100 ng of RNA was performed as described previously (Christine and Conlon, 2008). Expression levels were assessed using SYBR Green Master Mix (Sigma) on ABI 7900 Fast HT. RPS29 and miR-16 were used as the housekeeping references (de Jonge et al., 2007). Primer sequences can be found in Table 2.1. Following amplification, melting curves were generated to verify presence of a single amplification product. Each sample was analyzed in triplicate with a corresponding minus-RT control. Analysis generated Ct values based on thresholds determined by ABI 7900 software. Efficiency (E) of each primer set was determined based on a serial dilution DNA standard curve where E = 1/-slope. Data was analyzed by the Pfaffl method(Pfaffl et al., 2002) and is represented as relative fold change ±S.E.M.

Live Time-Lapse Imaging

Cells seeded on uncoated or Fibronectin-coated (10 µg/mL) 12-well dishes, or embedded in fibrin gel were imaged over 24 hr using Olympus IX70 inverted microscope encased in Plexiglas

housing to control internal environment (37°C, 5% CO_2 and relative humidity of 60%). Images were collected by Volocity 5.4.1 software.

Immunofluorescence

Cells seeded in chamber slides (BD Falcon) were fixed with cold methanol/acetone, blocked, and incubated with phospho-histone H3 (Millipore) or cleaved caspase-3 (Cell Signaling) overnight. Cells were incubated with rabbit anti-Cy3 (Sigma), stained with DAPI, and mounted (DakoCytomation). For cytoskeletal staining, cells were serum-starved (EBM-2+0.75% FBS) overnight, half of the cells were treated with 10μM Y-27632 (Sigma). Cells were fixed in 4% PFA, permeabilized with 0.1% Triton X-100, and incubated overnight with anti-vinculin (Sigma), and anti-phospho-paxillin (pY118, Invitrogen). Cells were incubated with fluorescent secondary antibodies, stained with FITC-conjugated phalloidin (Invitrogen) and DAPI, mounted and imaged (Zeiss 710 or 700 microscope).

Cell Morphology Quantification

Phase contrast images of live cells were taken at 4x magnification using the Olympus IX81. Cells were measured using ImageJ whereby two measurements of the length of each cell were taken to account for cell curvature followed by one measurement of the width of the cell (*n*=450 cells per condition). The sum of the two length measurements was then divided by the width to calculate the length-to-width (L:W) ratio.

FACS Analysis

HUVECs were grown in 10 cm tissue culture dishes (Greiner CELLSTAR) to subconfluence. Cells were dissociated with trypsin-EDTA (Gibco) and fixation was carried out at -20°C in 70% ethanol for 2 hr. Cells were stained in PBS containing 0.1% Triton X-100, 0.2 mg/mL RNase A (Sigma), and 20 µg/mL propidium iodide for 30 min at room temperature. 7,500-10,000 cells were analyzed on the Dako CyAn using Summit 4.3 software with the aid of the UNC Flow Cytometry Core Facility. Data analysis was performed using ModFit.

Sprouting Angiogenesis Assay

Sprouting assays were performed as described (Nakatsu et al., 2003; Sweet et al., 2012). Cells were stained with phalloidin (FITC) to visualize actin and with DRAQ5 to mark EC nuclei, and imaged (Olympus FLV500 inverted confocal microscope, 20x objective). Assay quantified measuring total length of protruding sprouts and number of branch points.

GST-RBD Pulldowns

Active RhoA-pulldown experiments were carried out as described (Guilluy et al., 2011). Briefly, shRNA-infected cells were serum starved for 16 hr then stimulated for 5 min with 1 U/mL thrombin (Calbiochem) and lysed in 50 mM Tris (pH 7.6), 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% DOC, 10 mM $MgCl_2$, and protease inhibitors (Roche). Lysates were clarified by centrifugation, rotated for 30 min with 30 µg purified GST-RBD (Rho binding domain) bound to glutathione-Sepharose beads (provided by Dr. K. Burridge, UNC-Chapel Hill), then washed twice and subsequently processed for SDS-PAGE. Blots were probed with anti-RhoA antibody (Santa Cruz. 1:250).

Generation of Egfl7 and miR-126 Adenoviruses

For construction of Ad-Egfl7, human *Egfl7* cDNA (Open Biosystems) was removed from pCMV-SPORT6 by digestion with Acc65I and NotI and inserted into the pAdTrack-CMV adenovirus vector (provided by UNC Gene Therapy Center) which drives expression of the inserted gene and GFP by two independent CMV promoters. For construction of Ad-miR-126, a 477 bp portion of intron 7 of human *Egfl7* containing the unprocessed stem loop of miR-126 was amplified from HUVEC genomic DNA and inserted into pAdTrack-CMV adenovirus vector. Adenoviral particles were packaged and generated by the UNC Gene Therapy Center. HUVECs, infected with lentivirus encoding shRNA, were co-infected with 25 MOI Ad-Egfl7 or Ad-miR-126 for 24 hr before being used in downstream assays (e.g. time-lapse imaging). To assess efficacy of Ad-Egfl7, a Western blot was conducted using anti-EGFL7 (Santa Cruz, N-14; 1:250). To assess efficacy of Ad-miR-126, qPCR using miR-126 specific primers was performed as described above.

Western Blotting

Western blots were performed with 50 µg protein using RhoA (Santa Cruz), phospho-myosin light chain 2 (Cell Signaling [Ser 19]), RhoC (Santa Cruz [K-12]).

Statistical Analysis

Data are expressed as means ± SEM as indicated. Statistical analysis was done by Student's t-test and $p<0.05$ was considered significant.

Table 2.1. Oligonucleotides used for quantitative real-time PCR.

Gene	Species	Forward	Reverse
Egfl7	Human	5' GGGATGACTGATTCTCCTCC	5' CACCAGAAGCCACATCAGCAG
Casz1	Human	5' CCTTCTCCAAAACAGACTCC	5' GTTTACATCTTCAGTGACGGC
miR-126	Human/*Xenopus*	5' TCGTACCGTGAGTAATAATGCG	5' GCGAGCACAGAATTAATACGAC
Flk1	Human	5' GAAACTGACTTGGCCTCGG	5' GACCCGAGACATGGAATCAC
RhoA	Human	5' CAGTTCCCAGAGGTGTATGTG	5' GCGATCATAATCTTCCTGCC
RhoB	Human	5' GACACCGACGTCATTCTCATG	5' GATGATGGGCACATTGGGAC
RhoC	Human	5' CTACGTCCCTACTGTCTTTG	5' GCAGTCGATCATAGTCTTCC
RPS29	Human	5' CGGTCTGATCCGGAAATATG	5' CCCGGATAATCCTCTGAAGG

Results

Analysis of *Casz1* expression in *Xenopus* embryos showed transcripts in the vitelline vein network (vvn) at a stage when the network is primarily made up of vascular ECs and devoid of smooth muscle cells (Figure 2.1A)(Christine and Conlon, 2008; Cox et al., 2006; Warkman et al., 2005) suggesting *Casz1* is expressed in vascular ECs. Furthermore, RT-PCR on vascular explants from late tailbud stage embryos (stage 32) demonstrated that *Casz1* is co-expressed in the vitelline vein region with vascular markers, *Msr* and *Erg* (Figure S2.1A). Consistently, we found CASZ1 co-localizes with the EC-specific marker PECAM in blood vessels of mouse embryos (Figure 2.1B). We have also cloned human *Casz1* and showed, as in *Xenopus* and mouse, *Casz1* is expressed in primary human umbilical vein ECs (HUVEC)(Figure 2.1C-D). These results taken together with sequence homology and synteny of CASZ1 across vertebrates (Christine and Conlon, 2008) demonstrate CASZ1 is an evolutionarily conserved transcription factor expressed in ECs.

To ascertain the function of CASZ1 in vascular development, we depleted CASZ1 in *Xenopus* embryos using a morpholino-based approach (Christine and Conlon, 2008). Whole-mount *in situ* hybridization using a panel of EC markers such as *Msr* and *Erg* (Baltzinger et al., 1999; Devic, 1996) demonstrated that at stages when ECs begin to migrate dorsally from ventral blood islands within the trunk region, there were no noticeable differences between control and CASZ1-depleted embryos (stage 29, Figures S2.1B, C, J, K). However, when ECs begin to assemble into cords (stage 32), defects became apparent whereby CASZ1-depleted embryos displayed a dramatically reduced vascular plexus (Figures 2.2A-D, S2.1D-E). Extension of intersomitic vessels from posterior cardinal veins, which is known to occur in *Xenopus* via sprouting angiogenesis in an anterior-to-posterior direction (Cleaver et al., 1997; Levine et al.,

2003), was also absent or significantly delayed in CASZ1-depleted embryos (Figure 2.2A, C, E, G). By mid- to late tadpole stages (stages 36 and 39), vascular networks of CASZ1-depleted embryos were comprised of cords which ran predominately in a dorsal-to-ventral pattern and underwent little to no branching or remodeling (Figures 2.2E-H, S2.1F-I, S2.1L-M). Overall, at stage 36, total length of the vitelline vein vasculature and number of branch points and intersomitic vessels were all significantly decreased in CASZ1-depleted embryos implying a role for CASZ1 in sprouting and remodeling of the vasculature (Figure 2.2I). While our studies focused on vein-derived sprouts, CASZ1-depletion unlikely disrupts arterial/venous differentiation due to proper specification and localization of the aortic arches (arterial) and posterior cardinal veins as well as due to the lack of arterial marker expression in veins (data not shown).

Noting the posterior cardinal veins of CASZ1-depleted embryos appeared thickened (stage 36), we sought to determine the time course of lumen formation in *Xenopus*. At stage 29, control and CASZ1-depleted ECs, as marked by *Msr*, were localized to positions of the future veins but had not yet undergone lumen formation (Figure 2.2J, N). By stage 32, ECs separated in control embryos but remained as aggregates in CASZ1-depleted embryos (Figure 2.2K, O). By stages 36 and 39, the veins of control embryos exhibited well-formed lumens surrounded by *Msr*-positive ECs while CASZ1-depleted veins remained closed and failed to open even at late stages (Figure 2.2L, M, P, Q). Collectively, these studies demonstrate CASZ1 is required for angiogenic remodeling and lumen morphogenesis during vertebrate vascular development.

Since we have previously established CASZ1 is required for heart development (Christine and Conlon, 2008), we determined if the vascular requirements for CASZ1 were secondary to its role in cardiac tissue. Taking advantage of the amenability of *Xenopus* embryos to organotypic culture (Mandel et al., 2010), we removed anterior regions of embryos, including all cardiac tissue at a stage prior to heart formation. Culture of explants showed in the absence of cardiac tissue, control explants formed vascular networks, as marked by the EC-specific gene *Ami* (Inui and Asashima, 2006), that were indistinguishable from un-manipulated embryos, demonstrating blood flow is not essential for correct patterning of the early vasculature (Figure S2.1N, P). Critically, we observed severe defects in vascular networks of CASZ1-depleted explants, strongly suggesting the role of CASZ1 in vascular development is independent of its role in cardiogenesis (Figure S2.1O, Q).

To examine vascular development in more detail and determine if the function of CASZ1 is evolutionarily conserved, we depleted CASZ1 in HUVECs by lentiviral-mediated short hairpin RNA (shRNA, 16-fold decrease in *Casz1* mRNA; Figure 2.3A). CASZ1-depleted cells displayed a thin and elongated morphology in stark contrast to characteristic cobblestone appearance of uninfected HUVECs or HUVECs infected with control shRNA (Figure 2.3B). To determine the precise requirement for CASZ1 and to characterize the dynamics of CASZ1-depleted HUVECs in real time, we coupled time-lapse imaging with quantitative analysis. Time-lapse movies showed that CASZ1-depleted cells initially adhered to plastic or fibronectin-coated substrates but a proportion of cells rounded up and detached (33%, Figure 2.3C, Movie S1, data not shown). Of CASZ1-depleted HUVECs that adhered, live imaging demonstrated the elongated morphology resulted from defects in contractility whereby the leading edge of CASZ1-depleted cells moved

forward without retraction of the trailing edge (Figure 2.3C, Movie S1). CASZ1-depleted cells also stopped dividing (0% shCasz1 vs. 20% control) and consistently, we failed to detect any phospho-histone H3 (pH3)-positive CASZ1-depleted cells (vs. 3% control cells; Figure S2.2A). Using fluorescence-activated cell sorting (FACS), we determined CASZ1-depleted ECs were blocked at the G1/S transition as seen by the significantly reduced number of cells in S-phase (Figure S2.2B). Blockage in G1/S progression was not associated with programmed cell death as determined by cleaved caspase-3 staining (Figure S2.2C). Furthermore, inability of CASZ1-depleted cells to maintain adhesion to the substrate was not a secondary consequence of cell cycle arrest as wildtype HUVECs chemically treated with mitomycin C did not divide yet remained attached (data not shown). Taken together, these results indicate CASZ1 has a conserved role in vascular development and is required for EC adhesion, contractility, and G1/S cell cycle progression.

To assess how adhesive and morphological defects of CASZ1-depleted cells manifested themselves in vessel assembly, we assessed sprouting using a sprouting angiogenesis assay in which HUVEC-coated beads were placed in fibrin. While control cells displayed elongated sprouts with multiple branch points, similar to our findings in *Xenopus*, CASZ1-depleted HUVECs had strikingly few sprouts and branches (Figure 2.3D). Live time-lapse imaging further showed that CASZ1-depleted cells extended out from beads to initiate sprout formation but then abruptly detached, indicating that CASZ1 is required for proper EC adhesion to promote angiogenic sprouting (Movie S4).

To identify direct cardiac and endothelial transcriptional targets of CASZ1, we generated a CASZ1-specific antibody and performed cloning chromatin immunoprecipitation (ChIP) from dissected cardiovascular *Xenopus* tissue (stages 27-29, i.e. same time and tissue requiring CASZ1)(Figure 2.4A). We identified 110 putative transcriptional targets including *Egfl7*, an ECM protein specifically secreted by ECs shown to be associated with similar cellular processes as we observe for CASZ1; EC adhesion and vessel tubulogenesis (Figure 2.4B, Table S2.2)(Nichol et al., 2010; Nikolic et al., 2010; Parker et al., 2004).

Recently, studies in mouse and zebrafish identified the evolutionarily conserved miR-126 contained within intron 7 of *Egfl7* (Fish et al., 2008; Kuhnert et al., 2008; Wang et al., 2008). We cloned *Egfl7* and miR-126 from *Xenopus* and showed EGFL7 is 47% identical to human and 45% identical to mouse EGFL7, while *Xenopus* miR-126 is completely (100%) conserved between mouse and human (Fitch et al., 2004)(Figure S2.3A). Genome analysis and characterization of *Xenopus* BACs corresponding to the *Egfl7*/miR-126 locus confirmed that like other model systems, miR-126 is located within intron 7 of the *Xenopus Egfl7* locus (Figure 2.4B). Expression analysis revealed *Egfl7* is expressed in all major vessels at all stages of vasculogenesis in *Xenopus*, as reported for zebrafish, mouse, and human (Figure 2.4C, E, G, I) (Fitch et al., 2004; Parker et al., 2004). Though *Egfl7* and miR-126 have been reported to be co-transcribed and co-expressed in the vasculature, we found *Xenopus* miR-126 has an expression pattern distinct from *Egfl7* as well as that reported in other species (Fish et al., 2008; Wang et al., 2008). Most notably, miR-126 expression is initiated in ECs at stage 32, slightly later than *Egfl7* (stage 29), and we observed strong expression of miR-126, but not *Egfl7*, in the developing somites (Figure S2.3B-I). Taken together these data show that the sequence, genomic

arrangement, and vascular expression of *Egfl7* and miR-126 are evolutionarily conserved, however in *Xenopus,* miR-126 has developed additional levels of regulation distinct from *Egfl7*.

Consistent with *Egfl7* being a direct target of CASZ1, analysis in CASZ1-depleted embryos showed *Egfl7* was initiated but not maintained in vascular tissues at all stages analyzed (Figure 2.4D, F, H, J). Consistently, *Egfl7* levels were significantly reduced in CASZ1-depleted HUVEC (3-fold; Figure 2.4K). We further observed a dramatic reduction in miR-126 (9-fold) in CASZ1-depleted HUVECs (Figure 2.4K). These effects were specific as the EC marker *Flk1* was unaltered (Figure 2.4K). However, we did not observe reduction in miR-126 in CASZ1-depleted *Xenopus* embryos at mid- and late-tadpole stages (stages 32-39, Figure S2.3E, G, I). Collectively, these studies demonstrate regulation of *Egfl7* and miR-126 expression in human ECs is dependent on CASZ1 while *Xenopus* miR-126 appears to have evolved a CASZ1-independent mechanism of regulation.

To determine whether CASZ1 directly regulates *Egfl7*, we performed *in vivo* transcriptional assays where single copies of intronic regions of *Egfl7* corresponding to the putative CASZ1-bound region (introns 2-5) were placed upstream of a basal promoter driving luciferase activity. Of these intronic regions, only the 5' half of intron 3 (*E1*) resulted in a dose-dependent, reproducible increase in transcriptional activity in response to CASZ1 (Figure 2.4L-M). To determine if CASZ1 endogenously binds to this element *in vivo* and to further refine the region within *E1,* we performed ChIP of endogenous CASZ1 from early embryos (stage 32), a time point when CASZ1 is required for *Egfl7* expression. Results showed CASZ1 binds to nucleotides 59-173 of intron 3 (*E1.2*) but not the most 5' end of *E1* (*E1.1*)(Figure 2.4N, data not

shown). Thus, CASZ1 directly binds to the *Egfl7* locus *in vivo* and can activate *Egfl7* transcription through a regulatory element within intron 3 of the *Egfl7* locus.

To determine if CASZ1 acts through EGFL7 to regulate EC development, we depleted *Egfl7* in embryos using a morpholino that left miR-126 expression intact (Figure S2.4A-E). Strikingly, EGFL7-depletion resulted in vascular phenotypes that phenocopied CASZ1-depletion (Figures 2.5A-H, S2.4F-S). As with CASZ1-depleted embryos, we found a reduction in density of ECs within the vitelline vein network (stage 32)(Figures 2.5A-D, S2.4F-G, S2.4J-M). Furthermore, while blood vessels did form in EGFL7-depleted embryos, there was significantly reduced branching and intersomitic vessel sprouting at later stages (Figures 2.5E-H, S2.4H-1, S2.4N-Q). Quantification of vitelline vein networks at stage 36 revealed results similar to CASZ1-depletion whereby the total length of the vasculature and number of branch points and intersomitic vessels of EGFL7-depleted embryos was significantly decreased compared to controls (Figure 2.5I). Furthermore, like CASZ1-depleted embryos, and in accordance with reports of EGFL7-depletion in zebrafish (Parker et al., 2004), lumens of posterior cardinal veins of EGFL7-depleted embryos failed to form (Figures 2.5J-M). Morpholinos targeting the Dicer cleavage site of *Xenopus* pri-miR-126 had no effect on *Egfl7* expression (Figures S2.5A-E) and did not result in gross defects in vessel morphology or patterning, in accordance with reports in zebrafish (Figures S2.5F-U)(Fish et al., 2008). Consistently, lumens of posterior cardinal veins of miR-126-depleted embryos were indistinguishable from controls (Figures S2.5V-Y). Taken together these results imply EGFL7 functions downstream of CASZ1 to regulate angiogenesis and vascular remodeling

Similar to our studies in *Xenopus*, time-lapse imaging of HUVECs revealed depletion of EGFL7 by shRNA (shEgfl7, 11-fold decrease in mRNA; Figure 2.5N) phenocopied CASZ1-depletion. Most notably, EGFL7-depleted cells demonstrated similar adhesion and proliferation defects as those of CASZ1-depleted cells (Figure 2.5O, Movie S2) and also displayed an elongated morphology similar to but not as extensive as observed in CASZ1-depleted cells. The sprouting angiogenesis assay further demonstrated similar defects to CASZ1-depleted cells whereby the total length of sprouts and number of branch points were significantly decreased in EGFL7-depleted cells compared to controls (Figure 2.5P). Live imaging showed failure to sprout was due to the inability of EGFL7-depleted cells to maintain adhesion (Movie S5). Results were specific since we observed minimal effect on expression of *Casz1* or *Flk1* (Figure 2.5N). Collectively, these studies show *Egfl7* functions downstream of CASZ1 to control vascular morphogenesis in *Xenopus* and humans. In addition, miR-126 does not function downstream of CASZ1 in *Xenopus* vascular development.

To test if CASZ1 functions through *Egfl7* or miR-126 to regulate vascular development and morphogenesis in human ECs, we assessed if restoration of *Egfl7* or miR-126 levels rescues defects associated with CASZ1-depletion. While 29% of cells infected with shCasz1 rounded up and detached compared to 3% of control cells, infection of CASZ1-depleted cells with adenoviral constructs expressing either human *Egfl7* (Ad-Egfl7) or miR-126 (Ad-miR-126) recovered proper adhesion as only 7.6% and 1% of cells detached, respectively (Figures 2.6A, S2.6A-B, Movie S3). However, restoration of *Egfl7*, but not miR-126, partially rescued EC morphology associated with CASZ1-depletion as measured by length-to-width ratio (L:W)(Figures 2.6B, S2.6C). Restoration of either *Egfl7* or miR-126 failed to rescue proliferation

defects (data not shown). Taken together, these studies demonstrate that CASZ1 acts to control EC adhesion through direct transcriptional regulation of *Egfl7* and miR-126. These data further indicate EGFL7 and miR-126 have distinct functions in regards to cell morphology since EGFL7 but not miR-126 can rescue cell shape changes, and further imply CASZ1 control of G1/S progression occurs by *Egfl7*- and miR-126-independent pathways.

Past studies have provided evidence that two of the physiological outputs of the CASZ1/EGFL7 pathway, cell adhesion and morphology, are directly controlled by activation of Rho GTPases. Specifically, RhoA is required for actomyosin-dependent cell contractility and formation of focal adhesions (FA) (Burridge and Wennerberg, 2004; Katoh et al., 2011; Parsons et al., 2010). To identify mechanisms that mediate the physiological function of the CASZ1/EGFL7 pathway, we aimed to determine the relationship between CASZ1/EGFL7 and RhoA. We found RhoA activity and levels were dramatically diminished in CASZ1 and EGFL7-depleted cells, however, levels of other Rho proteins such as RhoC were unaltered (Figure 2.6C, S2.6D-E). The decrease in RhoA protein occurred at the transcriptional level as RhoA but not RhoC mRNA levels were markedly diminished in both CASZ1 and EGFL7-depleted ECs (Figure 2.6D). However, RhoB levels were slightly increased possibly indicating a compensatory mechanism for loss of RhoA (Figure 2.6D).

We reasoned reduction of RhoA expression in CASZ1- and EGFL7-depleted cells would result in concomitant impaired downstream activity. Consistently, phosphorylation of regulatory subunit of myosin light chain (p-MLC) was decreased in CASZ1-depleted cells (Figure S2.6F). To further assess whether decreased RhoA activity directly plays a role in the adhesion and cell

shape defects we observe in CASZ1-depleted cells, we analyzed a panel of cytoskeletal and FA markers. Control cells displayed discrete bundles of F-actin stress fibers, as assayed by phalloidin staining, that contained short or punctate bands of FA markers vinculin and phosphorylated paxillin (Figure 2.6E, left panels). However, CASZ1-depletion mimicked treatment with the Rho kinase (ROCK) inhibitor Y-27632 resulting in diffuse actin networks devoid of stress fibers and failure of vinculin and phosphorylated paxillin to properly localize to adhesion sites on the periphery of the cell (Figure 2.6E, middle panels)(Narumiya et al., 2000). This phenotype was partially rescued by restoration of *Egfl7* levels in CASZ1-depleted cells (Figure 2.6E, right panels). Taken together these studies show CASZ1 acts through the direct transcriptional regulation of EGFL7 to control EC adhesion and shape via the RhoA pathway.

Discussion

In this work we demonstrate an essential and conserved role for the CASZ1/EGFL7/RhoA pathway in vascular patterning. Collectively, these data support a mechanism whereby CASZ1 directly binds to and maintains expression of *Egfl7* in ECs. EGFL7 is then localized extracellularly where it modulates signals between ECs and the underlying ECM. This in turn is responsible for transcriptional upregulation and activity of the RhoA GTPase which ultimately mediates EC contractility and adhesive properties to promote assembly, lumen formation, and functionality of the vasculature (Figure 2.7).

During embryogenesis, ECs assemble into cord-like structures that undergo further remodeling to form the primary vascular plexus. We have shown CASZ1 acts through the direct transcriptional regulation of EGFL7 to promote two critical processes during this period of embryogenesis: sprouting angiogenesis and lumen morphogenesis. The presence of thickened

cords lacking lumens in CASZ1-depleted embryos, together with the observation that CASZ1-depleted ECs fail to sprout properly or adhere to extracellular substrates, strongly implies that CASZ1 functions through the EGFL7/RhoA pathway to maintain proper EC adhesion during vessel assembly.

RhoA has been shown to be a central component of pathways which facilitate vascular remodeling. In this regard, RhoA has been demonstrated to be required for stress fiber formation, acting through the actomyosin contractile machinery, and for FA formation (Burridge and Wennerberg, 2004; Chrzanowska-Wodnicka and Burridge, 1996; Katoh et al., 2011; Nobes and Hall, 1995b). In accordance with CASZ1 acting through RhoA to promote proper EC adhesion, we find that depleting CASZ1 phenocopies inhibition of RhoA and leads to a lack of discrete stress fibers, decreased myosin II activity, and strikingly, the absence of FA markers at sites of substrate contact. Moreover, these alterations in EC adherence can all be rescued in a CASZ1-depleted background by restoration of *Egfl7*.

The role of RhoA in tubulogenesis has been controversial. While reports have demonstrated RhoA activity is central for lumen formation, for example in the formation of the mouse dorsal aorta (Strilic et al., 2009), other reports have shown that in culture RhoA is not required for lumen formation but rather, for maintenance of patent vessels (Bayless and Davis, 2002). However, the *in vivo* relevance of the latter observation is yet to be determined since the mechanism by which lumens arise in 3D collagen culture differs significantly from that of cord hollowing *in vivo* (Davis et al., 2007; Strilic et al., 2009). Paradoxically, it has been recently demonstrated RhoA and myosin II activity must be suppressed to promote lumen formation of

mouse vessels via negative regulation by Ras interacting protein 1 (RASIP1) (Xu et al., 2011). Collectively these studies along with our present findings imply that a precise level of RhoA activity is required for lumen formation with either too low or too high activity leading to failure of vascular cords to hollow and form lumens.

The finding that RhoA protein and mRNA expression are significantly diminished in CASZ1 and EGFL7-depleted HUVECs indicates transcriptional control of RhoA is impaired. Very few studies to date have focused on how RhoA transcription is controlled. While EGFL7 has been shown to interact with lysyl oxidases responsible for vascular elastogenesis as well as some Notch receptors on both ECs and neural stem cells, it is still unclear how these interactions may be influencing RhoA transcription or activity (Lelievre et al., 2008; Nichol et al., 2010; Schmidt et al., 2009). However, our results would support a mechanism by which CASZ1 modulates RhoA signaling through EGFL7 as restoration of *Egfl7* levels in CASZ1-depleted cells rescues adhesion, proper FA marker localization, and stress fiber formation. It was recently shown that RhoA is directly activated by the Myc-Skp2-Miz1-p300 transcriptional complex (Chan et al., 2010). Given a role for c-Myc in embryonic vascular development and later angiogenic remodeling (Baudino et al., 2002; Kokai et al., 2009; Rodrigues et al., 2008), it will be interesting to investigate whether CASZ1 transcriptional regulation of *Egfl7* directly plays into Myc-mediated transcriptional control of RhoA.

Studies on *Egfl7* and miR-126 have identified a 5.4Kb upstream sequence that is sufficient to drive EC-specific expression of a reporter gene in mouse, and mutation of two evolutionarily conserved Ets binding sites within this element eliminated expression in culture

(Wang et al., 2008). We have demonstrated CASZ1 is required for expression of *Egfl7* where CASZ1 is endogenously bound to intron 3 of the *Egfl7* locus in developing embryos and depletion of EGFL7 phenocopies CASZ1-depletion in embryos and HUVEC. Consistently, we note CASZ1 is not required for onset of *Egfl7* expression in embryos but rather its maintenance. Based on our studies, we favor a model by which Ets factors activate *Egfl7* via an upstream element and CASZ1 binds to intron 3 and functions to maintain *Egfl7* levels. This model is complementary to findings for the role of Ets factors in regulating the spatial pattern of *Egfl7* in mouse (Wang et al., 2008).

Our finding that EGFL7 and miR-126 are both regulated in a CASZ1-dependent manner in humans is congruent with reports in mouse. However, despite complete conservation of miR-126 across species, we also demonstrate miR-126 has undergone an evolutionarily divergent and unique means of regulation exemplified by our findings that *Xenopus* miR-126 is expressed in domains mutually exclusive to *Egfl7* (i.e. somites) and expression of *Xenopus* miR-126 in the vasculature is CASZ1-independent. These observations are broadly consistent with recent reports that miR-126 transcription can occur independently of host gene transcription through differential intronic promoters (Monteys et al., 2010).

The vascular system arises via concerted efforts of individual ECs to harness their unique behaviors to assemble into tubular structures. The molecular and cellular mechanisms by which the vasculature arises are still unclear but we have identified previously unknown roles for CASZ1 in regulating sprouting and morphogenesis. While endothelium becomes stabilized and quiescent after embryonic development, vessels retain sensitivity to changes in environment due

to injury, inflammation, or improper cardiac output thus making them susceptible to vascular dysfunction. In this regard, it is interesting that human CASZ1 has been linked to adult vascular diseases such as hypertension (Takeuchi et al., 2010). Events resulting in vascular dysfunction during this disease are associated with aberrant ECM remodeling, proliferation, and adhesion, all of which we have demonstrated to also be dysregulated upon depletion of CASZ1 (Lemarie et al., 2010). Therefore it will be intriguing to examine mechanisms by which CASZ1 itself is regulated and identify additional transcriptional targets that could trigger development of innovative therapeutic strategies in cardiovascular disease.

Figure 2.1. CASZ1 expression in vascular ECs is evolutionarily conserved. (A) *In situ* analysis of *Casz1 Xenopus* embryos (stage 41). Lateral view with anterior to left. *Casz1* is expressed in vascular structures including vitelline vein network (vvn, enlarged panel on right). **(B)** CASZ1 (green) co-localizes with PECAM (red) in neural blood vessels of E14.5 mouse embryos. **(C)** RT-PCR analysis of human *Casz1* in HUVEC cDNA. *Gapdh* was used as loading control. **(D)** Immunoprecipitation (IP) of CASZ1 from HUVECs. Control lane (left) represents IP with no antibody (Ab). Arrowhead represents 125kD human CASZ1.

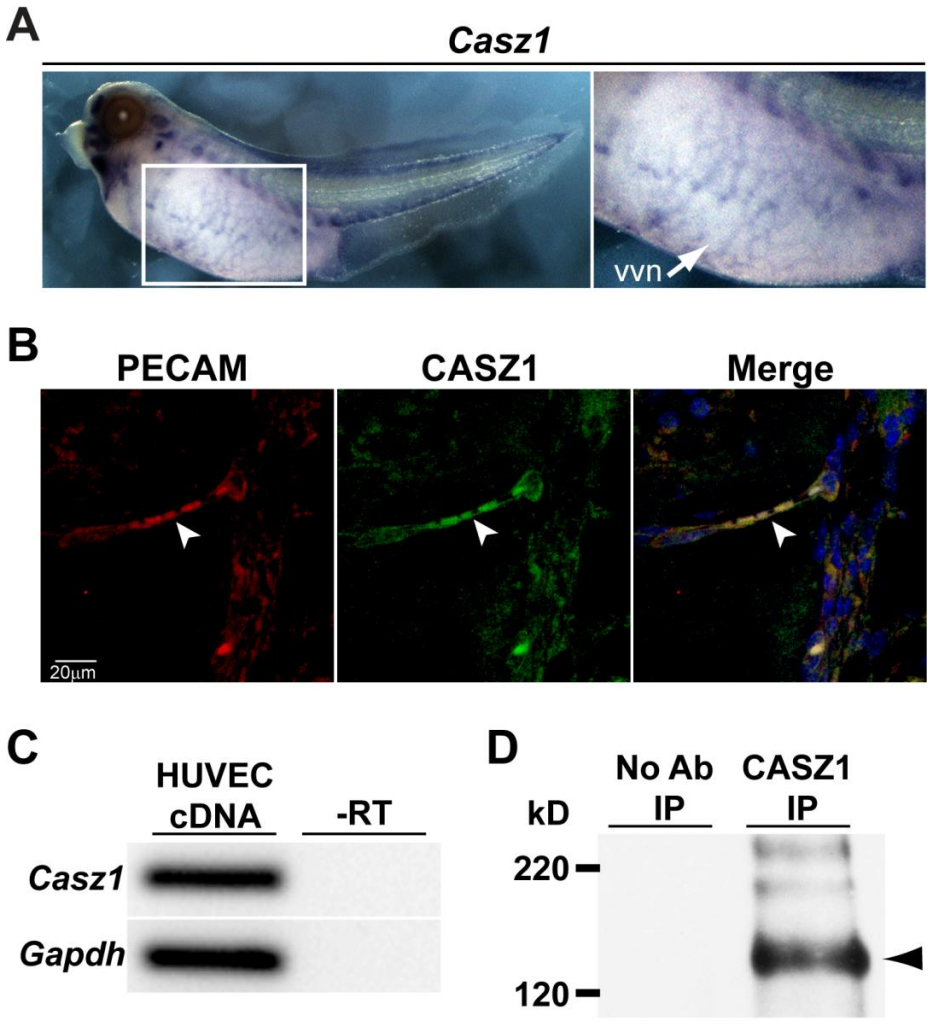


Figure 2.2. CASZ1 is required for vascular development and lumen formation. (A-H) *In situ* analysis with EC marker *Msr* control and CASZ1-depleted embryos (stages 32-36; lateral view with anterior to left). Vessel patterning and branching within vitelline vein network (vvn) are severely compromised at stage 32 in CASZ1-depleted embryos (A,C high magnification of vvn in B,D) and stage 36 (E,G high magnification of vvn in F,H). Note poor sprouting of intersomitic vessels (isv) in CASZ1-depleted embryos at both stages (enlarged box in A,C,E,G). (*n*=10 embryos/condition/stage, 3 independent experiments). **(I)** Quantification of vascular defects in control and CASZ1-depleted embryos (stage 36) representing combined total length of vessels, number of branch points within vvn, and number of isvs/embryo, respectively. Data represent mean ± SEM. (*n*=7 control and 10 Casz1 MO embryos). *: $p<0.05$; **:$p<0.01$; ***:$p<0.001$. **(J-Q)** Histological analysis illustrates time course of lumen formation in *Xenopus* from stages 29-39 (J-M). Note posterior cardinal vein (pcv) lumens begin to open between stages 32 and 36 in control embryos (K,L) but fail to form in CASZ1-depleted embryos (N-Q). Dorsal is top, ventral is bottom. Arrowheads correspond to positions of pcv which are enlarged in lower panels (*n*=2-5 embryos/ condition/stage).

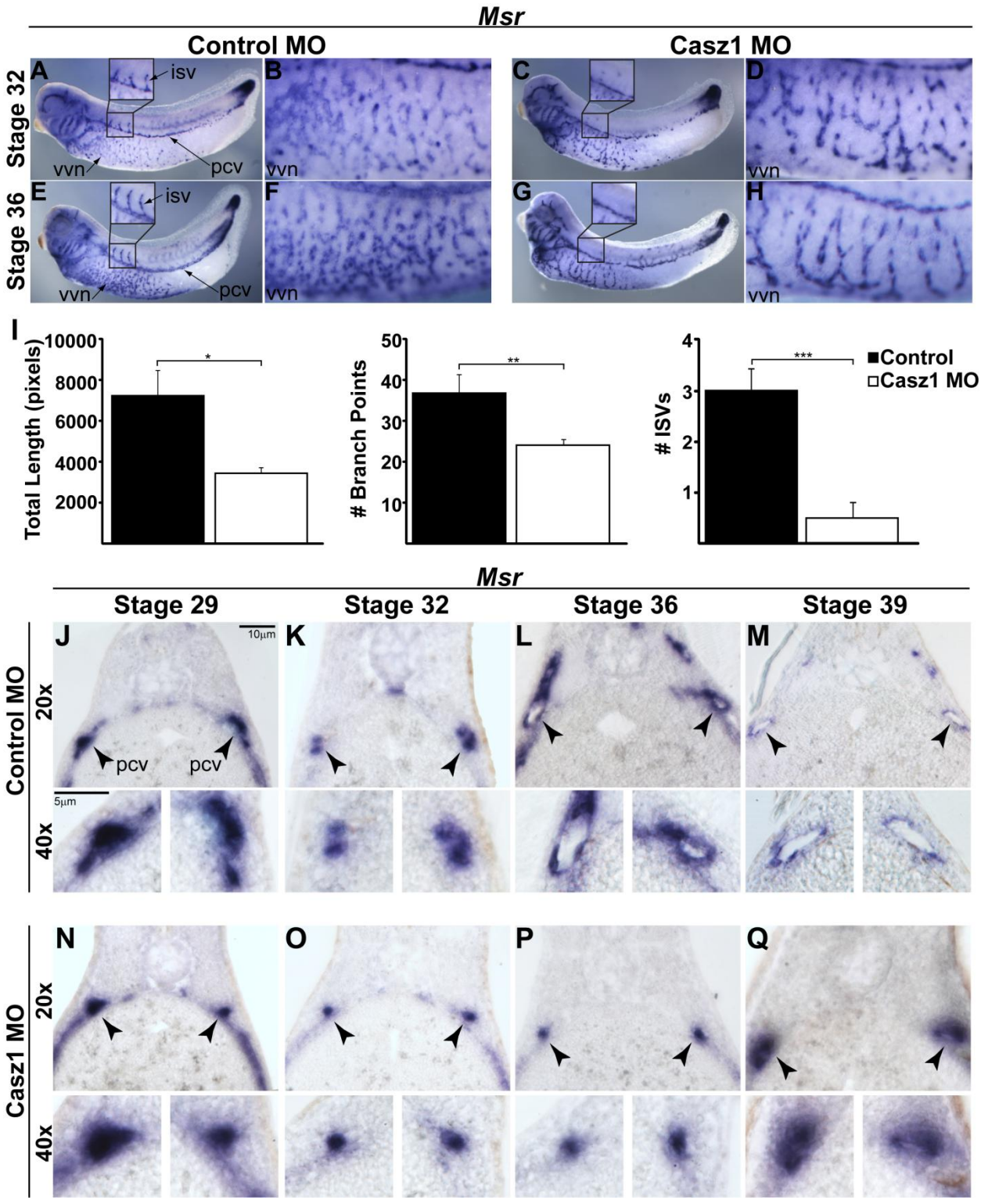

Msr
Control MO
Casz1 MO
Stage 32
Stage 36
A
B
C
D
E
F
G
H
isv
vvn
pcv
I
Total Length (pixels)
Branch Points
ISVs
Control
Casz1 MO
Msr
Stage 29
Stage 32
Stage 36
Stage 39
Control MO
Casz1 MO
20x
40x
J
K
L
M
N
O
P
Q
10μm
5μm

Figure 2.3. CASZ1 regulates EC behavior. (A) mRNA levels of *Casz1* after infection of HUVECs with shCasz1. *Casz1* is decreased by 16-fold. mRNA levels relative to *Rps29* ± SEM. ***: p<0.001. **(B)** Phase contrast images of control and shCasz1 HUVECs. Controls display cobblestone-like morphology while CASZ1-depleted cells are thin and elongated (red arrowheads for examples of each). **(C)** Time-lapse images of control and shCasz1 cells. Red arrowheads represent dividing control cell and rounded up CASZ1-depleted cell. Minutes elapsed presented at bottom right, taken every 5 min for 24 hr. Red arrow refers to elongated shCasz1 cell with trailing edge defects. Graph represents quantification of cells that round up and detach during imaging. Data represent mean ± SEM of 3 experiments conducted on independent batches of shRNA-infected cells (*n*=200 cells). **:p<0.01. **(D)** Sprouting angiogenesis assay was performed with control and CASZ1-depleted HUVEC. On day 6, cultures were fixed and stained for phalloidin (green) and DRAQ5 (blue). Graphs represent mean ± SEM of total sprout length and number of branch points/bead (*n*=11 beads/condition). Experiments were repeated twice on independent batches of shRNA-infected cells. **:p<0.01; ***p<0.001.

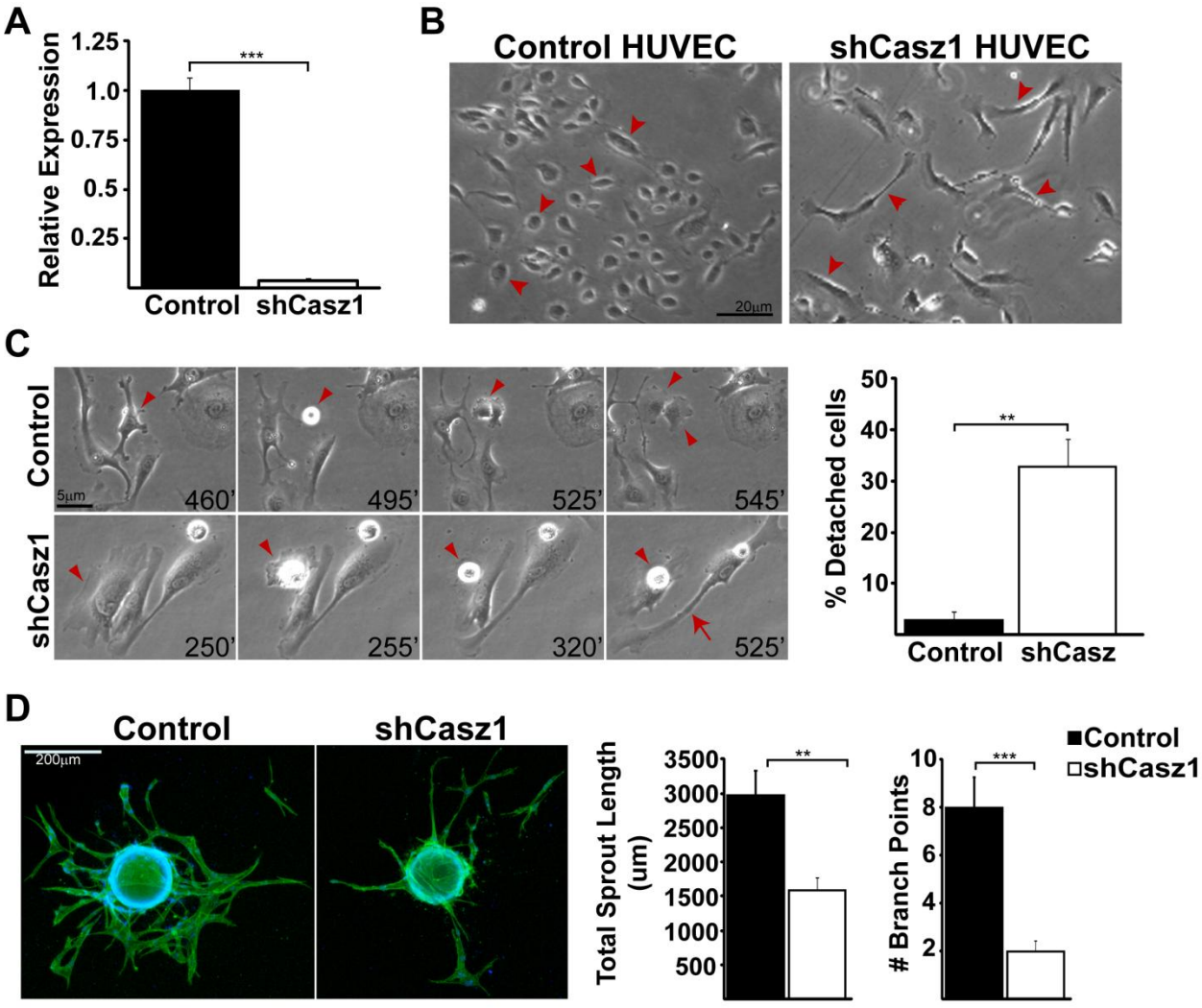
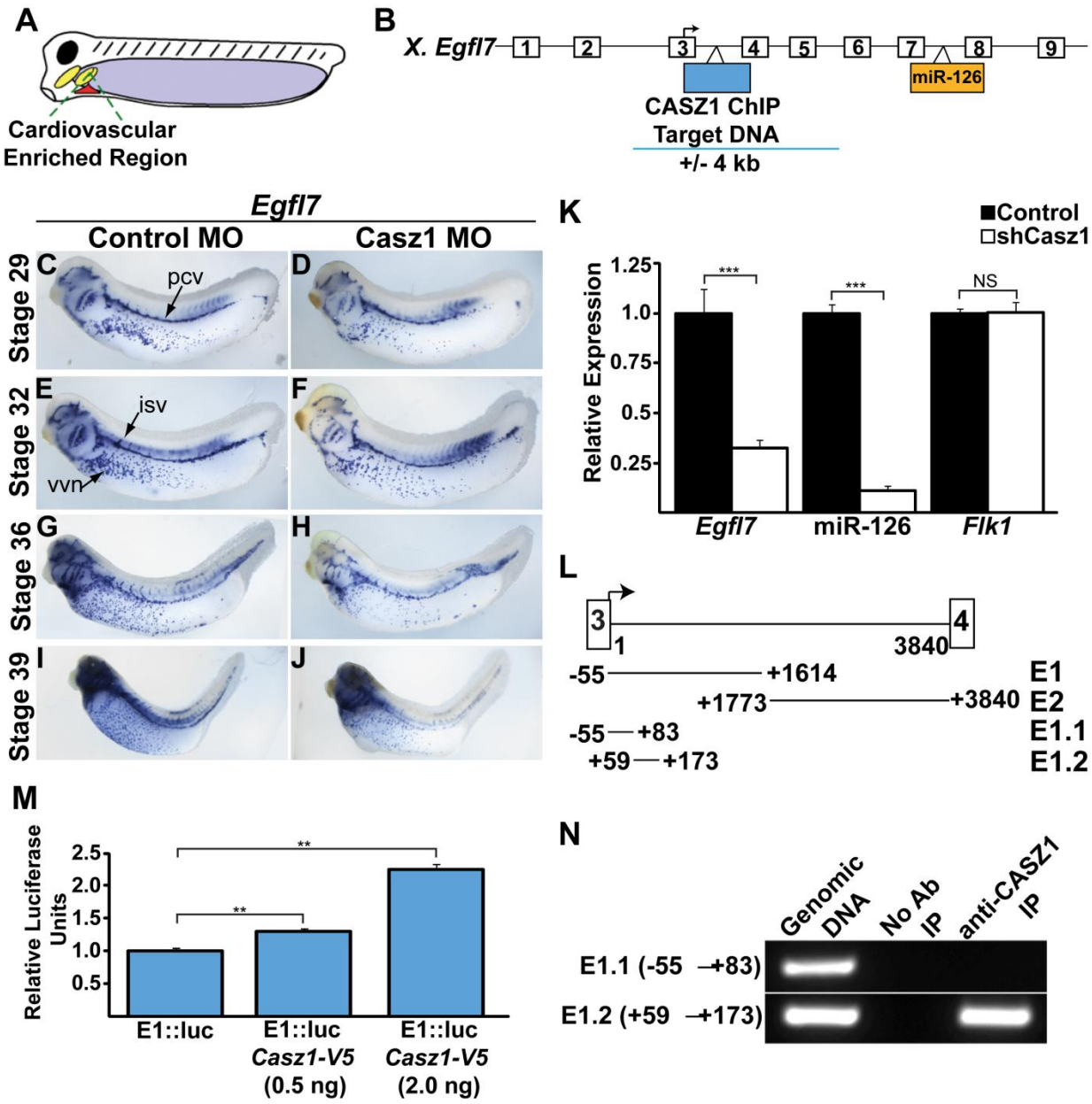

A
Relative Expression
1.25
1.0
0.75
0.5
0.25
***
Control
shCasz1
B
Control HUVEC
shCasz1 HUVEC
20μm
C
Control
shCasz1
5μm
460'
495'
525'
545'
250'
255'
320'
525'
% Detached cells
50
40
30
20
10
**
Control
shCasz
D
Control
shCasz1
200μm
Total Sprout Length (um)
3500
3000
2500
2000
1500
1000
500
**
Branch Points
10
8
6
4
2
***
Control
shCasz1

Figure 2.4. CASZ1 directly activates *Egfl7* transcription. (A) Illustration of cardiovascular-enriched region dissected from *X. tropicalis* for chromatin immunoprecipitation (ChIP). **(B)** Genomic structure of *Xenopus Egfl7* locus denoting CASZ1 ChIP fragment. White boxes: exons; shaded boxes, miR-126 in intron 7 and intronic region potentially containing CASZ1 element (+/- 4kb). **(C-J)** *In situ* analysis of *Egfl7* of stages 29-39 control and CASZ1-depleted embryos (lateral view with anterior to left). Note downregulation of *Egfl7* in vitelline vein network (vvn) and intersomitic vessels (isv) in CASZ1-depleted embryos. Pcv-posterior cardinal vein. **(K)** Relative mRNA expression of *Egfl7*, miR-126, and *Flk1* after infection of HUVECs with shCasz1. mRNA levels relative to *Rps29* ± SEM. ***:$p<0.001$; NS: not significant. **(L)** Schematic demarcating *Egfl7* genomic DNA regions (in bp) tested for transcriptional activation. *E1* (-55–1614) within intron 3 but not *E2* (1773–3840) resulted in increased luciferase (luc) activity. **(M)** *Egfl7* genomic region *E1* in presence or absence of *Casz1*. Bars represent fold increase in activity relative to control ± SEM. Experiments were repeated twice on independent batches of embryos, **:$p<0.01$. **(N)** Identification of 90bp region endogenously bound by CASZ1located within non-overlapping region of *E1.2* (113-227) PCR amplicon.


A
Cardiovascular
Enriched Region
B
X. Egfl7
1
2
3
4
5
6
7
8
9
miR-126
CASZ1 ChIP
Target DNA
+/- 4 kb
Egfl7
Control MO
Casz1 MO
Stage 29
Stage 32
Stage 36
Stage 39
C
D
E
F
G
H
I
J
pcv
isv
vvn
K
Control
shCasz1
Relative Expression
1.25
1.0
0.75
0.5
0.25
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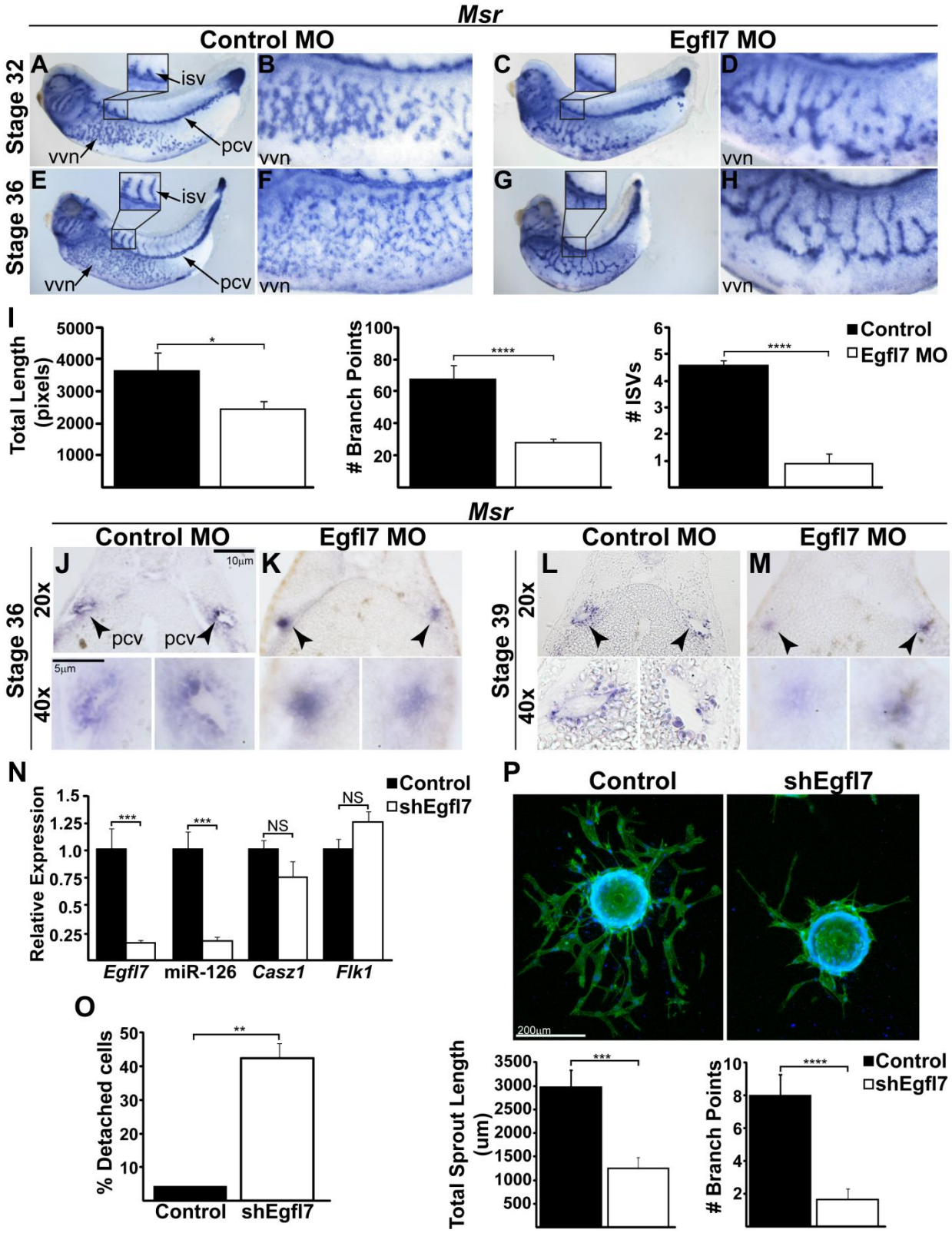
***
NS
Egfl7
miR-126
Flk1
L
3
4
1
3840
-55
+1614
+1773
+3840
-55
+83
+59
+173
E1
E2
E1.1
E1.2
M
Relative Luciferase Units
2.5
2.0
1.5
1.0
0.5
**
**
E1::luc
E1::luc
Casz1-V5
(0.5 ng)
E1::luc
Casz1-V5
(2.0 ng)
N
Genomic DNA
No Ab IP
anti-CASZ1 IP
E1.1 (-55 →+83)
E1.2 (+59 →+173)

Figure 2.5. EGFL7-depletion in embryos and HUVECs phenocopies CASZ1-depletion.

(A-H) *In situ* analysis with EC marker *Msr* control and EGFL7-depleted embryos (stages 32-36, lateral view with anterior to left). Note lack of branching in vitelline vein network (vvn) at stage 32 (A,C high magnification of vvn in B,D) and stage 36 (E,G high magnification of vvn in F,H). Intersomitic vessel (isv) sprouting is also impaired (A,C,E,G). (*n*=10 embryos/condition/stage, 3 independent experiments). **(I)** Quantification of vascular defects control and EGFL7-depleted embryos (stage 36) representing total vessel length, number of branch points within vvn, and number of isvs/embryo. Data represent mean ± SEM. (*n*=7 control and 10 Egfl7 MO embryos). *: $p<0.05$; **:$p<0.01$; ****:$p<0.0005$. **(J-M)** Histological analysis reveals lumenless posterior cardinal veins (pcv) in stage 36 (K) and stage 39 (M) Control and EGFL7-depleted embryos (J,L), dorsal top, ventral bottom, arrowheads correspond to pcv positions enlarged in lower panels (*n*=3 embryos/condition/stage). **(N)** mRNA expression of *Egfl7,* miR-126*, Casz1, and Flk1* after infection of HUVECs with shEgfl7. *Egfl7* is decreased 11-fold. mRNA levels relative to *Rps29* ± SEM. ***:$p<0.001$; NS: not significant. **(O)** Quantification of cells that round up and detach during imaging. Data represent mean ± SEM of 2 experiments conducted on independent batches of shRNA-infected cells. (*n*=100 cells). **:$p<0.01$. **(P)** Sprouting angiogenesis assay was performed with control and EGFL7-depleted HUVEC. On day 6, cultures were fixed and stained for phalloidin (green) and DRAQ5 (blue). Graphs represent mean ± SEM of total sprout length and number of branch points/bead (*n*=11 beads/condition). Experiments were repeated twice on independent batches of shRNA-infected cells. **:$p<0.01$; ***$p<0.001$.


Msr
Control MO
Egfl7 MO
Stage 32
Stage 36
A
B
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G
H
isv
pcv
vvn
I
Total Length (pixels)
Branch Points
ISVs
Control
Egfl7 MO
Msr
Control MO
Egfl7 MO
Control MO
Egfl7 MO
Stage 36
Stage 39
20x
40x
J
K
L
M
10μm
5μm
pcv
N
Relative Expression
Control
shEgfl7
Egfl7
miR-126
Casz1
Flk1
NS
O
% Detached cells
Control
shEgfl7
P
Control
shEgfl7
200μm
Total Sprout Length (um)
Branch Points

Figure 2.6. EGFL7 and miR-126 play distinct roles downstream of CASZ1. (A) Quantification of cells co-infected with shCasz1 and Ad-Egfl7 or Ad-miR-126 that round up and detach versus shCasz1 or control shRNA alone. Data represent mean ± SEM of 2 average experiments conducted on independent batches of shRNA-infected cells. (*n*=100 cells). **:p<0.01; ****:p<0.0005. **(B)** Quantification of cell morphology determined by measuring length-to-width (L:W) ratio. Morphology was improved in cells co-infected with shCasz1 and Ad-Egfl7 but not in cells co-infected with Ad-miR-126. Data represent mean ± SEM of 3 experiments conducted on independent batches of shRNA-infected cells. (*n*=300-600 cells). **:p<0.01; ****:p<0.0005. **(C)** RhoA protein expression in shRNA-infected HUVECs. RhoA levels were markedly decreased by depletion of CASZ1 and EGFL7. Graph of densitometry of RhoA levels relative to GAPDH. **(D)** Relative *RhoA, RhoB,* and *RhoC* mRNA expression after shRNA infection. mRNA levels relative to *Rps29* ± SEM. *:p<0.05; ***:p<0.001; NS: not significant. **(E)** Stress fibers and FAs disrupted in CASZ1-depleted HUVECs resemble cells treated with ROCK inhibitor Y-27632 (10μM). Restoration of *Egfl7* in CASZ1-depleted cells rescues proper FA localization. Phalloidin marks F-actin filaments (green), vinculin (red) and phosphorylated paxillin (yellow) mark FAs.

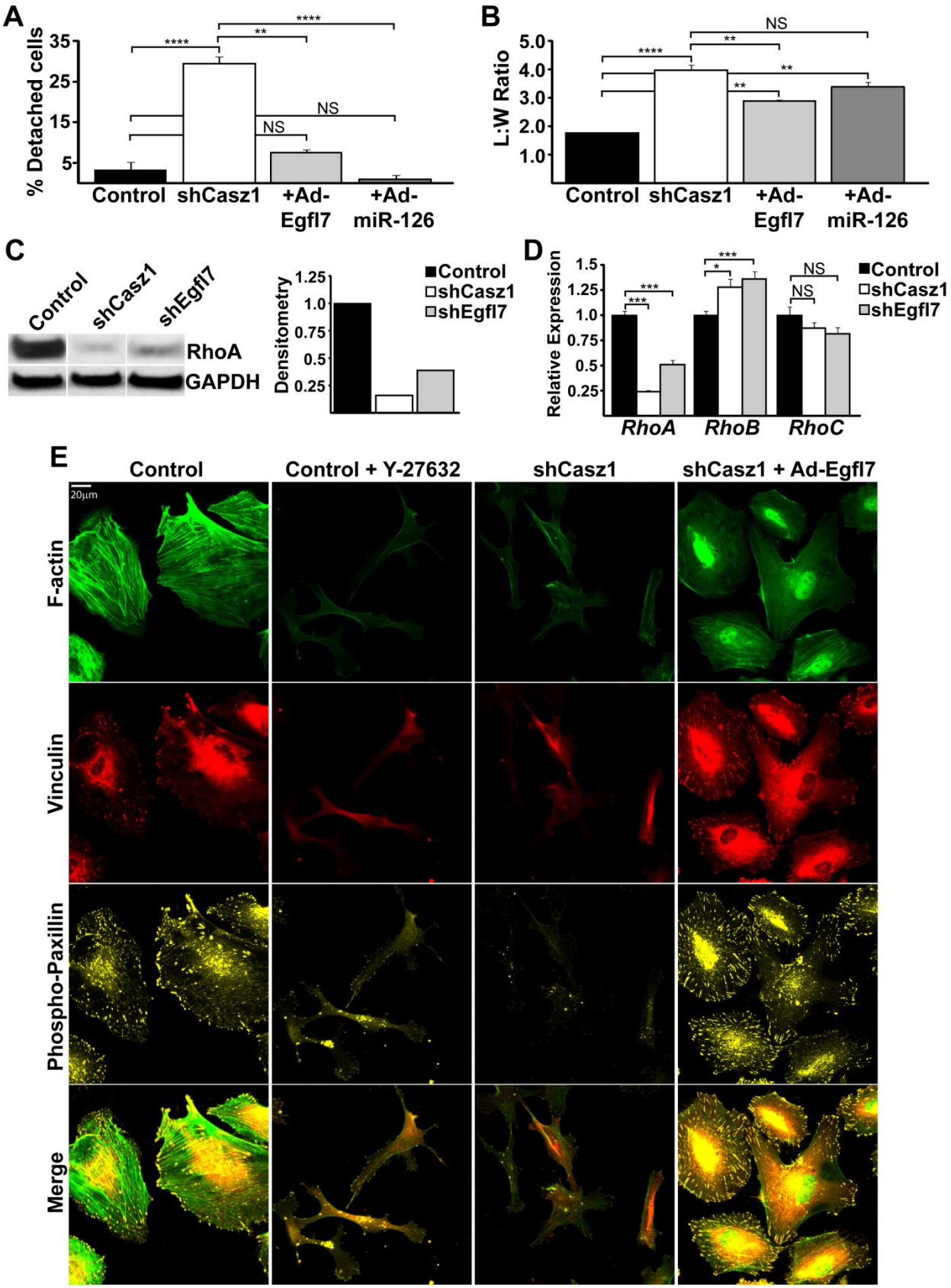
A
% Detached cells
35
25
15
5
****
**
****
NS
NS
Control
shCasz1
+Ad-Egfl7
+Ad-miR-126
B
L:W Ratio
5.0
4.0
3.0
2.0
1.0
NS
**
****
**
**
Control
shCasz1
+Ad-Egfl7
+Ad-miR-126
C
Control
shCasz1
shEgfl7
RhoA
GAPDH
Densitometry
1.25
1.0
0.75
0.5
0.25
Control
shCasz1
shEgfl7
D
Relative Expression
1.5
1.25
1.0
0.75
0.5
0.25
***
***
*
***
NS
NS
Control
shCasz1
shEgfl7
RhoA
RhoB
RhoC
E
Control
Control + Y-27632
shCasz1
shCasz1 + Ad-Egfl7
20μm
F-actin
Vinculin
Phospho-Paxillin
Merge

Figure 2.7. A model describing CASZ1 function in endothelial cells. CASZ1 functions by binding to an intronic element within the Egfl7 locus to activate proper levels of Egfl7 in endothelial cells. EGFL7 is then secreted to the extracellular matrix (ECM) where it likely binds cell-surface receptors which signal downstream to activate RhoA expression. Consequently, RhoA signaling modulates endothelial cell behaviors such as adhesion and contractility to promote vessel assembly and morphogenesis.

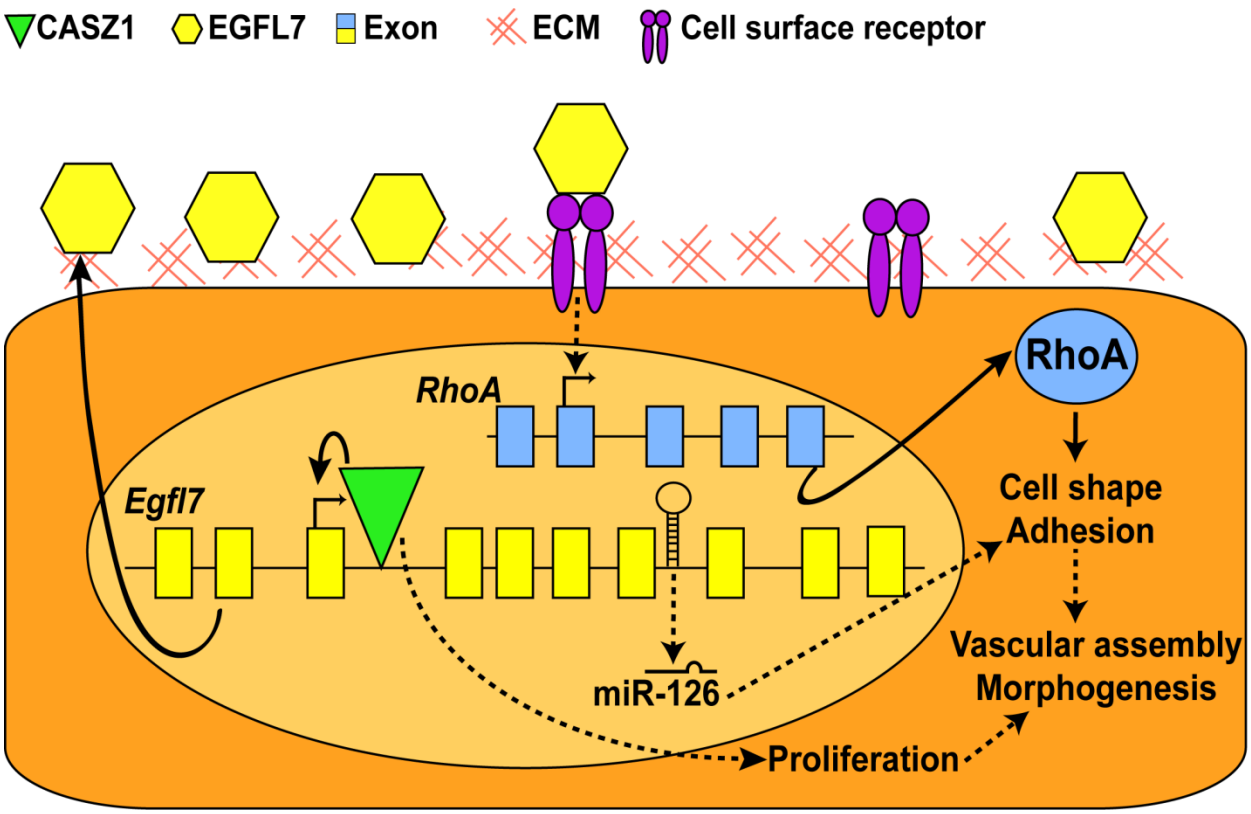

Figure S2.1. CASZ1 is required for vascular development. (A) Vascular explants excluding the head, heart, and somitic region were dissected from stage 32 embryos for RNA isolation and cDNA synthesis. RT-PCR showed that *Casz1* is co-expressed in this region with other vascular markers, *Msr* and *Erg*. *Eomesodermin* (*Eomes*), a marker of the forebrain and primitive mesendoderm, was a negative control while *Ef1α* was a loading control. **(B-I)** Whole-mount *in situ* analysis with EC marker *Erg* of stages 29-39 control and CASZ1-depleted embryos (lateral view with anterior to left). *Erg* was a secondary marker used to confirm vascular defects were not specific to *Msr.* Vessel branching and patterning were severely compromised in CASZ1-depleted embryos. Note lack of branching within vitelline vein network (vvn) as well as reduced sprouting of intersomitic vessels (isv). Posterior cardinal vein (pcv). (*n*=10 embryos/condition/stage, 3 independent experiments). **(J-M)** Whole-mount *in situ* analysis with EC marker *Msr* of stage 29 and 39 control and CASZ1-depleted embryos. (*n*=10 embryos/condition/stage, 3 independent experiments). **(N-Q)** The anterior-most region including the cardiac portion was dissected from early tailbud stage 29 control and CASZ1-depleted embryos prior to heart tube formation and therefore circulation. Explants were cultured until early tadpole stage 36 when the embryo has an established circulation and assayed by *in situ* hybridization with the endothelial marker *Ami*. (N,P) In the absence of functional circulation, control explants form a dense grossly normal vascular network similar to un-manipulated embryos (lateral view with anterior to the left). (O,Q) In the absence of CASZ1-depleted cardiac tissue and circulation, we observe an identical vascular phenotype in the trunk explants as we do in the un-manipulated embryo indicating the vascular defects in the CASZ1-depleted embryos are independent of the cardiac defects.

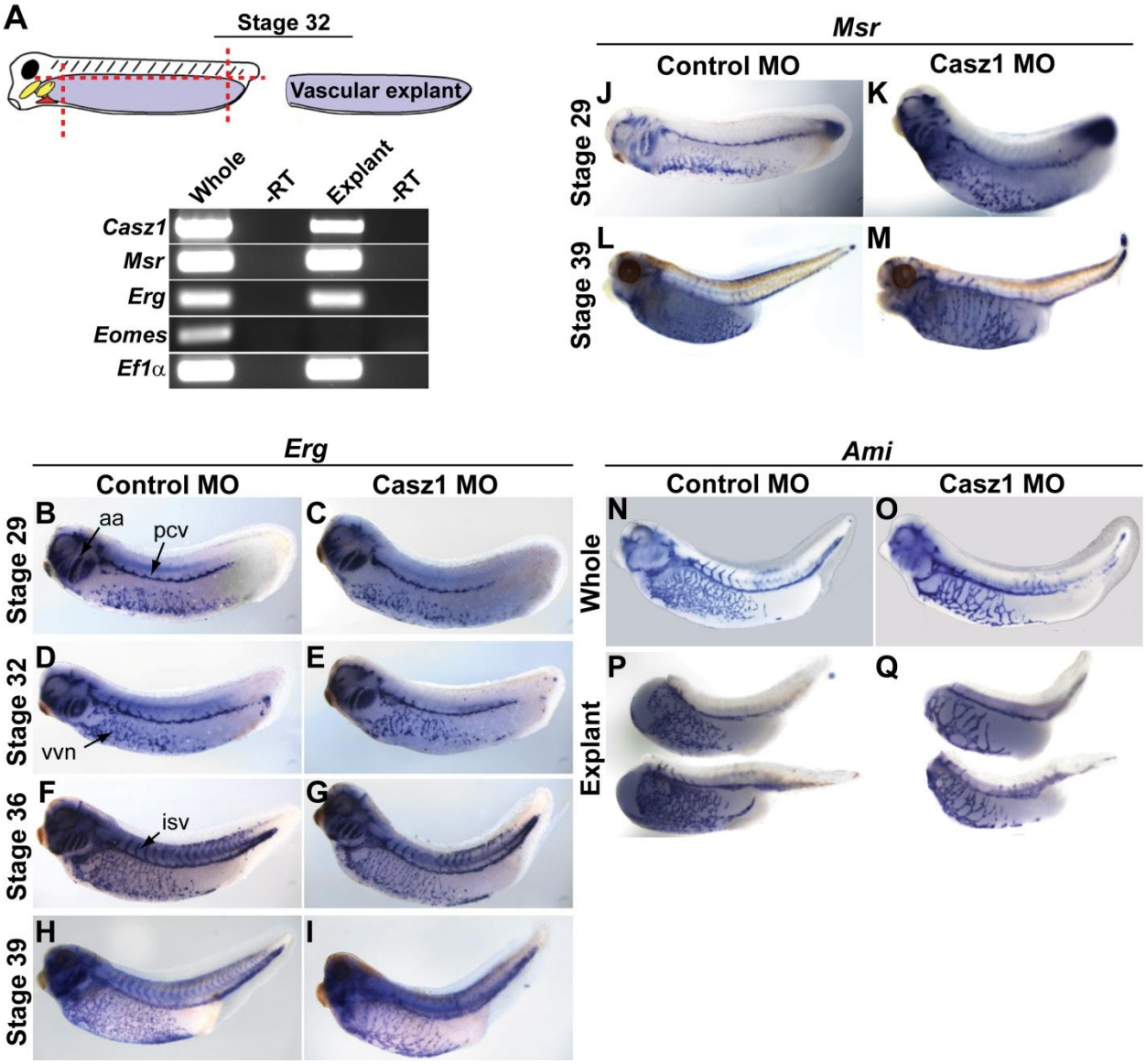
A
Stage 32
Vascular explant
Whole
-RT
Explant
-RT
Casz1
Msr
Erg
Eomes
Ef1α
Msr
Control MO
Casz1 MO
Stage 29
J
K
Stage 39
L
M
Erg
Control MO
Casz1 MO
Stage 29
B
aa
pcv
C
Stage 32
D
vvn
E
Stage 36
F
isv
G
Stage 39
H
I
Ami
Control MO
Casz1 MO
Whole
N
O
Explant
P
Q

Figure S2.2. CASZ1 is required for EC proliferation. (A) Control and shCasz1 cells were stained with anti-phospho-histone H3 (pH3-red) and DAPI (blue) to identify mitotic cells. Images were taken at 25x magnification while far right panels were taken at 63x. White arrowheads indicate pH3-positive cells. Graph represents quantification of pH3-positive cells. Data represent mean ± SEM of 3 independent experiments. (*n*=1000-2500 cells). **(B)** FACS analysis showing CASZ1-depleted cells arrest at G1/S with reduced proportion of cells in S phase compared to controls. **(C)** Control and CASZ1-depleted HUVECs were stained with anti-cleaved caspase-3 (red) and DAPI (blue) to identify apoptotic cells (top two panels). As a positive control, wildtype HUVECs were serum starved for 24 hr to induce apoptosis (lower panels). Images were taken at 25x magnification while far left panels were taken at 63x magnification. White arrowheads indicate cleaved caspase-3-positive cells. Graph represents quantification of cleaved caspase-3-positive cells. Data represent mean ± SEM of 2 independent experiments. (*n*=200-1000 cells).

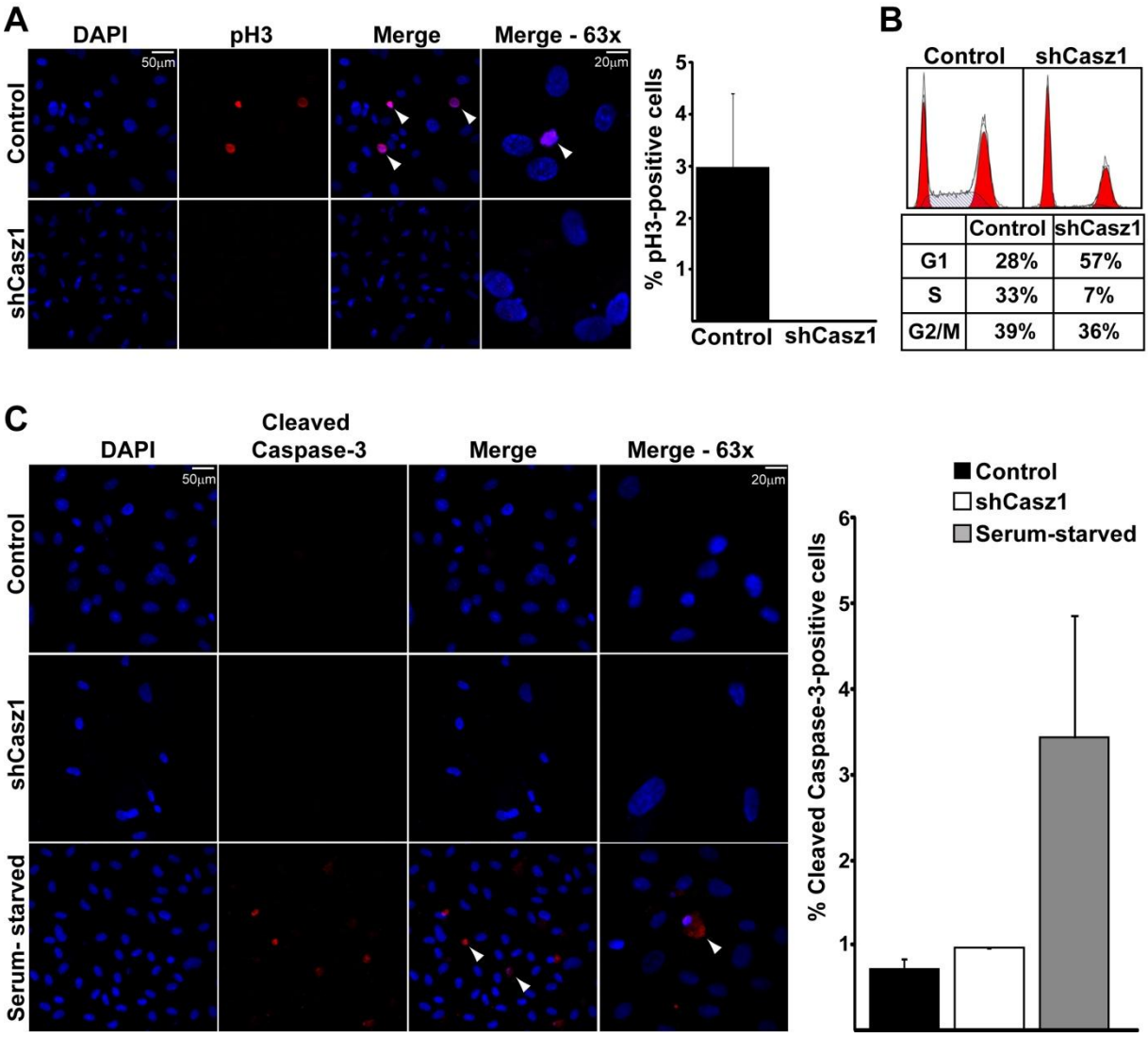
A
DAPI
pH3
Merge
Merge - 63x
50μm
20μm
Control
shCasz1
% pH3-positive cells
5
4
3
2
1
Control
shCasz1
B
Control
shCasz1
Control
shCasz1
G1
28%
57%
S
33%
7%
G2/M
39%
36%
C
DAPI
Cleaved Caspase-3
Merge
Merge - 63x
50μm
20μm
Control
shCasz1
Serum- starved
Control
shCasz1
Serum-starved
% Cleaved Caspase-3-positive cells
6
5
4
3
2
1

Figure S2.3. Sequence alignment of miR-126 and expression of miR-126 in CASZ1-depleted embryos. (A) *Xenopus* miR-126 was cloned from genomic DNA and confirmed to be located in *Egfl7* intron 7. Evolutionary conservation of mature miR-126 is shown. **(B-I)** Whole mount *in situ* analysis of miR-126 of stage 29-39 control and CASZ1-depleted embryos (lateral view with anterior to the left). miR-126 expression commences in the vasculature including the vitelline vein network (vvn) at stage 32 (D,E) and remains intact at all stages thereafter even in the absence of CASZ1 suggesting that it may be differentially regulated than its host gene. Note the robust expression of miR-126 in somites (s). (*n*=10 embryos/condition/stage, 3 independent experiments).

A

	5′	3′
H.sapiens	UCGUACCGUGAGUAAUAAUGCG	
M.musculus	UCGUACCGUGAGUAAUAAUGCG	
D.rerio	UCGUACCGUGAGUAAUAAUGCG	
X.tropicalis	UCGUACCGUGAGUAAUAAUGCG	
X.laevis	UCGUACCGUGAGUAAUAAUGCG	

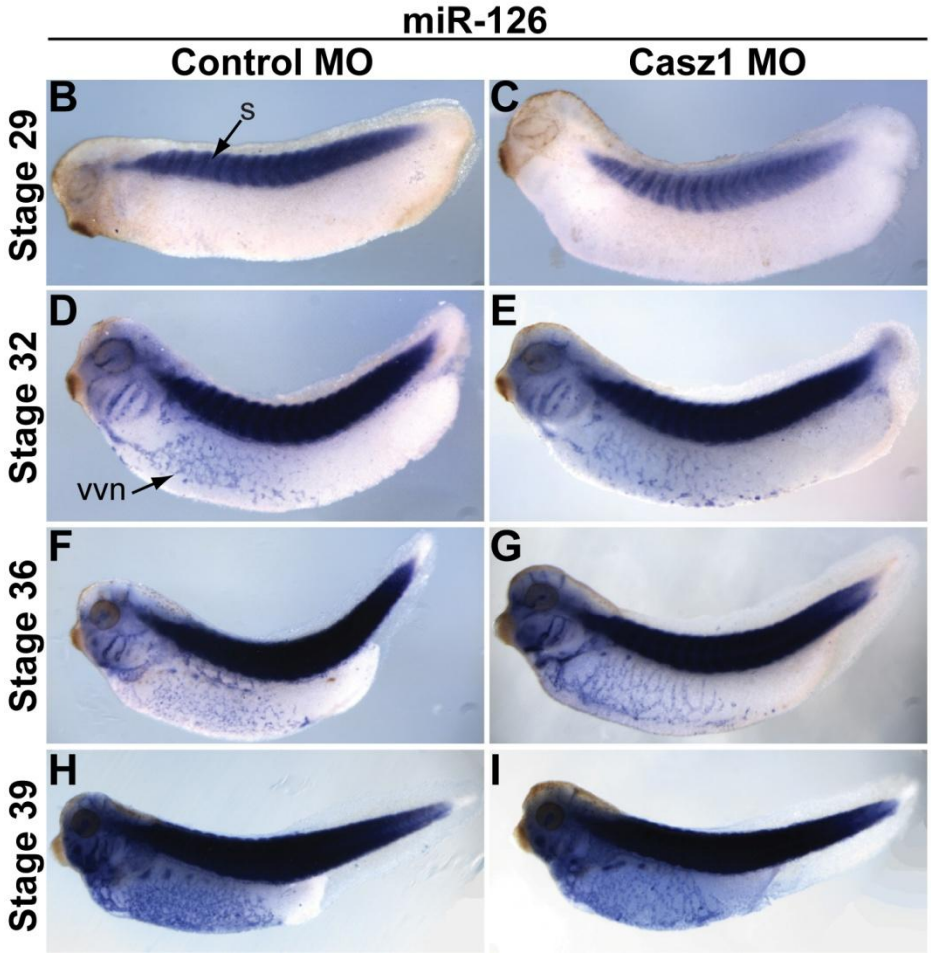

Figure S2.4. EGFL7-depletion phenocopies CASZ1-depletion during vascular development. (A) RT-PCR analysis of early tailbud stage 29 embryos injected at the one-cell stage with 40 ng Egfl7 MO demonstrating inhibition of proper splicing of *Egfl7* pre-mRNA. Control MO-injected embryos are negative controls and genomic DNA is a positive control for PCR amplification. **(B-C)** Whole mount *in situ* analysis of miR-126 expression in control and EGFL7-depleted early tadpole stage 36 embryos. There are no detectable alterations in miR-126 expression in EGFL7-depleted embryos. **(D-E)** Whole mount *in situ* analysis of *Casz1* expression in control and miR-126-depleted early tadpole stage 36 embryos. There are no detectable changes in *Casz1* expression in miR-126-depleted embryos. **(F-I)** Whole-mount in situ analysis with EC marker *Msr* of stage 29 and 39 control and EGFL7-depleted embryos. (*n*=10 embryos/condition/stage, 3 independent experiments). **(J-Q)** Whole-mount *in situ* analysis with EC marker *Erg* of stages 29-39 control and EGFL7-depleted embryos (lateral view with anterior to left). Vascular branching and patterning were severely disrupted in EGFL7-depleted embryos. Vitelline vein network-vvn, posterior cardinal vein- pcv, intersomitic vessels- isv. (*n*=10 embryos/condition/stage, 3 independent experiments).

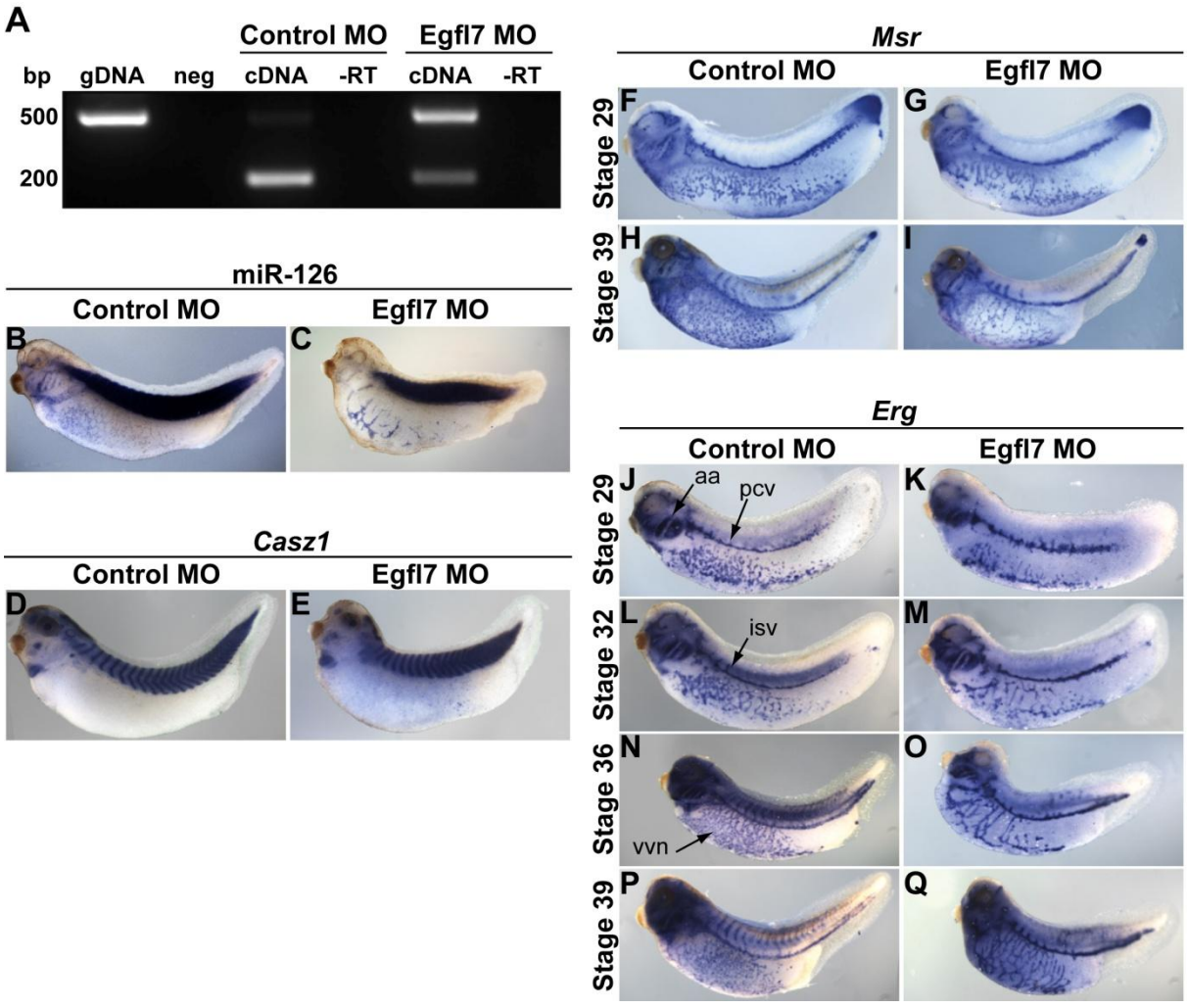
A
Control MO
Egfl7 MO
bp
gDNA
neg
cDNA
-RT
cDNA
-RT
500
200
miR-126
Control MO
Egfl7 MO
B
C
Casz1
Control MO
Egfl7 MO
D
E
Msr
Control MO
Egfl7 MO
Stage 29
F
G
Stage 39
H
I
Erg
Control MO
Egfl7 MO
Stage 29
J
aa
pcv
K
Stage 32
L
isv
M
Stage 36
N
vvn
O
Stage 39
P
Q

Figure S2.5. miR-126 depletion does not phenocopy CASZ1-depletion during vascular development. (A) Relative expression of miR-126 in embryos injected with miR-126 MO compared to control. Bars represent expression relative to miR-16 ± SEM. *:$p<0.05$. **(B-C)** Whole mount *in situ* analysis of *Egfl7* expression in control and miR-126-depleted early tadpole stage 36 embryos. There are no detectable alterations in *Egfl7* expression in miR-126-depleted embryos. **(D-E)** Whole mount *in situ* analysis of *Casz1* expression in control and miR-126-depleted early tadpole stage 36 embryos. There are no detectable changes in *Casz1* expression in miR-126-depleted embryos. **(F-U)** Whole mount *in situ* analysis of *Msr* (F,G,J,K,N,O,R,S) *and Erg* (H,I,L,M,P,Q,T,U) of stages 29-39 control and miR-126-depleted embryos (lateral view with anterior to the left). The vasculature forms normally in miR-126-depleted embryos with no apparent disruptions in vascular patterning or assembly. Vitelline vein network- vvn, posterior cardinal vein- pcv, intersomitic vessels- isv. (*n*=10 embryos/condition/stage, 3 independent experiments). **(V-Y)** Histological analysis shows normal lumens of pcv in stage 36 (W) and stage 39 (Y) miR-126-depleted embryos compared to controls (V,X). Dorsal is top, ventral is bottom. Arrowheads correspond to positions of pcv which are enlarged in lower panels. (*n*=2 embryos/condition/stage).

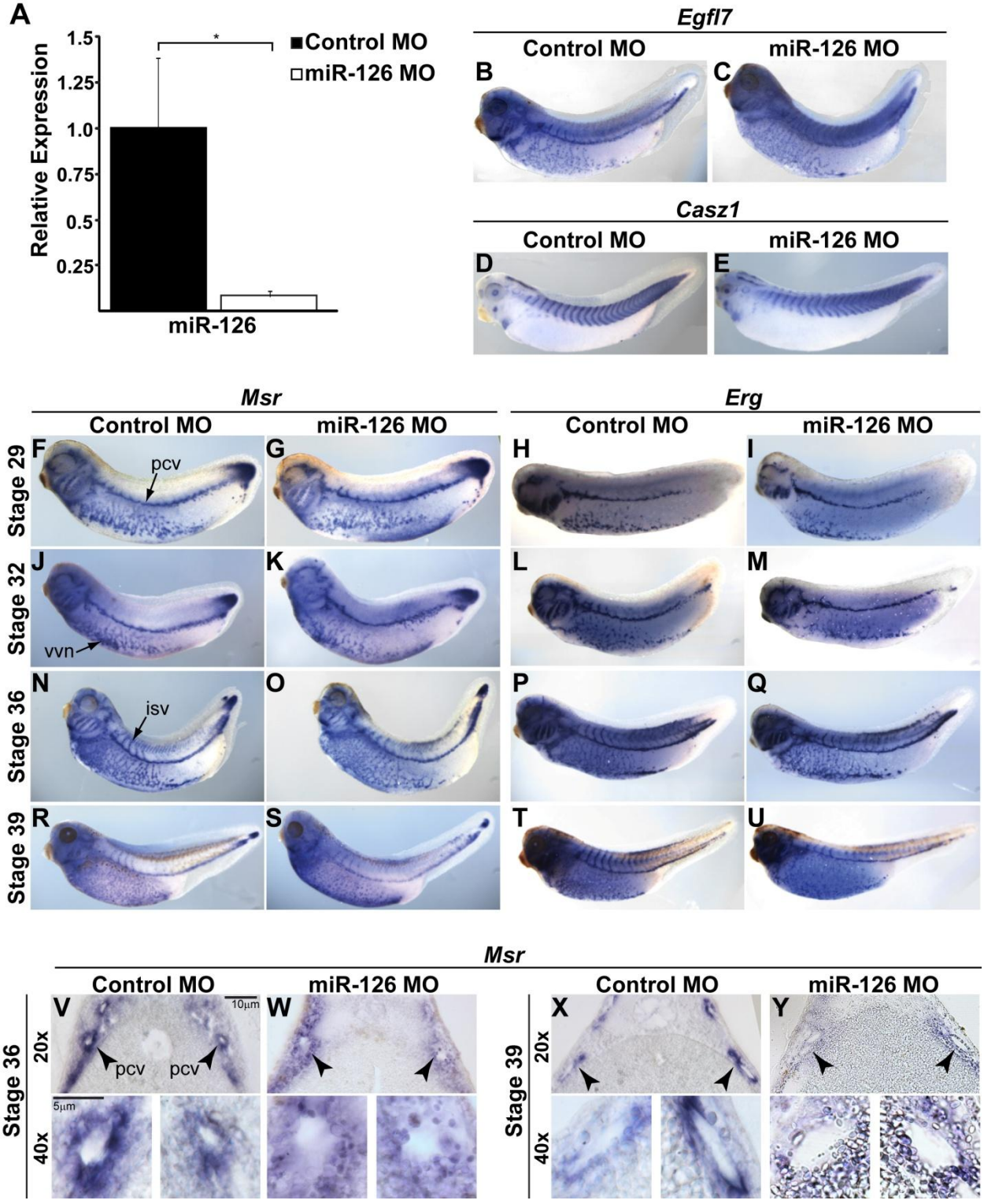
A
Relative Expression
1.5
1.25
1.0
0.75
0.5
0.25
*
Control MO
miR-126 MO
miR-126
Egfl7
Control MO
miR-126 MO
B
C
Casz1
Control MO
miR-126 MO
D
E
Msr
Control MO
miR-126 MO
Erg
Control MO
miR-126 MO
Stage 29
Stage 32
Stage 36
Stage 39
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
pcv
vvn
isv
Msr
Control MO
miR-126 MO
Control MO
miR-126 MO
Stage 36
Stage 39
20x
40x
V
W
X
Y
10μm
5μm
pcv
pcv

Figure S2.6. Efficacy of Ad-Egfl7 and Ad-miR-126. (A) Western blot of EGFL7 protein in HUVECs infected with control shRNA alone or co-infected with 25 MOI (multiplicity of infection) Ad-Egfl7. **(B)** Relative expression of miR-126 in HUVECs co-infected with shCasz1 and 25 MOI Ad-miR-126 compared to shCasz1 alone. mRNA levels are relative to miR-16 ± SEM. *:$p<0.05$. **(C)** Phase contrast images demonstrate elongated shCasz1-infected cells become more uniform in length and width after co-infection with Ad-Egfl7 but remain thin and elongated after co-infection with Ad-miR-126. **(D)** RhoA activity in un-stimulated (-) and Thrombin-stimulated (Thr) shRNA-infected HUVECs. RhoA activity was markedly decreased by depletion of CASZ1 and EGFL7. **(E)** Western blot representing unaltered RhoC protein levels in HUVECs infected with shCasz1, shEgfl7, or control shRNA. **(F)** Western blot showing decreased phosphorylation of myosin light chain (p-MLC) in CASZ1-depleted HUVECs versus control. Graph represents densitometry of p-MLC relative to GAPDH.

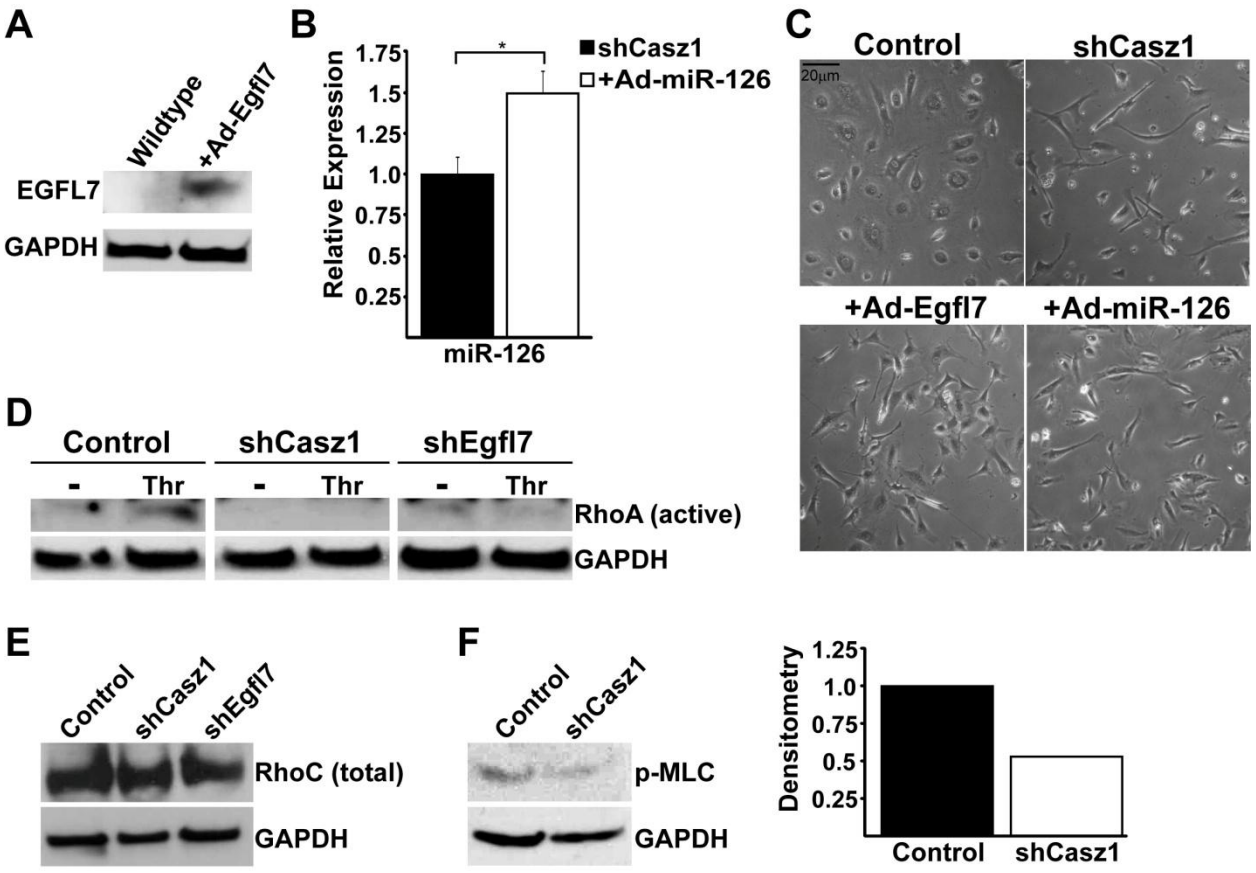
A
Wildtype
+Ad-Egfl7
EGFL7
GAPDH
B
Relative Expression
1.75
1.5
1.25
1.0
0.75
0.5
0.25
*
shCasz1
+Ad-miR-126
miR-126
C
Control
shCasz1
20μm
+Ad-Egfl7
+Ad-miR-126
D
Control
shCasz1
shEgfl7
-
Thr
-
Thr
-
Thr
RhoA (active)
GAPDH
E
Control
shCasz1
shEgfl7
RhoC (total)
GAPDH
F
Control
shCasz1
p-MLC
GAPDH
Densitometry
1.25
1.0
0.75
0.5
0.25
Control
shCasz1

Table 2.2. Putative direct targets of CASZ1.

Putative CASZ1 Targets	Target DNA Location	General Function
N-myc Downregulated Gene 1 (Ndrg1)	3743 bp 3'	Cell Growth Pathway
Jumonji Domain Containing 1A	Internal	Cell Growth Pathway
Cyld	8075 bp 5'	Cell Growth Pathway
MDM2	4868 bp 3'	Cell Growth Pathway
MDM4	Internal	Cell Growth Pathway
Cyclin L2	16 bp 3'	Cell Growth Pathway
Inhibitor of Growth 1 (Ing1)	309 bp 5'	Cell Growth Pathway
Gli2	3680 bp 3'	Cell Growth Pathway
Plexin A2	Internal	Migration/Adhesion
Neurexin 3	Internal	Migration/Adhesion
FAT-J	Internal	Migration/Adhesion
Wnt1 Induced Secreted Pathway 1 (Wisp1)	Internal and 2867 bp 3'	Wnt Signaling Pathway
Novel Band 4.1-like Protein 4 (NBL4)	6788 bp 5'	Wnt Signaling Pathway
Shisa2	Internal	Wnt Signaling Pathway
Prickle1	Internal	Wnt Signaling Pathway
Msx1	8775 bp 5'	Patterning
Alk2/ACVR1	9351 bp 5'	Patterning
Troponin T Type 3 (Tnnt3)	4654 bp 5'	Structural
Myosin 5C	internal	Structural
Cytochrome b5	internal	Metabolism
Cytochrome b5 reductase isoform 2	10,000 bp 3'	Metabolism
Cytochrome C Oxidase Subunit VIII-Heart specific isoform	3859 bp 3'	Metabolism

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CHAPTER 3: VASCULAR LUMEN FORMATION IN XENOPUS

Introduction

The formation of a functional vascular system during embryogenesis is critical for growth and survival. The development of a majority of organs and tissues first requires the proper establishment of a closed circulatory loop capable of transporting blood and nutrients, removing waste, and facilitating gas exchange. Blood vessels initially arise via vasculogenesis, which is characterized by the de novo assembly of endothelial precursors called angioblasts into primitive capillary-like networks (Flamme et al., 1997). Further expansion and remodeling of this primary plexus occurs by angiogenesis, or the sprouting of new vessels from preexisting vessels. Sprouting angiogenesis is the primary means by which a hierarchically branched and perfusable vascular system comprised of veins, arteries, and capillaries is ultimately formed (Risau, 1997).

Vascular development appears to be well-conserved from fish to mammals. An inherent feature that makes *Xenopus* an ideal model in which to study the early events associated with endothelial cell assembly is their external development thus enabling one to easily visualize embryonic blood vessel formation with minimal physical manipulation. Some of the earliest vessels that arise via vasculogenesis in *Xenopus* include the paired posterior cardinal veins, the

dorsal aorta, the vitelline vein network, the aortic arches, and the endocardium, all of which are specified from the mesoderm (Cleaver et al., 1997). However, the cellular origins of these vessels differ as has been demonstrated by fate-mapping experiments. Ciau-Uitz and colleagues demonstrated that by the 32-cell stage, the endothelial lineage becomes restricted to four blastomeres each of which give rise to different endothelial cell populations (Ciau-Uitz et al., 2000; Walmsley et al., 2002). For example, the vitelline vein network originates from progenitors distinct from those of the posterior cardinal veins. Angioblasts of the vitelline vein network form in close association with hematopoietic precursors in clusters called the ventral blood islands. The blood islands are considered to be the initial source of embryonic blood but a second wave of hematopoiesis from a distinct mesodermal source has also been shown to occur during the development of adult blood (Dzierzak and Speck, 2008; Flajnik et al., 1984; Kau and Turpen, 1983; Maeno et al., 1985; Turpen and Knudson, 1982). The dorsal lateral plate mesoderm (DLPM) contributes to the posterior cardinal veins, which run bilaterally along the length of the embryo, and the dorsal aorta, which runs along the midline ventral to the notochord. Adult hematopoietic cells form from the floor of the dorsal aorta, hence the distinction between the sources of embryonic and adult blood (Dzierzak and Speck, 2008; Leung et al., 2013).

The posterior cardinal veins and the dorsal aorta constitute the major embryonic vessels and begin forming shortly after neurulation. DLPM angioblasts first align into two bilateral strips in an anterior-to-posterior direction beginning at stage 20 (Walmsley et al., 2002). Between stages 27 and 33, a subset of these cells migrates over the underlying endoderm to the midline where they settle just beneath the hypochord to ultimately form the dorsal aorta (Cleaver and Krieg, 1998). It has been shown that this wave of angioblast migration occurs in response to

vascular endothelial growth factor (VEGF) expressed by the hypochord (Cleaver and Krieg, 1998). It is still unclear whether DLPM cells are the sole contributors to the dorsal aorta as studies in chick have also demonstrated a contribution from somitic mesoderm (Pardanaud et al., 1996; Sato et al., 2008). Migration is completed by stage 33/34 thereby specifying the positions where the posterior cardinal veins and dorsal aorta will later differentiate. Tubular vessels become apparent beginning at stage 36 concomitant with the commencement of circulation.

Very few studies to date have examined how vessels generate lumens in *Xenopus*, although Vokes and colleagues demonstrated that the endoderm is required specifically for tube formation but not angioblast specification (Vokes and Krieg, 2002). Indeed, vascular lumen formation is still an incompletely understood process in other species as well. Studies using cultured human endothelial cells, zebrafish, and mouse have elucidated some important molecular determinants of vascular lumen formation (Bayless and Davis, 2002; Blum et al., 2008; Davis and Camarillo, 1996; Helker et al., 2013; Herwig et al., 2011; Strilic et al., 2009; Wang et al., 2010). The predominant mechanism by which lumens are generated has been termed cord hollowing or extracellular lumen generation indicating a cell-cell separation event with the luminal compartment lying between multiple cells. Previous studies in the mouse elegantly demonstrated that angioblasts of the dorsal aorta are initially in close contact with each other displaying multiple junctions along the contact region (Strilic et al., 2009). Prior to cell-cell separation, these vascular cords establish apicobasal polarity with specific proteins including moesin, CD34 sialomucins, and non-muscle myosin II localizing to the apical/ luminal surface. It is believed that this polarization event drives the redistribution of junctions away from the cord center (Strilic et al., 2010a; Strilic et al., 2009). Furthermore, Rho kinase (ROCK)-dependent

signaling facilitates the appropriate cell shape changes needed to accommodate the developing lumen. Similar cord hollowing mechanisms have been observed in zebrafish vessels however additional mechanisms including intracellular lumen formation via coalescence of pinocytic vacuoles or apical membrane invagination and lumen ensheathment have also been demonstrated thus signifying that different species and/or different vascular beds may use a number of diverse ways to generate as well as maintain vascular lumens (Blum et al., 2008; Helker et al., 2013; Herwig et al., 2011; Kamei et al., 2006; Wang et al., 2010)[Reviewed in Chapter 1].

Epidermal Growth Factor-Like domain 7 (EGFL7) is a small protein of 20-30 kD that is highly expressed in endothelial cells actively engaging in vessel assembly during embryonic development, injury, pregnancy, and tumor growth (Campagnolo et al., 2005; Fan et al., 2013; Fitch et al., 2004; Parker et al., 2004). Structural analysis of EGFL7 has revealed it to contain an Emilin-like domain characteristic of secreted proteins and indeed, EGFL7 is secreted by endothelial cells and deposited into the extracellular matrix (ECM) (Fitch et al., 2004; Lelievre et al., 2008). Numerous studies have implicated EGFL7 in vascular lumen morphogenesis as well as sprouting angiogenesis. In zebrafish, EGFL7 was shown to be required for the establishment of vascular lumens likely by providing a permissive environment for endothelial cell adhesion and migration (Parker et al., 2004). In accordance with these studies, we have shown that EGFL7 is also required for vascular lumen formation in *Xenopus* and modulates endothelial cell behaviors such as cell shape and adiesoin to promote vessel sprouting (Charpentier et al., 2013a). Overexpression studies in mouse have further indicated that EGFL7 plays a role in vessel patterning and remodeling potentially through its interaction with Notch receptors (Nichol et al., 2010). Finally, EGFL7 is a currently being investigated in phase II clinical trials by Genentech

for its role in promoting tumor angiogenesis. Generation of anti-EGFL7 antibodies has been shown to augment the efficacy of anti-VEGF therapies in pruning and damaging tumor vessels in non-small cell lung cancer (Johnson et al., 2013). Thus, understanding the function and regulation of this protein is of therapeutic value.

For the first time, we demonstrate the cellular and molecular mechanisms underlying vascular lumen formation in *Xenopus* using the posterior cardinal veins and the dorsal aorta as models. While lumens do appear to be generated via cord hollowing, we note some distinct features in *Xenopus* such as the absence of apicobasal polarity. Furthermore, we investigated the underlying defects associated with impaired lumen formation in EGFL7-depleted embryos and determined that cells fail to undergo proper cell shape changes and resolve cell-cell junctions in order to accommodate the luminal compartment.

Materials and Methods

Xenopus Embryo Manipulation and Collection

Xenopus embryos were prepared and collected as previously described (Showell et al., 2006). Embryos were staged according to Nieuwkoop and Faber (1967). *Egfl7* and control morpholinos were used as previously described (Charpentier et al., 2013a). Five hundred pg of mRNA encoding membrane-GFP/pCS2 (Provided by John Wallingford lab, UT Austin) was co-injected with 35 ng of *Egfl7* or control morpholino in 1-cell stage embryos. For explant assays, the anterior region of stage 28 embryos consisting of the head and heart was removed using sterilized forceps. Explants were cultured for 24 hours in 0.1X Modified Barth's Solution (MBS) containing 10 ug/mL gentamycin before fixation.

Whole Mount *in situ* Hybridization

In situ hybridization was carried out as previously described using an anti-sense probe against *Erg* (Charpentier et al., 2013a; Harland, 1991). For experiments in which in situ hybridization was coupled with immunohistochemistry, changes to the published in situ protocol were made as follows: 1.) Embryos were fixed in 4% paraformaldehyde for 2 hours at room temperature or 4° overnight then stored in 1X phosphate buffered solution (PBS); 2.) Fixed embryos were rinsed three instead of five times in PBS-T (0.1% Tween-20) prior to Proteinase K treatment; 3.) Embryos were incubated in10 ug/mL Proteinase K (Roche) for 10 minutes; 4.) Embryos were rinsed once in PBS-T following Proteinase K treatment and were then immediately fixed in 4% PFA for 20 minutes; 5.) Following color reaction in BM Purple (Roche), embryos were washed several times in 1X PBS and then immediately processed for histology and immunostaining.

Histology

For time course analysis of lumen formation, in situ hybridization was performed as above using the *Erg* probe. After completion of BM purple color reaction, embryos were dehydrated gradually into methanol for at least 24 hours. Embryos were subsequently rehydrated in PBS and taken through a gradient of PBS/glycerol before being embedded in a gelatin solution (0.4% Gelatin Type A, 27% BSA, 18% sucrose, PBS) mixed with glutaraldehyde. A Leica VT1200S vibratome was used to cut 20 um transverse sections. Sections were directly mounted onto slides and imaged on an Olympus IX81 inverted fluorescent microscope. For immunohistochemistry, following the modified in situ protocol above, embryos were embedded in 4% low melting point agarose (Promega) made in 1X PBS. A Leica VT1200S vibratome was used to cut 70 um transverse sections which were collected in 0.5X PBS.

Immunohistochemistry

Immunohistochemistry was carried out on sections in a 48-well plate beginning with three 30-minute PBS-T (1% Triton X-100) washes then incubation for one hour in PBS-T containing 10% fetal bovine serum (FBS). Primary antibodies as listed in Table 1 were applied overnight at 4°. The following day, sections were washes five times for one hour each in PBS-T before incubation with secondary antibodies as listed in Table 1 overnight at 4°. The following day sections were again rinsed five times in PBS-T, incubated with 200 ng/mL DAPI/PBS solution (Sigma) for 30 minutes, and mounted. Images and Z-stacks were taken with a Zeiss 700 confocal microscope.

Antibody	Catalog Number	Dilution Used
GFP	Molecular Probes, A6455	1:1000
GFP (JL8)	Clontech, 632381	1:1000
ZO-1	Invitrogen, 33-9100	1:100
Claudin-5	Santa Cruz Biotechnologies, sc-28670	1:500
Laminin	Sigma, L9393	1:250
Fibronectin	Sigma, F3648	1:250
Atypical PKC zeta	Santa Cruz Biotechnologies, sc-216	1:500
Occludin	Invitrogen, 42-2400	1:250
Alexa Fluor 488 Goat anti-Mouse	Molecular Probes, A-11001	1:1000
Alexa Fluor 488 Donkey anti-Rabbit	Molecular Probes, A-21206	1:1000
Alexa Fluor 546 Goat anti-Mouse	Molecular Probes, A21123	1:1000
Alexa Fluor 546 Goat anti-Rabbit	Molecular Probes, A-11010	1:1000

Table 3.1. List of antibodies used in immunohistochemistry.

Results

The mechanisms underlying vascular lumen formation in *Xenopus* are unknown. To begin to investigate this event, we first performed a time course analysis using in situ hybridization with an endothelial probe to identify the developmental window when lumens arise. We focused our studies on three major vessels, the paired posterior cardinal veins and the dorsal aorta, which can all be visualized simultaneously in transverse sections. Initially, angioblasts of the DLPM coalesce in two bilateral strips corresponding to the positions of the paired posterior cardinal veins (Ciau-Uitz et al., 2000; Walmsley et al., 2002). Beginning around stage 32, a subset of these angioblasts migrates over the underlying endoderm towards the

midline in response to VEGF signals emanating from the hypochord, a transient structure that lies directly ventral to the notochord (Cleaver, 1998; Cleaver et al., 2000). These angioblasts will ultimately contribute to the dorsal aorta which will form just beneath the hypochord. This wave of migration is completed by stage 33/34 when all three populations of angioblasts can be detected by in situ hybridization with the endothelial marker *Erg* (Figure 3.1A). At this stage, the angioblasts have not yet formed functional vessels and cells are coalesced into distinct clusters. However by stage 35/36, vascular lumens of both posterior cardinal veins and the dorsal aorta become evident indicating that they likely arise simultaneously (Figure 3.1B). As embryogenesis progresses, the lumens expand in size and are functional in their ability to circulate blood (Figure 3.1C). Therefore, we focused our studies on the stage prior to lumen formation (stage 33) and immediately following the emergence of lumens (stage 36) to examine the cellular and molecular events underlying the formation of functional vascular tubes.

Previous reports have indicated that circulation is required for vessel remodeling and integrity (Lucitti et al., 2007; Udan et al., 2013b; Zhong et al., 2011). To determine whether lumens can form in the absence of circulation, we took advantage of the ability to culture *Xenopus* embryo explants. We removed the anterior region of the embryo, including the head and the heart, at a stage prior to cardiac development (i.e. stage 28). Within six hours, this wound completely heals and the explants can be cultured to the desired developmental time point. We assessed the presence of vascular lumens at stage 36 using in situ hybridization with *Erg* followed by histology. The lumens of the posterior cardinal veins and dorsal aorta were indistinguishable between un-manipulated and explant embryos (Figure 3.1D-E). These results therefore strongly indicate that cardiac development and establishment of circulation are not

prerequisites for at least the initial establishment of vascular lumens. However, it remains plausible that in the continued absence of circulation, lumens may become unstable and collapse as has been demonstrated previously (Chen et al., 2012; Kochhan et al., 2013).

To investigate lumen morphogenesis at the cellular level, we next coupled whole mount in situ hybridization and immunohistochemistry to investigate the cellular morphology prior to and following lumen formation. In one-cell stage embryos, we first injected mRNA encoding GFP tagged to the small peptide CAAX which results in the ubiquitous sequestration of GFP to the cell membrane (MEM-GFP) and therefore enabled us to visualize cell-cell boundaries. We performed in situ hybridization with *Erg* to label our endothelial cell populations and at stage 33, we can identify angioblasts of the posterior cardinal veins based on expression of *Erg* (Figure 3.2A). Additionally, the posterior cardinal veins develop between the somites and kidneys, anatomical landmarks that have a characteristic appearance and therefore can also be used to identify the appropriate cells. Angioblasts of the dorsal aorta are more easily identified as they are arranged ventral to the hypochord and right on top of the endoderm (Figure 3.2B). At this stage, angioblasts can be seen in close contact to one another as a cord-like structure. In accordance with this observation, angioblasts of the mouse dorsal aorta are also initially arranged as a cord prior to lumen formation (Strilic et al., 2009). The angioblasts of the posterior cardinal veins appear to have a polygonal cobblestone-like morphology while dorsal aorta angioblasts appear slightly narrower and more elongated (Figure 3.2A-B). In agreement with the overall vessel morphology shown previously, at stage 36 vascular lumens of all vessels become discernible and are accompanied by alterations to endothelial cell morphology (Figure 3.2C-D). Similar to the mouse dorsal aorta, in *Xenopus*, the posterior cardinal vein and dorsal aorta cells

become narrower and more elongated likely in order to accommodate the developing luminal compartment. Taken together, thus far it appears that *Xenopus* lumen formation is facilitated by endothelial cell shape changes indicating this is a conserved event between amphibians and mammals.

In addition to cell morphology alterations, studies in human tissue culture models, zebrafish, and mouse have elucidated additional molecular determinants such as the role of cell-cell junctions and establishment of apicobasal polarity to play an important role during vascular lumen formation. To that end, we next examined the expression and distribution of cell junction molecules as a function of time. Tight junctions between endothelial cells during early stages of vessel assembly have been reported in zebrafish (Jin et al., 2005). We first looked at the expression of zonula occludens-1 (ZO-1), a well-established marker of tight junctions ((Dejana et al., 2009)+more). In addition to expression in endothelial cells, ZO-1 labels tight junctions in other cell types such as the kidney and epithelium. At stage 33, ZO-1 is expressed in a discrete dot-like pattern between *Erg*-expressing angioblasts of the posterior cardinal veins (Figure 3.3A). Multiple ZO-1-containing puncta are observed at the cell-cell interface indicating contacts between the cells. The junctions are redistributed but are maintained at points of cell-cell contact at stage 36 when lumens have formed (Figure 3.3B). In the dorsal aorta at stage 33, ZO-1 is also expressed in discrete points between angioblasts and sometimes also appears in a linear pattern potentially reflecting the more elongated morphology of the dorsal aorta angioblasts compared to those of the posterior cardinal veins (Figure 3.3C). At stage 36, the dorsal aorta luminal compartment is surrounded by endothelial cells maintaining ZO-1-containing tight junctions, similar to the veins (Figure 3.3D).

Claudin-5 is another marker of tight junctions that has been shown to be restricted to cell types of the cardiovascular lineage (Morita et al., 1999). Examination of Claudin-5 expression in the posterior cardinal veins and dorsal aorta reveals that in contrast to ZO-1, Claudin-5 is absent from tight junctions between angioblasts at stage 33 (Figure 3.4A, C). However, once lumens have formed, Claudin-5 is distinctly detected at points of cell-cell contact in both the veins and the aorta (Figure 3.4B, D). Taken together, these results indicate that tight junctions are hierarchically assembled, with expression of ZO-1 marking tight junctions prior to as well as following lumen formation and Claudin-5 marking tight junctions only once lumens are evident potentially denoting mature and differentiated vessels as opposed to immature angioblasts. A similar spatial and temporal expression pattern has also been noted in zebrafish vessels (Jin et al., 2005).

In mouse and zebrafish, it has been shown that the driving force between cell-cell separation during lumen formation is the establishment of apicobasal polarity. Specifically, the apical or luminal surface is defined by the localization of certain markers, including moesin and CD34 sialomucin podocalyxn-like protein (PODXL), to this surface even prior to cell-cell separation (Strilic et al., 2009; Wang et al., 2010). To address whether a similar mechanism takes place during *Xenopus* lumen formation, we examined the distribution of the apical marker, atypical protein kinase C-zeta (aPKCζ), which is known to be expressed in endothelial cells as well as epithelial tissues such as the kidney (Chen and Zhang, 2013; Joberty et al., 2000; Koh et al., 2008). At stage 33, aPKCζ can be detected on the apical surface of the kidney but is absent from the basal surface (Figure 3.5A). However, aPKCζ expression is absent from either surface in *Erg*-expressing endothelial cells of the posterior cardinal veins implying that apical polarity is

not established prior to the emergence of lumens (Figure 3.5A). In mouse and zebrafish, localization of markers to the apical surface persists after the cells separate and a lumen is formed. In contrast, in stage 36 *Xenopus* posterior cardinal veins, aPKCζ continues to be absent from the luminal surface however is maintained in the kidney (Figure 3.5B). aPKCζ expression is also absent at both stages of dorsal aorta lumen development (Figure 3.5C-D). These results show that apical polarity does not appear to be a determinant of vascular lumen formation in *Xenopus*.

In addition to apical polarity, a hallmark of mature vessels is the deposition of distinct proteins into the basement membrane surrounding the basal surface of endothelial cells. These proteins include laminin, collagen, and fibronectin which play a role in recruiting mural cells such as smooth muscle cells to endothelial cell-lined tubes as well as provide cues instructing vessels to stay quiescent or initiate sprouting (Davis and Senger, 2005; Hynes, 2007; Stratman et al., 2009). Laminin is expressed in epithelial tissues in addition to endothelial cells and can be detected on the basal surface of the kidney in *Xenopus* but not on the apical surface (Figure 3.6A). Intriguingly, in stage 36 posterior cardinal veins and aortas, laminin is not only expressed on the basal surface of the vessels but is also enriched on the apical surface (Figure 3.6A-B). Furthermore, staining of the veins and aorta with fibronectin also reveals both basal and apical expression (Figure 3.6C-D). Taken together with the aPKCζ staining, these results indicate that unlike other species, *Xenopus* vessels are not in fact polarized and lumens likely form via a mechanism independent of initial establishment of apicobasal polarity.

We and others have previously reported that the small ECM protein Epidermal Growth Factor-Like domain 7 (EGFL7) is required for vascular lumen formation (Charpentier et al., 2013a; Parker et al., 2004). However, the underlying basis for impaired lumen formation in the absence of EGFL7 remains unclear. Time course analysis by in situ hybridization with *Erg* indicates that angioblasts have properly migrated and are correctly positioned at the locations of the paired posterior cardinal veins and the dorsal aorta in stage 33 EGFL7-depleted *Xenopus* embryos indicating that initial specification and migration of angioblasts is not defective (Figure 3.7A). However, at stage 36, EGFL7-depleted angioblasts fail to separate and neither the posterior cardinal vein or dorsal aorta lumens form (Figure 3.7B). At the cellular level, EGFL7-depleted angioblasts of the posterior cardinal veins have a comparable polygonal cell morphology to control embryos prior to lumen formation at stage 33 (Figure 3.7C). In addition, depletion of EGFL7 did not affect the cellular morphology of dorsal aorta angioblasts at stage 33 and they remain in a slightly narrower, elongated shape similar to control cells (Figure 3.7E). However at stage 36, EGFL7-depleted posterior cardinal vein angioblasts fail to change their cell shape and remain cobblestone-like (Figure 3.7D). Accordingly, EGFL7-depleted dorsal aorta angioblasts also fail to undergo further elongation that typically accompanies lumen formation (Figure 3.7F). Taken together, these results indicate that EGFL7 is required for the proper cell shape changes underlying formation of vascular lumens.

To investigate the underlying basis for failed lumen formation in the absence of EGFL7, we first examined the distribution of cell junctions in EGFL7-depleted angioblasts. Staining of the posterior cardinal vein angioblasts with ZO-1 at stage 33 did not reveal any noticeable differences in tight junction localization compared to control embryos (Figure 3.8A). ZO-1-

containing junctions appeared as distinct puncta between adherent angioblasts. Similarly, ZO-1 localization appeared normal in stage 33 EGFL7-depleted dorsal aorta angioblasts (Figure 3.8C). However, at stage 36, EGFL7-depleted angioblasts continued to remain attached to each other as evidenced by the retention of tight junctions between cells (Figure 3.8B, D). Therefore, it appears that junctions do not properly redistribute away from the cord center in the absence of EGFL7. Further analysis of tight junctions by Claudin-5 expression indicated that prior to lumen formation, Claudin-5 is absent from tight junctions between angioblasts of the posterior cardinal vein and dorsal aorta, similar to control embryos (Figure 3.9A, C). Interestingly, despite the impaired formation of lumens in stage 36 posterior cardinal veins or dorsal aortas, Claudin-5-containing tight junctions become apparent between EGFL7-depleted angioblasts (Figure 3.9B, D). While the onset of Claudin-5 does not appear to be altered in the absence of EGFL7, these results raise the possibility that EGFL7-depleted angioblasts may be delayed in either undergoing appropriate cell shape changes or resolving ZO-1-containing tight junctions. Therefore, by the time lumens should be generated (stage 36), additional Claudin-5-containing tight junctions are formed potentially prohibiting any further attempts for the cells to separate and leaving them "stuck" together.

Discussion

The transition from cords of angioblasts into functional vascular tubes is a dynamic morphogenetic process requiring cells to alter their behaviors to promote the expansion of a luminal compartment. Here we have demonstrated some of the cellular and molecular determinants underlying lumen formation of major vessels in *Xenopus*. Following specification of the endothelial lineage from the dorsal lateral plate mesoderm and subsequent migration of angioblasts to the position of the dorsal aorta, angioblasts of the posterior cardinal veins and

aorta are initially found adhered to one another through the formation of ZO-1-containing tight junctions. In addition, the cells exhibit a cobblestone-like appearance at this stage further indicative of immature angioblasts. These initial events appear to be highly conserved as similar cord-like structures have been observed in zebrafish and mouse major vessels (Jin et al., 2005; Strilic et al., 2009). In all three species, the presence of lumens is accompanied by elongation of cell shape and redistribution of junctions away from the cord center. Furthermore in *Xenopus*, we have shown that lumen-containing vessels are also characterized by the formation of additional tight junctions expressing Claudin-5. In zebrafish, ZO-1 expression also precedes Claudin-5 expression potentially indicating that Claudin-5 marks more mature vessels (Jin et al., 2005). However, while Claudin-5 expression was restricted to the arterial lineage in zebrafish, we did not observe a difference in expression between arteries and veins in *Xenopus*.

The most surprising finding during *Xenopus* lumen formation is that apicobasal polarity does not seem to established. While enrichment of makers such as moesin, CD34 sialomucin podocalyxn-like protein (PODXL), and non-muscle myosin II to the apical surface is evident even as early as the vascular cord stage in mouse and zebrafish, we fail to ever detect apical expression of aPKC in vessels prior to or following lumen formation (Nielsen and McNagny, 2008; Strilic et al., 2009; Wang et al., 2010). This finding is fascinating for a number of reasons. First, depletion of proteins that localize to the apical membrane such as moesin and PODXL in mouse or zebrafish results in significantly impaired lumen formation indicating that their presence at the apical surface is required for cell-cell separation events (Strilic et al., 2009; Wang et al., 2010). Second, the adherens junction molecule VE-Cadherin has also been shown to play a role in establishing vessel apicobasal polarity and in the absence of VE-Cadherin, vessels display

non-specific and abnormal localization of apical and basal markers and lumens fail to form properly (Lampugnani et al., 2010; Strilic et al., 2009; Wang et al., 2010). Third, it was shown that the negative charge of the sialic acids on PODXL is the primary force responsible for cell-cell repulsion. Collectively, these previous studies implicate a strong role for polarity in dictating cell behavior and establishing proper vessel structure so our finding that lumens in *Xenopus* form despite enrichment of markers to the apical surface indicates that lumens must arise in a polarity-independent manner. A recent study that uncovered a novel mechanism of lumen formation in zebrafish in which endothelial cells collectively migrate to wrap around a luminal compartment also noted the absence of polarity markers thus supporting a polarity-independent mechanism (Helker et al., 2013). However, what then is the driving force underlying cell-cell separation in *Xenopus*? We also found that basement membrane markers are atypically deposited in *Xenopus* vessels. Laminin and fibronectin are not only expressed on the basal surface of the vessels but also on the apical surface in contrast to findings in zebrafish and mouse (Jin et al., 2005; Xu and Cleaver, 2011; Xu et al., 2011). Interestingly, invertebrates do not have endothelial cells but still contain a rudimentary vascular system which includes a dorsal aorta (Hartenstein and Mandal, 2006). In amphioxus, the dorsal aorta arises on top of a basement-membrane lined endoderm and unlike mammalian vessels, the lumen of the amphioxus aorta is lined with the basement membrane protein laminin (Kucera et al., 2009). These findings suggest that endothelial cells arose as a consequence of evolution to serve as a barrier between basal and apical surfaces. Thus, the absence of this polarized endothelium in *Xenopus* may signify the remnants of an early ancestor that survived the course of vertebrate evolution. The study in amphioxus further suggests that lumens may be generated by the action of phagocytic blood cells that degrade the extracellular matrix. It remains to be established whether this is the case in *Xenopus* or whether

cell-cell repulsion occurs via opposing charges similar to PODXL. It is also noteworthy that the major vessels in *Xenopus* arise in close association with basement membranes of other organs; for example, the kidney in the case of the posterior cardinal veins and the gut endoderm in the case of the dorsal aorta. It is therefore possible that these adjacent basal surfaces may play some role in the shaping of vascular tubes perhaps by providing a scaffold on which to assemble or in more direct ways by means of cell-cell crosstalk. Our results call for future studies aimed at examining the direct role of polarity, if any, in establishing vascular lumens in *Xenopus*. The continuous generation of specific antibodies in *Xenopus* will also help in addressing the localization of other known apical membrane markers.

We have further delineated the underlying basis for the lumen formation defects observed in EGFL7-depleted embryos. Angioblasts appeared indistinguishable between control and EGFL7-depleted embryos at stage 33 before the commencement of lumen formation. EGFL7-depleted angioblasts maintained tight junctions expressing ZO-1 between each other and exhibited the characteristic polygonal cellular morphology indicative of cord-like structures. However, in the absence of EGFL7, angioblasts fail to elongate and resolve their junctions away from the cord center resulting in the absence of vascular lumens in the majority of cases. These findings are in line with the published literature. In EGFL7-depleted zebrafish, angioblasts of the major vessels also fail to redistribute ZO-1-containing junctions and thus remain coalesced together (Parker et al., 2004). Although the exact mechanisms underlying cell shape remain to be elucidated, it has been shown that Rho GTPase signaling is a major factor in modulating the cytoskeleton. In fact, in the mouse dorsal aorta, impaired Rho kinase (ROCK) signaling was associated with failure of cells to properly elongate and subsequently form lumens (Strilic et al.,

2009). We have recently demonstrated that EGFL7 lies upstream of the RhoA pathway wherein EGFL7-depleted human endothelial cells exhibit significantly reduced RhoA mRNA as well as protein levels leading to altered cell morphology and aberrant focal adhesion formation (Charpentier et al., 2013a). Thus in line with these results, the failure of EGFL7-depleted angioblasts to undergo proper cell shape changes may be attributed to dysregulation of Rho signaling. However, it still remains to be determined how Rho signaling influences lumen formation in the context of the embryo.

The finding that Claudin-5 becomes expressed in tight junctions of EGFL7-depleted embryos despite the absence of a visible vascular lumen raises a number of questions and ideas. Does Claudin-5 mark mature vessels or differentiated endothelial cells? Thus the absence of Claudin-5 in stage 33 angioblasts and its onset at stage 36 in both control and EGFL7-depleted embryos would imply that Claudin-5 might serve as a marker of differentiated endothelial cells but not necessarily of functional vessels. This would therefore indicate that EGFL7 is not required for endothelial cell differentiation but rather the processes and behaviors associated with remodeling of the cells into distinct, perfusable vessels. However, studies on Claudin-5 strongly implicate its role in maintaining vascular barrier function, particularly that of the blood-brain-barrier (BBB). In zebrafish, Claudin-5 is highly expressed in endothelial cells of the brain vasculature at a stage when the BBB is established consistent with the presence of a vascular barrier (Xie et al., 2010). Furthermore, Claudin-5-null mice do not exhibit any defects in vasculogenesis but at later stages display selective BBB defects further implying that only mature vessels express and require Claudin-5 within tight junctions (Nitta et al., 2003). Finally, Jin et al. and colleagues demonstrated that zebrafish angioblasts express ZO-1 prior to tube

formation and the onset of Claudin-5 expression coincides with the emergence of vascular lumens (Jin et al., 2005). Therefore, it is more likely that Claudin-5 becomes expressed in tight junctions once vessels require a barrier that is impermeable to diffusion of soluble particles. However, EGFL7-depleted embryos do not even have vascular lumens in need of gate-keeping yet express Claudin-5. We propose that the onset of Claudin-5 is temporally regulated and its appropriate expression at stage 36 likely reflects a delay in EGFL7-depleted angioblasts from undergoing cell shape changes or resolving cell-cell junctions in a timely manner. It is possible that the formation of Claudin-5-expressing tight junctions may even further exacerbate the defects associated with EGFL7-depletion as they may in fact further restrict the cells from altering their morphologies or prevent them from re-localizing these new junctions in addition to the preexisting ZO-1-containing junctions away from the cord center. Consequently, EGFL7-depleted angioblasts may end up being "stuck" together and unable to generate lumens. It would be interesting to note whether there are differences in the stability of ZO-1 versus Claudin-5-containing junctions, and whether the intracellular mechanisms responsible for the break down and reformation of each are distinct.

We have for the first time demonstrated how vascular lumens arise in the major vessels in *Xenopus*. Although some of the steps in lumen formation are conserved between *Xenopus* and mammals, we have also discovered some novel features in *Xenopus* vessels, such as the lack of distinct apical and basal surfaces, that may predate back to the simple cardiovascular systems of invertebrates. Thus *Xenopus* may serve as a useful model in which to study the evolutionary basis of endothelial cell development. Furthermore, we have performed detailed analysis of the lumen formation defects associated with EGFL7-depletion. These studies have raised interesting

questions about the expression of tight junction molecules during early vascular development in the embryo and how the distribution of tight junctions may be affected by extracellular matrix factors.

Figure 3.1. Time course analysis of lumen formation in *Xenopus*. (A) In situ hybridization was performed using the endothelial-specific probe *Erg*. At stage 33, angioblasts are arranged in the positions of the future posterior cardinal veins (PCV; bottom squares) and dorsal aorta (DA; top square). **(B)** At stage 36, lumens surrounded by *Erg*-expressing cells are evident. **(C)** Lumens continue to expand in size by stage 39. **(D)** In situ hybridization using the *Erg* probe demonstrates that un-manipulated embryos at stage 36 display normal lumens of the posterior cardinal veins and dorsal aorta. **(E)** Explants in which the head and heart have been removed at a stage prior to heart development also display normal lumens by stage 36 indicating that circulation is not a requirement for initial establishment of vascular lumens. Boxes correspond to areas magnified to 40x as seen in the bottom panels.

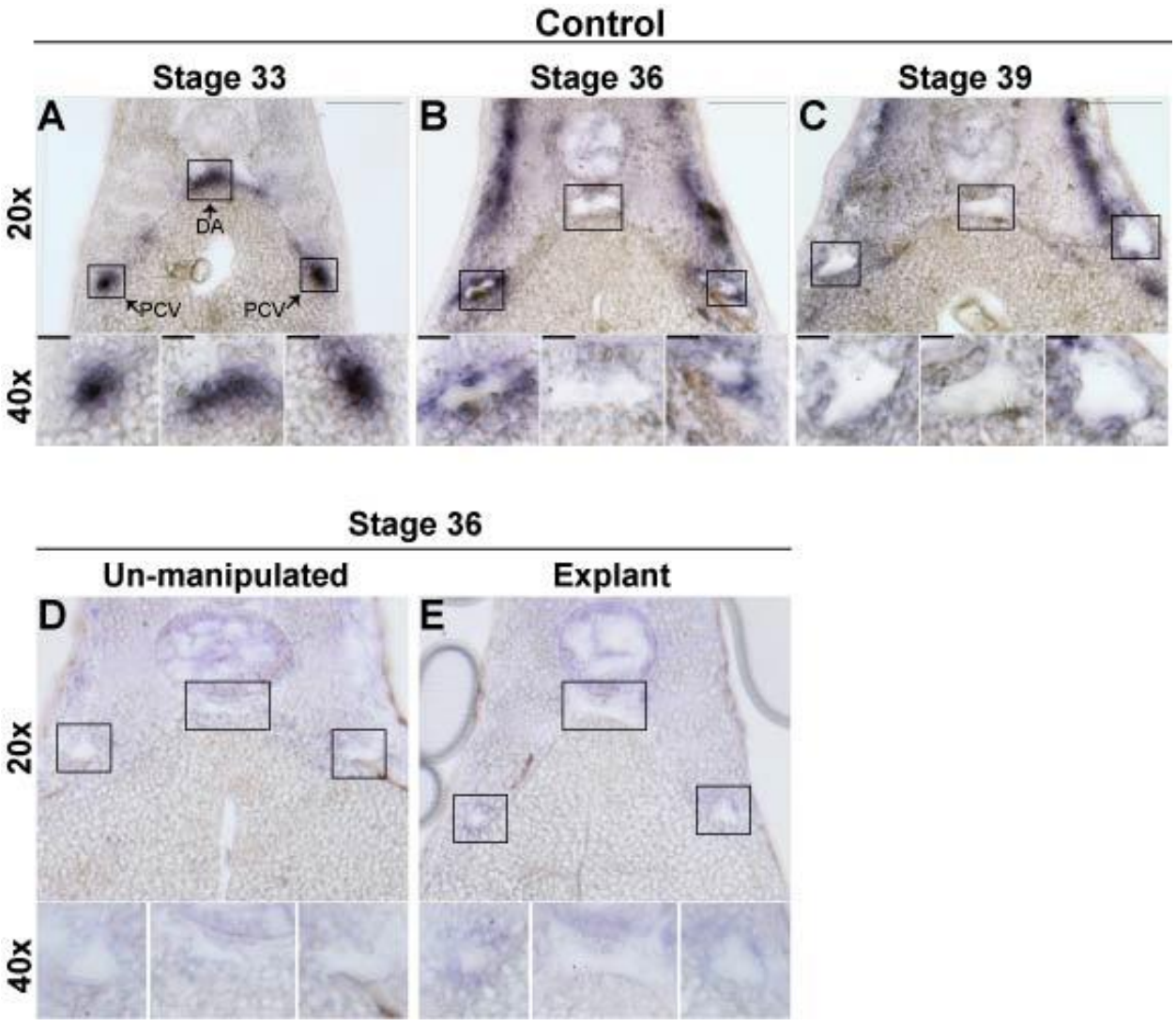

Control
Stage 33
Stage 36
Stage 39
A
B
C
20x
40x
DA
PCV
PCV
Stage 36
Un-manipulated
Explant
D
E
20x
40x

Figure 3.2. Cellular morphology of angioblasts undergoing lumen formation. (A) In situ hybridization using the *Erg* probe was performed on wildtype embryos injected with MEM-GFP RNA. At stage 33, polygonal cobblestone-like angioblasts can be detected in the *Erg*-expressing posterior cardinal vein (PCV) region. **(B)** At stage 36 when posterior cardinal vein lumens become discernible, angioblasts elongate their shape to accommodate the developing luminal compartment. Dotted lines correspond to angioblasts while solid lines correspond to the kidney. **(C)** In the dorsal aorta (DA), angioblasts appear slightly narrower in size compared to posterior cardinal vein angioblasts at stage 33. **(D)** Dorsal aorta angioblasts also undergo further elongation of their shape to accommodate the lumen. Dotted lines correspond to angioblasts while solid lines correspond to the hypochord.

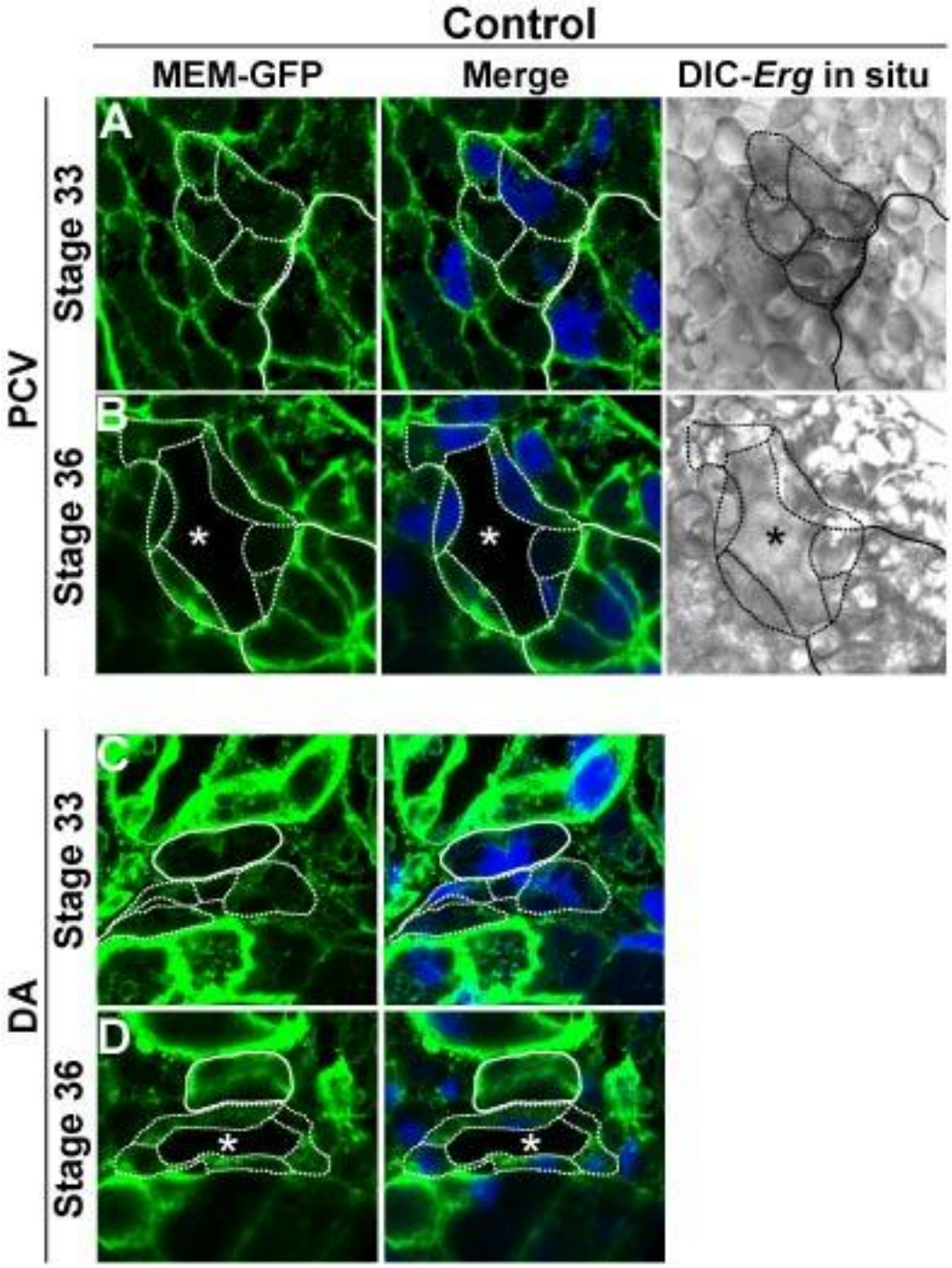

Control
MEM-GFP
Merge
DIC-*Erg* in situ
PCV
Stage 33
Stage 36
A
B
C
D
DA
Stage 33
Stage 36

Figure 3.3. Distribution of ZO-1 tight junctions during lumen formation. (A) In situ hybridization using the *Erg* probe (DIC) was performed on wildtype embryos injected with MEM-GFP RNA followed by immunohistochemistry using GFP (green) and ZO-1 (red) antibodies. Tight junctions can be found along cell-cell contact area between *Erg*-expressing angioblasts in stage 33 posterior cardinal veins (PCV). **(B)** ZO-1 tight junctions redistribute away from the cord center but remain between cells once posterior cardinal vein lumens have formed by stage 36. **(C)** Tight junctions are also found between angioblasts of the dorsal aorta (DA) at stage 33. **(D)** Tight junctions are redistributed between dorsal aorta cells during lumen formation at stage 36.

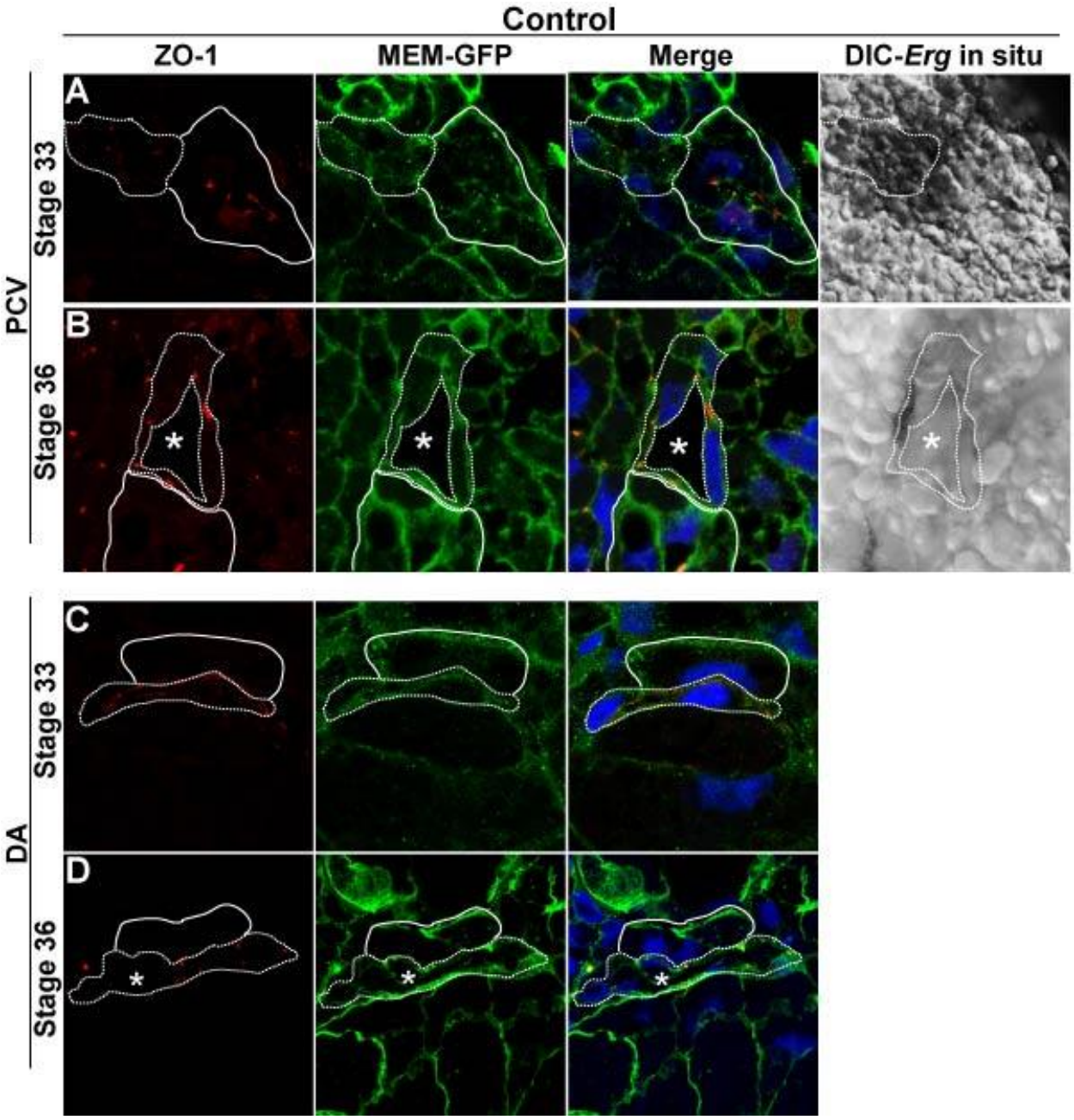

Control
ZO-1
MEM-GFP
Merge
DIC-Erg in situ
PCV
Stage 33
Stage 36
DA
Stage 33
Stage 36
A
B
C
D

Figure 3.4. Distribution of Claudin-5 tight junctions during lumen formation. (A) In situ hybridization using the *Erg* probe (DIC) was performed on wildtype embryos injected with MEM-GFP RNA followed by immunohistochemistry using GFP (green) and Claudin-5 (red) antibodies. Claudin-5 expressing tight junctions are absent from angioblasts of the posterior cardinal vein (PCV) at stage 33. **(B)** The formation of Claudin-5 containing tight junctions becomes apparent between posterior cardinal vein angioblasts once lumens are formed at stage 36. **(C)** Claudin-5 containing tight junctions are also absent from dorsal aorta (DA) angioblasts at stage 33. **(D)** Claudin-5-containing tight junctions are formed once dorsal aorta lumens develop.

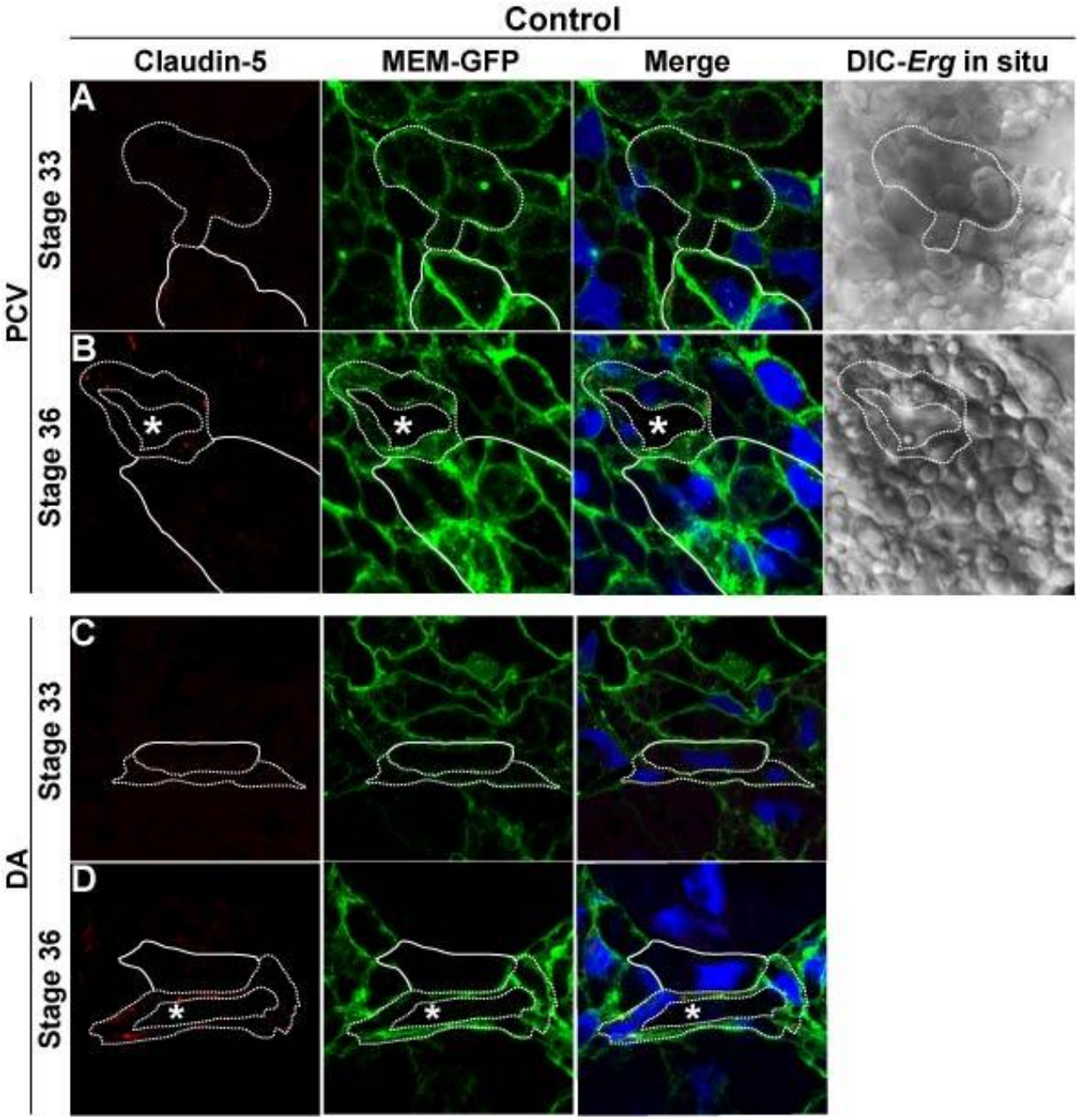

Control
Claudin-5
MEM-GFP
Merge
DIC-*Erg* in situ
PCV
Stage 33
Stage 36
DA
Stage 33
Stage 36
A
B
C
D

Figure 3.5. atypical PKCζ is not enriched on the apical surface of vessels in *Xenopus.* (A) In situ hybridization using the *Erg* probe (DIC) was performed on wildtype embryos injected with MEM-GFP RNA followed by immunohistochemistry using GFP (green) and atypical PKCζ (aPKCζ; red) antibodies. While aPKCζ is expressed on the apical surface of the kidney, apical enrichment in stage 33 angioblasts is not apparent in the posterior cardinal veins (PCV). **(B)** By stage 36, aPKCζ is still absent from the apical surface in posterior cardinal veins with lumens.

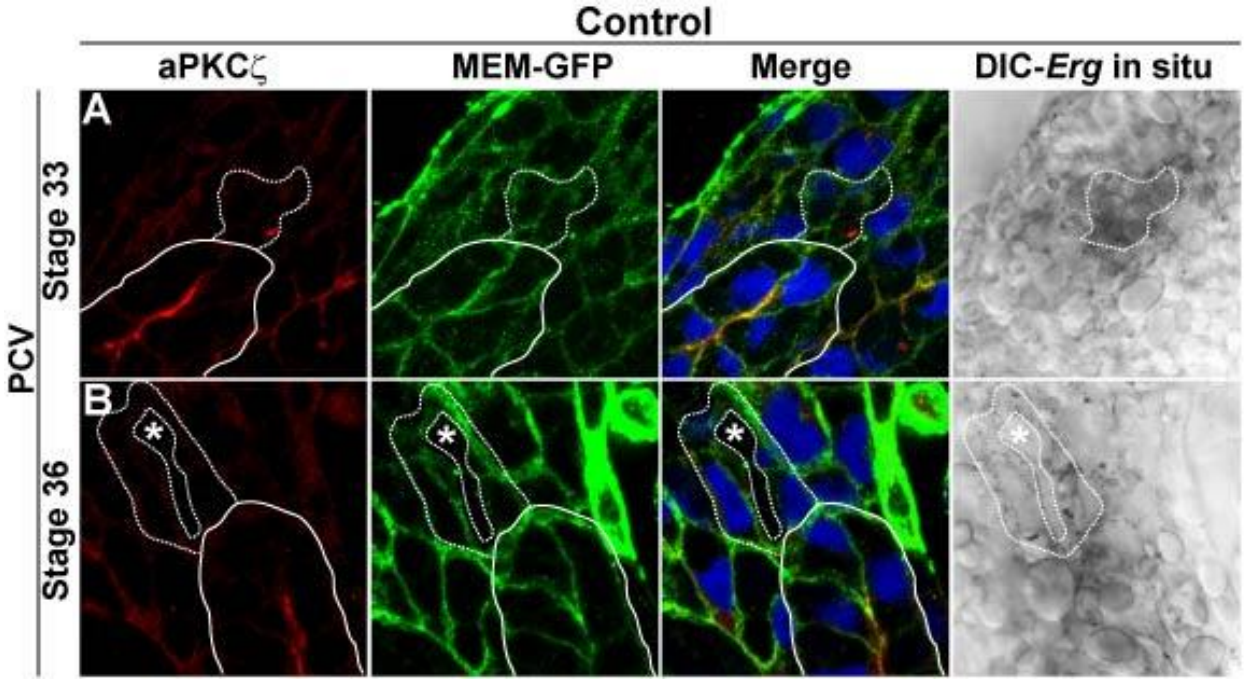


Figure 3.6. Basement membrane proteins are not exclusively deposited on the basal surface of vessels in *Xenopus*. (A) In situ hybridization using the *Erg* probe (DIC) was performed on wildtype embryos injected with MEM-GFP RNA followed by immunohistochemistry using GFP (green) and laminin (red) antibodies. Laminin can be detected on the basal surface of the kidney but is also deposited on the apical surface of the posterior cardinal veins (PCV) at stage 36. **(B)** Laminin can also be detected on the apical surface of the dorsal aorta (DA). **(C)** Fibronectin (red) is also deposited on the apical and basal surfaces of stage 36 posterior cardinal veins.

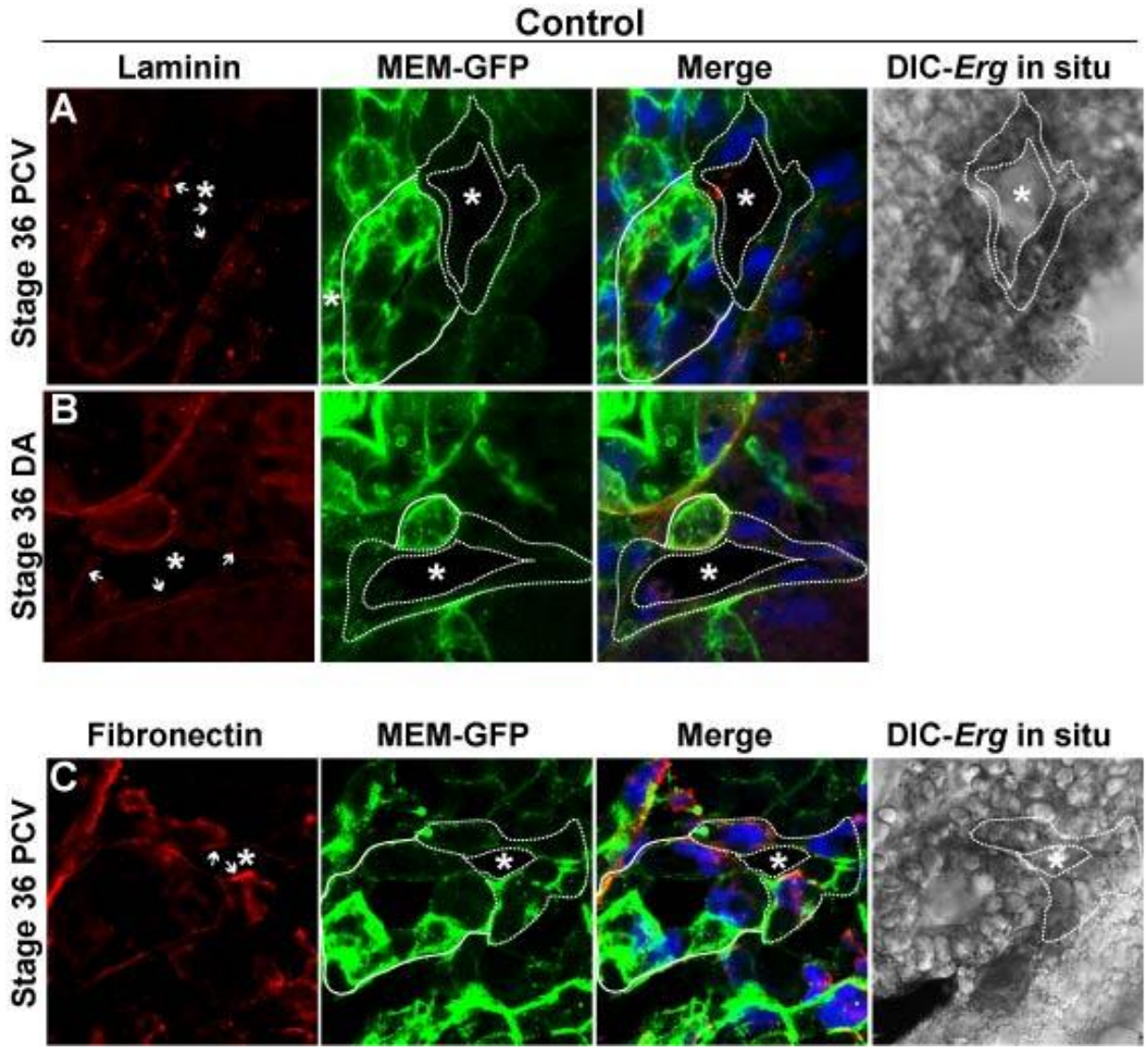


Figure 3.7. Time course analysis of lumen formation in EGFL7-depleted embryos. (A) In situ hybridization was performed using the endothelial-specific probe *Erg*. At stage 33, angioblasts of EGFL7-depleted embryos are correctly positioned at the future posterior cardinal veins (PCV; bottom squares) and dorsal aorta (DA; top square). **(B)** At stage 36, EGFL7-depleted angioblasts fail to properly separate and generate vascular lumens. **(C)** In situ hybridization using the *Erg* probe (DIC) was performed on EGFL7-depleted embryos injected with MEM-GFP RNA (green). At stage 33, EGFL7-depleted angioblasts of the posterior cardinal veins display a normal, polygonal morphology similar to controls. **(D)** At stage 36, EGFL7-depleted cells do not undergo proper cell shape changes and posterior cardinal vein lumens fail to form. **(E)** The cellular morphology of EGFL7-depleted angioblasts in stage 33 dorsal aortas is not significantly altered compared to control. **(F)** However in stage 36 EGFL7-depleted embryos at stage 36, the lack of discernible dorsal aorta lumens is accompanied by angioblasts that failed to elongate.

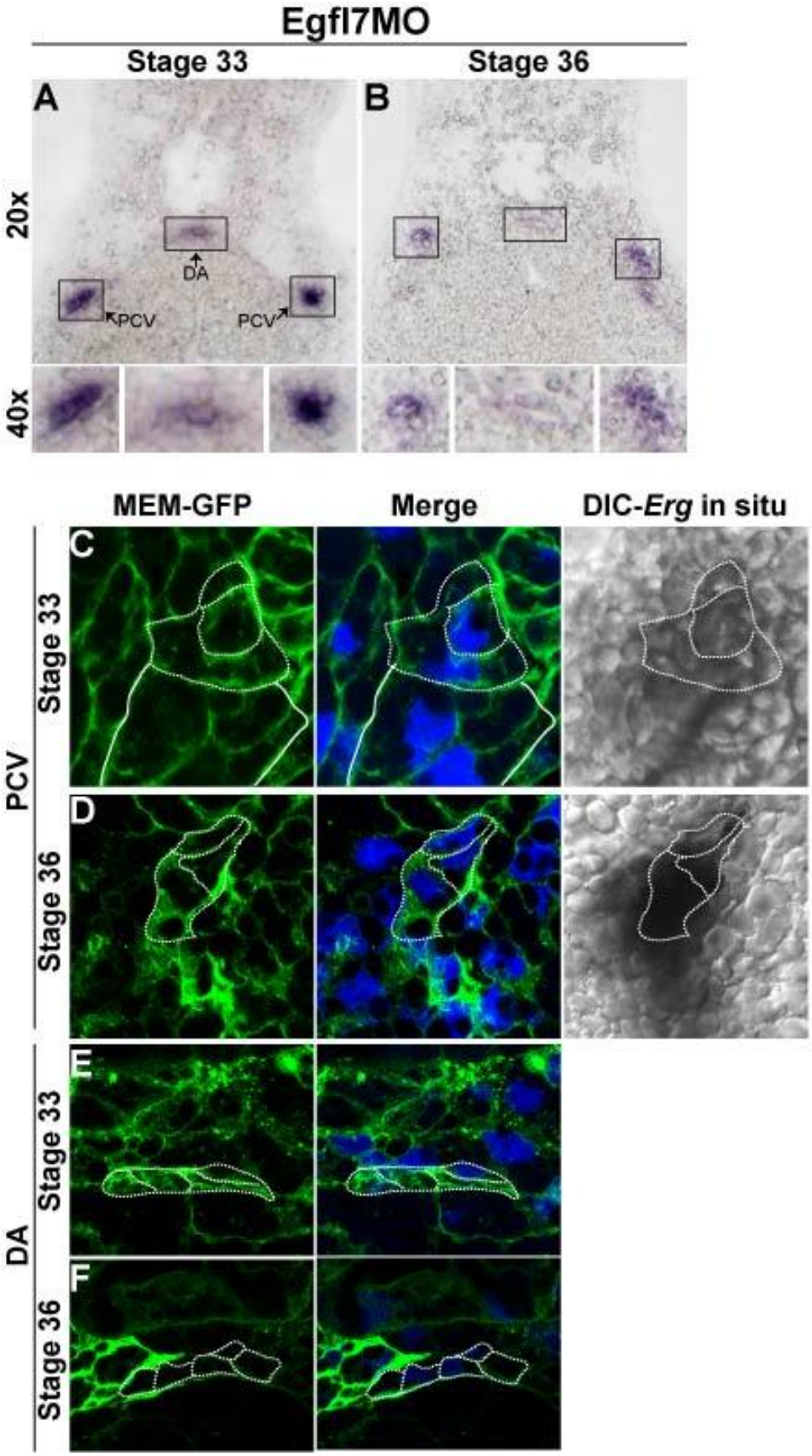

Egfl7MO
Stage 33
Stage 36
A
B
20x
40x
DA
PCV
PCV
MEM-GFP
Merge
DIC-Erg in situ
PCV
Stage 33
Stage 36
DA
Stage 33
Stage 36
C
D
E
F

Figure 3.8. Distribution of ZO-1 tight junctions in EGFL7-depleted vessels. (A) In situ hybridization using the *Erg* probe (DIC) was performed on wildtype embryos injected with MEM-GFP RNA followed by immunohistochemistry using GFP (green) and ZO-1 (red) antibodies. Tight junctions can be found along cell-cell contact area between *Erg*-expressing angioblasts in stage 33 posterior cardinal veins (PCV). **(B)** EGFL7-depleted angioblasts of the posterior cardinal veins fail to redistribute ZO-1 tight junctions away from the cord center and vascular lumens fail to form. **(C)** Tight junctions are also found between angioblasts of the dorsal aorta (DA) at stage 33. **(D)** Failed lumen formation in EGFL7-depleted dorsal aortas is accompanied by retention of ZO-1 tight junctions between angioblasts at stage 36.

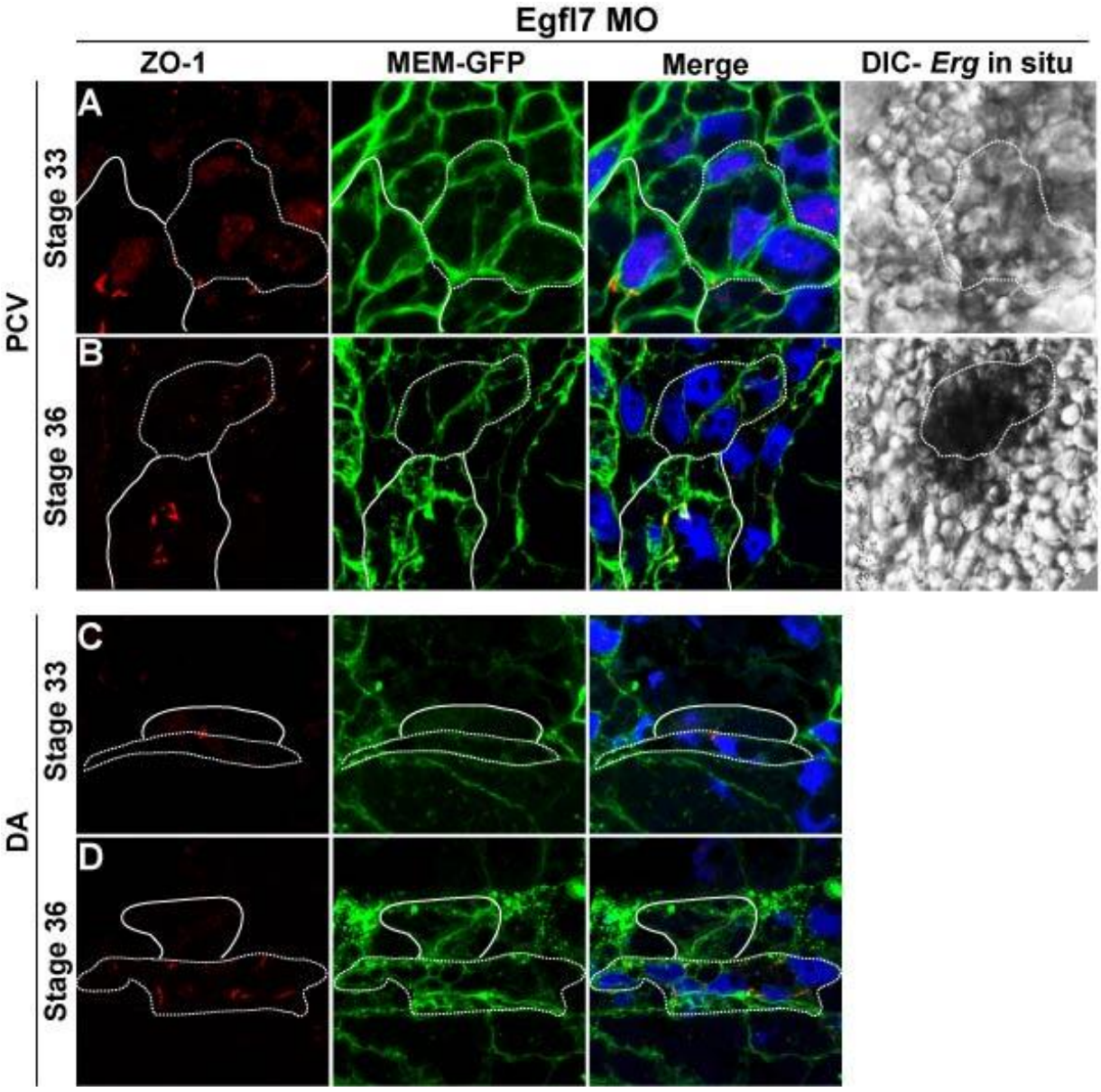
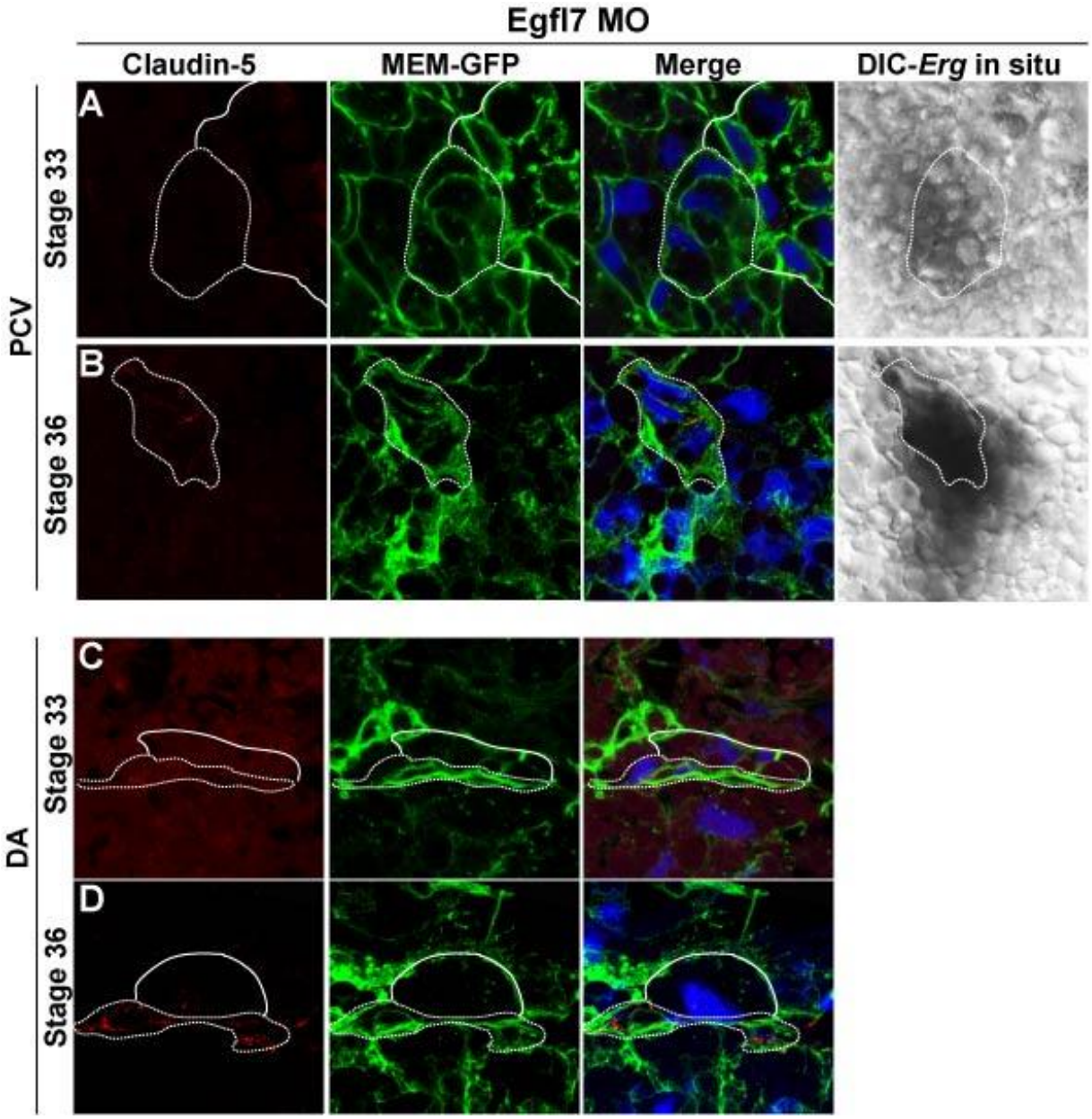

Egfl7 MO
ZO-1
MEM-GFP
Merge
DIC- *Erg* in situ
PCV
Stage 33
Stage 36
DA
Stage 33
Stage 36
A
B
C
D

Figure 3.9. Distribution of Claudin-5 tight junctions in EGFL7-depleted vessels. (A) In situ hybridization using the *Erg* probe (DIC) was performed on EGFL7-depleted embryos injected with MEM-GFP RNA followed by immunohistochemistry using GFP (green) and Claudin-5 (red) antibodies. Claudin-5 expressing tight junctions are absent from angioblasts of the posterior cardinal vein (PCV) at stage 33. **(B)** Claudin-5 tight junctions are apparent between EGFL7-depleted angioblasts of the posterior cardinal veins despite the lack of discernible lumens. **(C)** Claudin-5 expression is absent from tight junctions in stage 33 EGL7-depleted dorsal aortas (DA) similar to controls. **(D)** Claudin-5 tight junctions form between angioblasts in the absence of EGFL7 and in the absence of a vascular lumen


Egfl7 MO
Claudin-5
MEM-GFP
Merge
DIC-*Erg* in situ
PCV
Stage 33
Stage 36
A
B
DA
Stage 33
Stage 36
C
D

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CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

The formation of a highly branched and functional vascular system consisting of capillaries, veins, and arteries requires endothelial cells to carry out many dynamic behaviors including migration, proliferation, sprouting, changes in cell shape, and alterations in cell-cell and cell-matrix adhesion (Eilken and Adams, 2010; Geudens and Gerhardt, 2011; Udan et al., 2013a). Endothelial cells must harness their individual behaviors and unite as a cohort to properly align and pattern vascular structures. A complex interplay among growth factors, cytoskeletal modulators, transcription factors, and intracellular signaling pathways must be coordinated to elicit proper endothelial cell movements for each step of vascular assembly (Marcelo et al., 2013; Park et al., 2013; Patel-Hett and D'Amore, 2011; Vieira et al., 2010). Although many aspects of this interplay have been described, we have yet to uncover networks that more fully account for how perturbations in a given factor, such as a transcription factor, change the expression, distribution, and activity of downstream effectors that result in particular cellular phenotypes.

CASTOR (CASZ1) is an evolutionarily conserved para-zinc finger transcription factor expressed in the cardiovascular system of vertebrate species (Christine and Conlon, 2008; Liu et al., 2006). CASZ1 is required for cardiomyocyte differentiation during *Xenopus* cardiac

development, but further functional studies elucidating the role of CASZ1 in vertebrate development are still needed. In addition to the heart, *Casz1* transcripts are also found within developing *Xenopus* blood vessels, and recent genome-wide association studies have uncovered a link between the human *Casz1* locus and vascular disease risk factors including hypertension and high blood pressure (Christine and Conlon, 2008; Takeuchi et al., 2010). The purpose of this dissertation, therefore, was to examine the mechanisms by which CASZ1 regulates blood vessel development. Using *Xenopus* embryos and a primary human endothelial cell line, we found that CASZ1 is critical for endothelial cell behaviors including adhesion, proliferation, and changes in cell shape associated with vessel assembly, sprouting, and lumen morphogenesis. By performing a screen for CASZ1 transcriptional targets, we found that CASZ1 regulates blood vessel development through the extracellular matrix (ECM) factor *Epidermal growth factor-like domain 7* (*Egfl7*). Moreover, we discovered that the CASZ1/*Egfl7* transcriptional pathway regulates a major signaling cascade directly responsible for cell adhesion and morphology. This dissertation thus comprises a set of studies that more directly link the transcriptional regulation of gene expression to physiological outputs. Furthermore, this dissertation demonstrates that seemingly simple cellular processes, such as alterations in cell-matrix adhesion, require the combined efforts of transcriptional regulators, extracellular factors, and intracellular signaling networks.

Expression and Function of CASZ1 in Endothelial Cells

Consistent with the results of a previous study (Christine and Conlon, 2008), in Chapter 2 we confirmed that CASZ1 is expressed in the vasculature during embryonic development. In addition to its expression in *Xenopus* vessels, we showed that CASZ1 is also expressed in mouse and human endothelial cells, suggesting that the role of CASZ1 in vascular biology is evolutionarily conserved. CASZ1-depleted *Xenopus* embryos display several severe vascular

defects independent of the role of CASZ1 in cardiogenesis. First, the vitelline vein networks of CASZ1-depleted embryos are markedly underdeveloped. Vessels that do form run in a dorsal-to-ventral direction with little branching and intermingling, resulting in a poorly assembled vascular plexus. Second, sprouting of intersomitic vessels from the posterior cardinal veins, a process known to occur via angiogenesis, is delayed or completely stunted. Third, in the absence of CASZ1, major vessels such as the posterior cardinal veins and dorsal aorta fail to generate vascular lumens and thus are not properly remodeled into functional tubes. Studies in human umbilical vein endothelial cells (HUVECs) provide insights into the underlying basis of vascular defects *in vivo*. For example, CASZ1-depleted HUVECs fail to progress through the cell cycle, exhibit a dramatically elongated morphology, and are unable to maintain adhesion to the underlying substrate. Proliferation, cell shape, and cell-matrix adhesion are all critical for proper vascular assembly, sprouting, and lumen morphogenesis (Fryer and Field, 2005; Lebrin et al., 2004; Noseda et al., 2004; Strilic et al., 2009; Wary et al., 2012).

Outstanding questions remain as to whether CASZ1 has specific functions during different steps of vascular assembly that correlate with its expression. For example, although we showed that *Casz1* transcripts localize to vitelline veins, their expression is lower than that in other *Casz1*-expressing tissues such as somites and heart. Therefore, could CASZ1 be expressed in discrete subsets of endothelial cells depending on their current behavior? Is CASZ1 expressed in sprouting endothelial cells during active vascular expansion or is it more broadly distributed in the vasculature? In particular, it would be interesting to carefully examine CASZ1 expression in endothelial cells from the standpoint of angiogenic sprouting. Endothelial cells undergoing angiogenesis display unique characteristics depending on whether they are actively sprouting.

Endothelial "tip cells" guide new vessels by loosening cell-cell adhesions, degrading the surrounding ECM, extending filopodia, and migrating out to form new sprouts in response to a gradient of soluble VEGF (Benedito et al., 2009; Gerhardt et al., 2003; Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007). Endothelial cells that remain behind are known as "stalk cells" that are phenotypically distinct from tip cells, as they maintain the lumen and structure of the parent vessel and actively proliferate to allow the new sprout to extend. Thus, it would be fascinating to examine whether CASZ1 is preferentially expressed in tip versus stalk cells, as this would provide further clues to the precise function of CASZ1 during vascular assembly.

The vitelline vein network in *Xenopus* initially arises via vasculogenesis, but the extent to which this network expands and becomes remodeled by angiogenesis is unknown (Cleaver et al., 1997; Levine et al., 2003). Our studies indicate that angiogenesis occurs within this region based on the following observations: 1) CASZ1-depleted endothelial cells are properly specified and at least initially coalesce into vascular structures within the vitelline vein network; 2) CASZ1-depleted cells successfully migrate and coalesce at proper positions of major vessels; and 3) angiogenic sprouting of intersomitic vessels from properly patterned (but not remodeled) posterior cardinal veins is impaired in CASZ1-depleted embryos. Thus, we speculate that CASZ1 is not required for specification, migration, or initial patterning of vessels but is critical for further expansion by angiogenesis and vascular remodeling. Furthermore, based on the role of CASZ1 in the maintenance of adhesion, proliferation, and lumen morphogenesis, we speculate that CASZ1 may be expressed in endothelial stalk cells. Although CASZ1 expression throughout the major vessels may be required for lumen morphogenesis, CASZ1 expression could also be restricted in a network of small-caliber vessels such as vitelline veins. Although tip cells are

responsible for initiating and extending new sprouts, stalk cells are required for providing connectivity with the parent vasculature and proper vessel architecture, and impaired stalk cell function is associated with tip cell sprout regression (Phng et al., 2009; Sharghi-Namini et al., 2014). Thus, the inability of CASZ1-depleted cells to make new connections may be due to impaired stalk cell behaviors, such as reduced proliferation and failure to undergo proper changes in cell shape to maintain a tubular structure. CASZ1-depleted stalk cells may thus be unable to keep up with tip cells and thereby prevent new vessel formation. Additionally, the characteristic cobblestone appearance of HUVECs is significantly altered in the absence of CASZ1. Although filopodial extensions and motility were not examined in our study, it is tempting to speculate that our findings are indicative of a change in cell fate from a structural stalk cell to a more tip cell-like phenotype.

Although the lack of robust antibodies and methods to visualize the vitelline vein network outside of its endogenous location overlaying a yolky and autofluorescent endoderm creates challenges in precisely characterizing the expression of CASZ1 in *Xenopus* embryos, the sprouting angiogenesis assay described in Chapter 2 is one way to examine CASZ1 localization during multiple steps of angiogenesis (Nakatsu et al., 2003). A more *in vivo* approach would be to employ the mouse retina angiogenesis model that has been extensively used in studies of tip and stalk cell dynamics (Fruttiger, 2007; Gariano and Gardner, 2005; Stahl et al., 2010; Uemura et al., 2006). Nonetheless, the studies described in Chapter 2 reveal a novel role for CASZ1 during vascular assembly and provide a foundation for future studies aimed at deciphering the likely diverse roles played by CASZ1 during each step of vessel development.

CASZ1 Regulates Blood Vessel Assembly and Morphogenesis through its Direct Target *Egfl7*

The studies described in Chapter 2 also aimed to uncover the mechanisms by which CASZ1 regulates vascular development, specifically by validating a putative transcriptional target of CASZ1 identified by a cloning chromatin immunoprecipitation (ChIP) screen from cardiovascular-enriched tissue. *Egfl7* was chosen as the top candidate target given its previously established role in vascular lumen formation and sprouting angiogenesis and because intron 7 of *Egfl7* houses the endothelial-specific microRNA miR-126, which was once heavily investigated for its role in maintaining vascular integrity and promoting angiogenic signaling (Fish et al., 2008; Kuhnert et al., 2008; Parker et al., 2004; Schmidt et al., 2007; Wang et al., 2008). In addition to verifying that CASZ1 endogenously and directly binds to and activates transcription through a 114 bp enhancer element within intron 3 of the *Egfl7* locus, we surprisingly found that CASZ1 is required for *Egfl7* but not miR-126 expression in *Xenopus* endothelial cells. Furthermore, depletion of EGFL7 in *Xenopus* embryos phenocopies the vitelline vein branching and lumen formation defects of CASZ1-depleted embryos, but miR-126 depletion does not affect the initial assembly or remodeling of the vasculature. These studies strongly suggest that in *Xenopus* embryos, miR-126 may be differentially regulated from its host gene via a CASZ1-independent mechanism. Consistent with this possibility, previous studies also show that intronic microRNAs can be transcribed independently of their host genes through alternative promoters (Bell et al., 2010; Monteys et al., 2010).

We further determined whether the regulation of these targets by CASZ1 is evolutionarily conserved. We found that CASZ1 is required for both *Egfl7* and miR-126 expression in HUVECs and that depletion of EGFL7 in HUVECs phenocopies the adhesion and cell

morphology defects associated with the absence of CASZ1. However, only restoration of *Egfl7* levels rescued both adhesion and cell morphology, whereas restoration of miR-126 rescued only adhesion, indicating that these targets may function somewhat independently of each other while still relying on CASZ1 activity, at least in human cells. These results are in line with those of previous studies suggesting that EGFL7 functions early during vascular development to initiate lumen formation, whereas miR-126 functions later in development to maintain the integrity of vascular tubes (Fish et al., 2008; Parker et al., 2004; Wang et al., 2008).

Although we demonstrated that CASZ1 directly binds to and regulates *Egfl7* expression in *Xenopus*, CASZ1 seems to be required for the maintenance but not the onset of *Egfl7* in endothelial cells. Indeed, previous studies demonstrate that the *Egfl7* locus is regulated by additional factors including the Ets transcription factors and GATA-2 (Harris et al., 2010; Le Bras et al., 2010; Wang et al., 2008). Transcription factors responsible for endothelial gene expression can also function cooperatively to regulate transcription (De Val and Black, 2009; De Val S., 2008). It would therefore be fascinating to explore the possibility that CASZ1 functions with other transcription factors to fully govern *Egfl7* expression. Intron 3 of *Xenopus Egfl7* is not well conserved among vertebrate species, implying that CASZ1 may function through an alternative element in mammalian cells. Therefore, when further establishing the mechanisms by which CASZ1 regulates *Egfl7* in endothelial cells, it would be beneficial to map the region of the *Egfl7* locus that requires CASZ1 through luciferase and ChIP assays, preferably in HUVECs. By mapping this domain, we could identify other putative endothelial transcription factor binding sites nearby and test whether CASZ1 acts in tandem with these elements to cooperatively regulate *Egfl7* expression.

CASZ1/*Egfl7* Lies Upstream of the RhoA Signaling Pathway

Beyond characterizing the CASZ1/*Egfl7* transcriptional pathway, the studies described in Chapter 2 also examined how dysregulation of this pathway leads to aberrant endothelial cellular behaviors associated with adhesion and cell shape. These aberrant phenotypes are reminiscent of improper RhoA GTPase signaling, and, indeed, RhoA plays a major role in regulating cytoskeletal dynamics and cell-matrix contacts through the formation of stress fibers and focal adhesions (Burridge and Wennerberg, 2004; Chrzanowska-Wodnicka and Burridge, 1996; Katoh et al., 2011; Vouret-Craviari et al., 2002). We confirmed that in the absence of CASZ1 or EGFL7, RhoA mRNA and protein levels are significantly reduced, leading to impaired formation of stress fibers and mislocalization of focal adhesion proteins away from the cell periphery. Furthermore, restoration of *Egfl7* levels in CASZ1-depleted cells improves the formation of stress fibers and proper distribution of focal adhesion, indicating that the CASZ1/*Egfl7* transcriptional pathway is required for proper RhoA expression and activity. Although RhoA activity is dynamic, it is rare to observe dramatic differences in steady-state levels of total RhoA. Thus, our studies provide a unique model in which to study the mechanisms of *RhoA* transcriptional regulation. The only transcription factor known to directly activate *RhoA* is Myc. Thus, it would be intriguing to examine whether CASZ1/*Egfl7* is responsible for *RhoA* expression through a Myc-mediated pathway. Furthermore, it will be critical to determine whether RhoA is important for vascular assembly and lumen morphogenesis in ways similar to CASZ1 and EGFL7 in *Xenopus*. Because RhoA is not restricted to endothelial cells and plays major roles in morphogenetic cell movements very early during embryogenesis, examining the function of RhoA in *Xenopus* endothelial cells is not simple (Bement et al., 2005; Drechsel et al., 1997; Kim and Han, 2005; Tanegashima et al., 2008). Recent technologies employing a

photoactivatable form of RhoA, however, may improve our ability to modulate RhoA activity in specific tissues at specific time points (Morckel et al., 2012). It would be especially interesting to investigate the role of RhoA during lumen morphogenesis given recent reports indicating that Rho kinase (ROCK) signaling in the mouse dorsal aorta is required for proper changes in cell shape that accompany the formation of the vascular lumen (Strilic et al., 2009).

Distinct Mechanisms of Lumen Formation in *Xenopus*

In Chapter 3, we focused on elucidating the molecular and cellular mechanisms underlying a more specific step during blood vessel development—vascular lumen formation. Although the studies described in Chapter 2 indicate that CASZ1 and EGFL7 are required for vascular lumen formation, virtually nothing is known about how lumens are generated in *Xenopus*. However, recent studies in zebrafish and mice reveal that similar sets of determinants and mechanisms are at play in these species. Endothelial cells initially coalesced into cord-like structures must undergo a series of morphogenetic movements, including the redistribution of cell-cell junctions away from the cord center and the alteration of cell shape to accommodate the luminal compartment. Although the driving force behind these movements appears to be the establishment of apicobasal polarity in the mouse dorsal aorta, we revealed that endothelial cell polarization is not a prerequisite for lumen formation in *Xenopus* major vessels. However, similar to zebrafish and mice, we observed that *Xenopus* endothelial cells reorganize their junctions and elongate during lumen formation, raising the question of what promotes cell-cell separation and cell shape changes in this species? Although our studies suggest that EGFL7 is required for changes in cell shape and proper redistribution of junctions, it is still not clear how EGFL7 accomplishes these functions.

Current imaging techniques and the availability of endothelial-specific transgenic lines make zebrafish a top model for imaging the dynamics associated with vascular development, including lumen formation (Blum et al., 2008; Helker et al., 2013; Herwig et al., 2011; Kamei et al., 2006; Lenard et al., 2013; Wang et al., 2010). The ability to image lumen formation in *Xenopus* would greatly enhance our understanding of the endothelial cell behaviors needed to form functional tubes. Although we lack vascular-specific reporter lines at the time of lumen formation, one possible way to image lumen formation of posterior cardinal veins would be to use an injectable endothelial cell-specific fluorescent dye. As shown in Chapter 3, embryos at the one-cell stage could be injected with an mRNA encoding a form of GFP that sequesters to cell membranes throughout the embryo. Another mRNA encoding GFP tagged to histone H2B could also be co-injected to label cell nuclei. Prior to lumen formation at stage 33, embryos could be anesthetized with a low dose of tricaine and injected with DiI-acetyled (Ac)-LDL, which is specifically taken up by endothelial cells and fluoresces red along the length of the posterior cardinal vein (Levine et al., 2003). We could then immediately visualize dye uptake along this region using a fluorescent microscope, and as lumens open and circulation commences, we could visualize the distribution of the dye in the vasculature. Because posterior cardinal veins form just below the overlying epithelium, we would need to use confocal microscopy to image embryos at the level of veins. Using DiI-Ac-LDL to label endothelial cells and GFP to mark cell membranes and nuclei, we could image the opening of vessel lumens over the course of about six hours (i.e., the time between stages 33 and 36 when embryos are cultured at room temperature). These potential experiments would require optimization, as we do not know how embryos would tolerate being under a laser for an extended amount of time or how easily it would be to visualize lumens forming on the lateral surface of embryos rather than tissue sections. However, these

studies would provide valuable insights into the dynamics and complexity of this process, which currently cannot be gleaned from mere snapshots.

The importance of studying this specific step of vascular development arises from the fact that the delivery of therapeutics to tumor sites is often hindered by a lack of functional and efficient vessels. Tumor vessels are abnormal, leaky, tortuous, and prone to metastatic dissemination of cancer cells (Carmeliet, 2003; Carmeliet and Jain, 2011a). Thus, recent studies are exploring the process of tumor vessel normalization, in which instead of attempting to damage and prune back tumor vessels, the vasculature is conditioned to become more "normal" to permit more efficient drug delivery directly to the tumor (Carmeliet and Jain, 2011b). Understanding the basis for initiating and maintaining vascular lumens may therefore enhance vessel normalization efforts.

Future Directions

Our studies clarify that CASZ1 has a critical function in the assembly and remodeling of blood vessels, in part by regulating its direct target *Egfl7.* In endothelial cells, CASZ1 appears to act as a transcriptional activator. However, the CASZ1 expression domain extends not only to the vasculature but also to the heart, neural tissue, and muscle (Christine and Conlon, 2008; Liu et al., 2006). It is also important to note that the function of CASZ1 in endothelial cells appears to differ from that in the heart. Whereas CASZ1 is critical for endothelial cell proliferation, CASZ1 appears to promote terminal differentiation over progenitor self-renewal in cardiomyocytes (Christine and Conlon, 2008). Furthermore, CASZ1 is described as a tumor suppressor in neuroblastoma cell lines, inhibiting cell growth while inducing differentiation (Liu et al., 2011c; Liu et al., 2011d). Thus, CASZ1 likely acts on unique sets of transcriptional targets

within each type of tissue. As transcription factors can act as activators or repressors depending on the context (Fujimoto et al., 2000; Peng and Jahroudi, 2002; Sakabe et al., 2012), it would be interesting to identify further transcriptional targets of CASZ1 in endothelial cells and other cell types such as cardiomyocytes to determine whether CASZ1 exhibits context-specific functions. Although isolating endothelial cells in *Xenopus* embryos is currently problematic due to difficulty in excising tissue consisting exclusively of vessels and a lack of endothelial-specific reporters, one way to identify additional CASZ1 targets would be to use HUVECs. Data from ChIP-Seq experiments of wildtype HUVECs and RNA-Seq experiments of control versus CASZ1-depleted HUVECs (by shRNA) could be combined to examine the elements to which CASZ1 directly binds and whether CASZ1 regulates these targets via activation or repression mechanisms. Such studies may provide further information about the targets through which CASZ1 may regulate cell cycle progression in HUVECs, as restoration of *Egfl7* levels in CASZ1-depleted cells is unable to rescue proliferation deficits.

Moreover, our understanding of how CASZ1 mediates transcriptional activity is still extremely limited. Processes underlying heart development, such as specification of cardiac progenitor cells, cardiomyocyte differentiation, and patterning and growth, are known to rely on the combined actions of multiple transcription factors in a temporal fashion (Hiroi et al., 2001; Junion et al., 2012; Stennard et al., 2003; Vincentz et al., 2008). Likewise, transcription factors driving endothelial gene programs are known to function cooperatively (De Val and Black, 2009; De Val S., 2008; Pham et al., 2007; Sacilotto et al., 2013; Wei et al., 2009). It would therefore be fascinating to identify the core components of the CASZ1 transcription complex in each of these cellular contexts. These studies would contribute to our understanding of the mechanisms by

which CASZ1 regulates its target genes and may help explain apparent discrepancies in CASZ1 function in different cell types. Proteomic studies greatly facilitate the identification of components of other transcription factor complexes (Kaltenbrun et al., 2013). Thus, the ability to isolate the CASZ1 transcription complex, especially from HUVECs, is not out of reach. Furthermore, the generation of new tools in *Xenopus*, such as the isolation of nuclei tagged in specific cell types (INTACT) method, will enable the enrichment of nuclear CASZ1 transcriptional complexes, particularly from cardiac tissue. Genome editing tools, including TALEN and CRISPR platforms, have also garnered interest in many model organisms including bacteria, yeast, *Drosophila*, *C. elegans*, zebrafish, and mice given their ability to silence or to enhance and tag specific genes. Indeed, the capability of tagging endothelial-specific genes with fluorescent reporters would be valuable for generating a transgenic line in which pure endothelial cell populations can be visualized and isolated for downstream applications such as ChIP-Seq, RNA-Seq, and proteomics.

Another area of interest stemming from this thesis work is elucidating the pathways downstream of EGFL7 activity. Although associated with the ECM, the mechanism by which EGFL7 modulates cellular behavior is still unclear. Moreover, the discovery of the intronic miR-126 in the *Egfl7* locus complicates the analysis of phenotypes associated with loss of EGFL7 (Kuhnert et al., 2008). Previous studies demonstrate that EGFL7 interacts with lysyl oxidases to negatively regulate the deposition of elastin fibers, suggesting that EGFL7 may remodel the ECM so that it is amenable to certain endothelial cell behaviors such as migration or adhesion (Lelievre et al., 2008). Another likely possibility is that EGFL7 functions as a ligand for cell-surface receptors responsible for mediating intracellular signaling cascades. Indeed, EGFL7

competes with delta-like ligand-4 for Notch receptor binding, and a recent study shows that EGFL7 also interacts with integrin αvβ3 (Nichol et al., 2010; Nikolic et al., 2013; Schmidt et al., 2009). Both Notch and integrin signaling are implicated in numerous endothelial cell processes (Brooks et al., 1994; Davis and Bayless, 2003; Friedlander et al., 1995; Funahashi et al., 2008; Hellstrom et al., 2007; Serini et al., 2006; Shawber et al., 2003; van der Flier et al., 2010). In addition to exploring the existence of other molecules that interact with EGFL7, it would be interesting to pursue the outcomes of these interactions. For example, what effectors are downstream of EGFL7/Notch or EGFL7/integrin αvβ3 interactions? One way to address this question would be to map the domains of EGFL7 that are required for interaction. EGFL7 is relatively small, composed of only 278 amino acids, with delineated domains that are implicated in protein-protein interactions of other factors (i.e., EGF) (Fitch et al., 2004). Initially, these studies could be performed by co-immunoprecipitating overexpressed proteins corresponding to EGFL7 deletion constructs with or without Notch or αvβ3 receptors in HEK293 cells, which are much easier to transfect than HUVECs. After the mapping of a domain, we could perform site-directed mutagenesis to abolish the interaction and generate an adenovirus expressing mutated EGFL7. As HUVECs can be successfully co-infected with lentiviral shRNAs and adenovirus as demonstrated in Chapter 2, we could then knock down endogenous EGFL7 levels in HUVECs and introduce mutated EGFL7 via adenovirus, which would presumably prevent the endogenous interaction and fail to rescue a portion of the cellular defects. We could then screen for the effects of this impaired interaction through a proteomics-based comparison between control and mutated EGFL7 cells to identify the downstream effectors responsible for mediating EGFL7-dependent processes. These studies would more clearly define the role of EGFL7 in specific cell behaviors and clarify the mechanisms by which EGFL7 primarily functions in endothelial cells.

Studies in mice reveal that EGFL7 is dispensable for embryonic development and that previously noted functions of EGFL7 in vascular development are in fact ascribed to miR-126 (Kuhnert et al., 2008; Schmidt et al., 2007). The disparity in EGFL7 loss-of-function phenotypes among mice, zebrafish, and *Xenopus* could be attributed to functional redundancy with its paralog EGFL8, which may not be conserved between amphibians and fish (Charpentier et al., 2013a; Nikolic et al., 2010; Parker et al., 2004; Subhan et al., 2013). However, mouse studies do not discredit the role of EGFL7 in disease. Expression profiling reveals that EGFL7 is significantly upregulated in multiple cancer cell lines and tissues, especially in solid tumors (Delfortrie et al., 2011; Fan et al., 2013; Huang et al., 2010; Wu et al., 2009). The growth and survival of tumors depends on the establishment of a vascular network, although tumor vessels are often abnormal and inefficient. One major driver of tumor angiogenesis is an oxygen-depleted microenvironment, primarily functioning through hypoxia inducible factor (HIF) signaling, which promotes the expression of numerous pro-angiogenic factors such as VEGF (Hirota and Semenza, 2006; Lee and Simon, 2012; Skuli et al., 2012). Interestingly, EGFL7 is upregulated in response to hypoxia in tissue culture and exerts a protective effect on endothelial cell survival under stressful conditions (Xu et al., 2008). This heightened activity in response to stress prompted investigators from the biotechnology company Genentech to pursue EGFL7 as a potential anti-angiogenesis target in cancer therapy (Yeung et al., 2009). Although anti-VEGF approaches have been at the forefront of anti-angiogenesis therapeutics for the last decade, progressive resistance to these treatments has been documented (Meadows and Hurwitz, 2012; Welti et al., 2013). Thus, efforts to identify an ideal cocktail of angiogenesis inhibitors are continuing. The generation of anti-EGFL7 antibodies can actually accentuate the efficacy of anti-VEGF in damaging and pruning tumor vessels in murine models of non-small cell lung cancers,

pushing anti-EGFL7 therapies into Phase II clinical trials (Johnson et al., 2013). These studies show that although EGFL7 may not play a major role in quiescent endothelial cells, it is critical for endothelial function in times of stress. Therefore, it may be of value to re-examine EGFL7-null mice and their response to stress-induced pathological remodeling.

The study of cancer biology from a developmental standpoint reveals that many of the pathways and factors required for embryonic development are often aberrantly reactivated or shut off during cellular transformation and subsequent cancer progression. This certainly appears to be the case for EGFL7, which is normally downregulated in vessels after embryonic development while becoming highly activated during physiological neoangiogenesis in response to injury and also in tumor vessels. Although this thesis attempted to further address the role of CASZ1 during embryonic development, CASZ1 also emerged as an important modulator of neuroblastoma. Indeed, CASZ1 expression is downregulated in humans with neuroblastoma as well as primary cell lines, and its restoration in cells inhibits growth and tumorigenicity, indicating a tumor suppressive role for CASZ1 (Liu et al., 2011c; Liu et al., 2011d).

Given that CASZ1 is an important regulator of *Egfl7* expression during endothelial cell development, it would be interesting to address whether this transcriptional pathway is similarly required during pathological remodeling. We do not know the function of CASZ1 in adult endothelial cells, but we hypothesize that because *Egfl7* is significantly downregulated in the vasculature during adulthood, CASZ1 may be unnecessary for *Egfl7* regulation or may regulate the expression of other targets. The pro-angiogenic cascade that is enhanced during cancer includes upregulation of *Egfl7*, raising the possibility that CASZ1 may lie downstream of stimuli

driving this cascade. One immediate way to test this possibility would be to examine CASZ1 expression in HUVECs in response to treatment with angiogenic growth factors such as VEGF, FGF, and TGF-β or to determine whether CASZ1 is induced in a hypoxia-dependent manner by exposing cells to low oxygen. Ultimately, the generation of a conditional CASZ1 knock-out mouse is necessary to shed light on the potential function of CASZ1 during mammalian embryogenesis and pathological remodeling. Indeed, mice that stably express a floxed allele of *Casz1* and endothelial-specific and inducible Cre-drivers are now available for future work on the role of CASZ1 in vasculature.

In summary, the studies within this dissertation comprise a foundation for future studies relating to the transcriptional control of vascular development and disease. We have shown that blood vessel assembly and lumen morphogenesis are driven by the transcriptional activation of an ECM factor, which mediates downstream signaling directly involved in the modulation of endothelial cell behaviors associated with proper adhesion and morphology. Understanding the complexity of the pathways involved in vascular development will provide a better foundation for studying and modifying these networks in disease and may help identify key targets amenable to therapeutic manipulation.

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APPENDIX 1: TRANSCRIPTIONAL REGULATION OF BLOOD VESSEL FORMATION[3]

Formation of a functional vascular system is critical for the delivery of nutrients, the removal of waste, and gas exchange. During vasculogenesis, the *de novo* formation of blood vessels, mesodermal cells differentiate into endothelial cell precursors that proliferate and migrate to specified locations in the embryo before assembling into cord-like structures to form the primary vascular plexus.(Herbert and D.Y., 2011) Redistribution of junctional molecules, establishment of apicobasal polarity, and cell morphology changes all facilitate the opening of vessel lumens. These primitive blood vessels are further pruned and remodeled by angiogenesis when new vascular branches form by sprouting from pre-existing vessels. Additionally, endothelial cells become specified to contribute to either the venous or arterial vasculature.(Herbert and D.Y., 2011) Understanding the molecular and cellular mechanisms underlying endothelial cell behavior will enable us to develop more efficacious therapies for diseases, such as atherosclerosis, rheumatoid arthritis, and tumorigenesis.

The specification of the cardiovascular lineage and the subsequent morphogenesis of the heart and vessels depend on the combined activities of a number of transcription factors. Work within our lab revealed a novel role for the transcription factor CASZ1 in *Xenopus* cardiogenesis by regulating cardiomyocyte differentiation.(Christine and Conlon, 2008) In a recent study, we also showed that CASZ1 is required for blood vessel branching and lumen formation in *Xenopus* embryos, independent of its role in cardiac development.(Charpentier et al., 2013a) At the

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cellular level, depletion of CASZ1 in primary human endothelial cells results in impaired adhesion to the underlying substrate, aberrant contractility, and G1/S cell cycle arrest, indicating that CASZ1 is necessary for promoting endothelial cell behaviors associated with proper vascular assembly.(Charpentier et al., 2013a) Utilizing a chromatin immunoprecipitation-cloning screen, we found that CASZ1 modulates these endothelial cell behaviors by activating the expression of its direct transcriptional target, epidermal growth factor-like domain 7 (*EGFL7*). Depletion of EGFL7, an endothelial-secreted extracellular matrix (ECM) protein, resulted in poorly arborized vascular networks that were devoid of vessel lumens, indicating a requirement for EGFL7 in angiogenesis and lumen morphogenesis in accordance with previous reports.(Nichol and Stuhlmann, 2012; Nikolic et al., 2013) Moreover, the EGFL7-deficient vascular networks were similar to those in CASZ1-depleted embryos. We linked this CASZ1/*EGFL7* transcriptional hierarchy to the RhoA GTPase signaling pathway, which directly controls the cellular outputs we observed to be defective under CASZ1 and EGFL7-depleted conditions. RhoA transcript levels and activity were significantly diminished in the absence of either CASZ1 or EGFL7. Consequently, formation of actin-based stress fibers and localization of focal adhesion markers at sites of substrate contact were aberrant in CASZ1-depleted cells, but these defects were rescued by reintroduction of EGFL7.

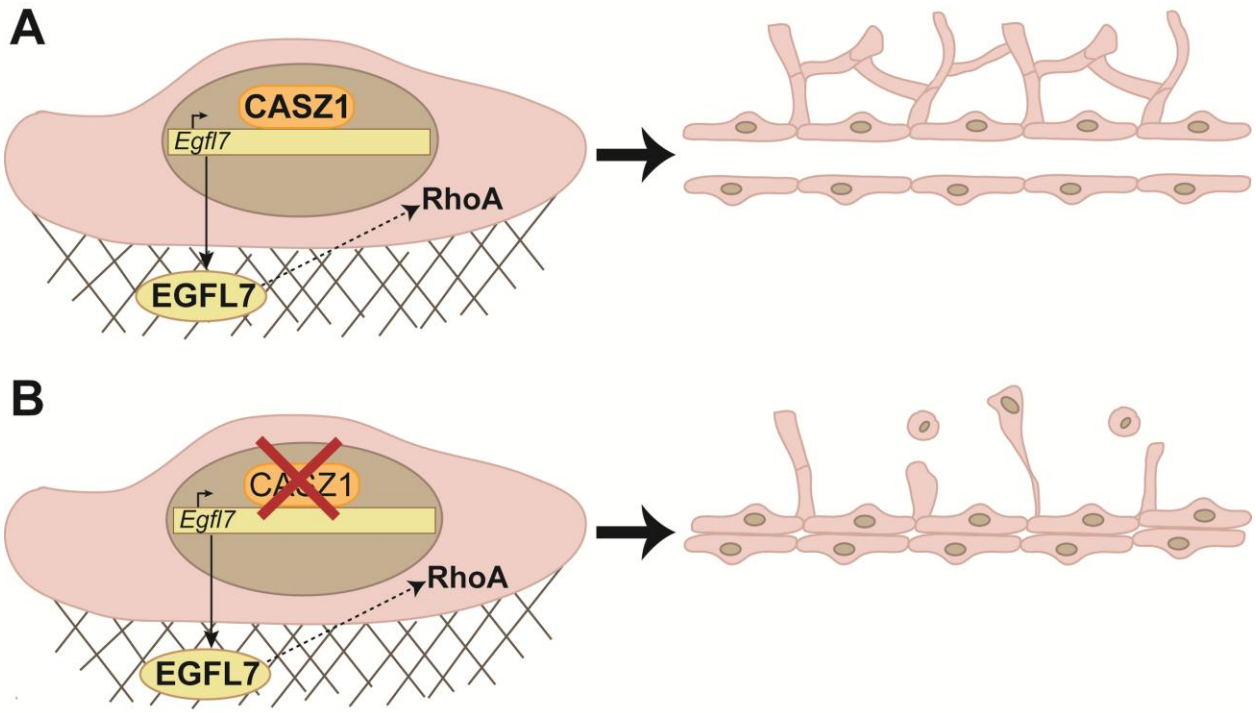
From these results, we propose a model whereby CASZ1 binds to and activates *EGFL7* gene expression in endothelial cells in order to release EGFL7 into the ECM. Through yet unknown mechanisms, we hypothesize that EGFL7 binds to cell-surface receptors, such as integrins, to activate a signaling cascade necessary for RhoA transcription and its subsequent activity. RhoA then directly modulates endothelial cell behaviors, such as adhesion and

contractility, to promote the proper assembly of vascular networks (Figure A1.1). While we have shown that activation of this pathway is necessary for the formation of a functional vascular system from a developmental standpoint, it is not surprising that improper activation of such a pathway could lead to pathological vascular remodeling in adult tissues, such as during tumorigenesis. While highly expressed in developing vessels, EGFL7 is downregulated in the quiescent vasculature of the adult, but is upregulated again in response to injury or cellular stress.(Nichol and Stuhlmann, 2012) Indeed, high EGFL7 levels are correlated with several tumors and cancer cell lines, and EGFL7 monoclonal antibodies are currently being tested in clinical trials for use in vascular tumor therapies (http://www.gene.com/medical-professionals/pipeline).(Nichol and Stuhlmann, 2012) RhoA has been shown to be required for lumen formation, but increased RhoA activity also induces vascular permeability, which potentially associates RhoA with the unstable, leaky vasculature characteristic of tumors.(Karlsson et al., 2009) Therefore, uncovering the molecular networks underlying embryonic development may provide novel targets for the design of therapeutics to treat patients with cancer.

There have been limited studies on mammalian CASZ1 in both development and disease. Recently, the human ortholog of *Casz1* was identified and shown to be highly expressed in adult heart tissue.(Liu et al., 2006) The evolutionary role of CASZ1 in cardiovascular development is further emphasized by a recent genome-wide association study, demonstrating a genetic association between the *Casz1* locus and both blood pressure and hypertension.(Takeuchi et al., 2010) However, despite the essential role of CASZ1 in cardiovascular development and human disease, the cellular requirements and molecular mechanisms by which CASZ1 regulates cardiac

development remain unknown. To address these issues, we generated a *Casz1* knockout mouse that will provide a means to understand mechanistically how this transcription factor functions in cardiovascular disease. Future studies include identifying additional transcriptional targets of CASZ1 in the heart and vasculature and investigating how CASZ1 regulates transcription. To that end, we are undertaking a number of proteomics-based approaches to determine how CASZ1 itself is regulated, to identify cardiac and/or vascular-specific co-factors with which CASZ1 interacts to regulate transcription, and to uncover novel downstream pathways dependent on CASZ1 function.

Figure A1.1. Function of CASZ1 in endothelial cells. (A) Proper expression and activity of CASZ1 in endothelial cells results in transcriptional activation of *Egfl7* and subsequent RhoA activity thereby promoting the assembly of a well-branched, lumenized vascular system. **(B)** Disruption of CASZ1 function results in cords of endothelial cells lacking a central lumen and angiogenic sprouts. Branches that are apparent consist of thin, elongated cells that are unable to maintain adhesion to the underlying matrix or existing vasculature.



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APPENDIX 2: THE CASZ1/EGFL7 TRANSCRIPTIONAL PATHWAY IS REQUIRED FOR RHOA EXPRESSION IN VASCULAR ENDOTHELIAL CELLS[4]

The cardiovascular system is the first organ system to develop and function during embryonic development. Proper establishment of the circulatory system is essential for meeting the demands of a rapidly growing embryo in need of nutrients, gas exchange, and waste removal. Blood vessel development occurs via vasculogenesis and angiogenesis. During vasculogenesis, endothelial cells, the building blocks of blood vessels, are first specified from the mesoderm whereby they subsequently migrate to precise locations within the embryo to assemble into primitive vascular structures.(Patan, 2000; Patel-Hett and D'Amore, 2011) From this point on, most of vascular development proceeds via angiogenesis in which vessels branch or sprout from pre-existing vessels ultimately giving rise to a specialized hierarchy of veins, arteries, and lymphatic vessels.(Geudens and Gerhardt, 2011; Lawson et al., 2001) The coordination of endothelial cell behaviors including migration, proliferation, and adhesive properties between cells and between the underlying extracellular matrix (ECM) is critical for these key steps of vascular assembly, and hence dysregulation of these behaviors can lead to a number of disease states such as atherosclerosis, stroke, rheumatoid arthritis, and tumorigenesis.(Carmeliet and Jain, 2011a; Chappell and Bautch, 2010) An understanding of the molecular and cellular mechanisms underlying endothelial cell behavior is therefore necessary to provide a basis for the design of therapeutics.

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Endothelial cells are highly sensitive to influences from the environment and alter their behaviors in response to signaling cascades typically initiated extracellularly through cell surface receptors. One key family of proteins known to play a critical role in signal transduction is the Rho family of small GTPases. The Rho GTPases, including members of the Rho, Rac and Cdc42 sub-families, are critical regulators of the actin cytoskeleton and promote the formation of distinct actin bundles such as actin stress fibers, lamellipodia, or filopodia respectively.(Heasman and Ridley, 2008) Like all GTPases, RhoA is regulated by GTP binding and cycles between the active GTP-bound form and the inactive GDP-bound form. RhoA activity is tightly controlled by Guanine nucleotide Exchange Factors (GEFs) that facilitate exchange of GDP for GTP, GTPase Activating Proteins (GAPs) that facilitate RhoA's intrinsic GTPase activity (to inhibit RhoA), and RhoGDIs which sequester RhoA into an inactive sub-cellular fraction. In its GTP-bound form, RhoA interacts with a variety of effector molecules that mediate its effects on the actin cytoskeleton including the Rho-kinases (ROCK 1 and II), diaphanous-related formins (mDia1 and mDia2), protein kinase N, citron kinase, rhophilin, and Rhotekin. The mDia proteins directly catalyze actin polymerization in cooperation with the actin binding protein, profilin; while ROCK stimulates actin polymerization by inhibiting the disassembly of actin polymers through LIM-kinase-dependent inhibition of cofilin. ROCK also inhibits myosin phosphatase to stimulate actin-myosin based contraction, which promotes actin bundling and stress fiber formation.(Heasman and Ridley, 2008)

Recent studies indicate that besides regulating cell shape changes, Rho-dependent signals can directly impact gene expression. Indeed, important SRF-cofactors including Myocardin-related transcription factors (i.e. MRTF-A and MRTF-B) have been shown to contain monomeric

(G)-actin binding domains, and association with G-actin masks a nuclear localization targeting sequence resulting in their cytoplasmic sequestration. Thus, upon RhoA-induced G-actin polymerization, the fall in free cytoplasmic G-actin results in G-actin release from MRTFs therefore promoting nuclear accumulation and facilitating MRTF-dependent gene transcription.(Guettler et al., 2008; Miralles et al., 2003) The Rho/MRTF/SRF pathway is a key means by which the smooth muscle cell phenotype is regulated(Lockman et al., 2004) and recent studies indicate that it may also be particularly important for determining endothelial cell fates as perturbation of the Rho-MRTF axis impairs endothelial cell differentiation from mesenchymal stem cells and conversely, the endothelial to mesenchymal transition.(Mihira et al., 2012; Wang et al., 2013) RhoA-mediated signaling has also been implicated in NF-κB-dependent gene transcription in various cell types including endothelial cells thereby promoting angiogenesis, at least in part, by up-regulating VEGFR2 levels.(Sivaraj et al., 2013)

Accumulating evidence has highlighted significant roles for RhoA in vascular biology including angiogenesis, lumen formation, and endothelial barrier function.(Bryan et al., 2010; Spindler et al., 2010; Xu et al., 2011). We have recently further implicated RhoA-mediated signaling in vascular development by showing that RhoA lies downstream of a transcriptional cascade involving the transcription factor CASTOR (CASZ1) and its direct target *Epidermal Growth Factor-like Domain 7* (*Egfl7*).(Charpentier et al., 2013a; Charpentier et al., 2013b) CASZ1 is an evolutionarily conserved transcription factor originally characterized in *Drosophila* to maintain neural stem cell identity.(Cui and Doe, 1992; Mellerick et al., 1992) In addition to expression domains in the brain, *Casz1* transcripts have been identified in cardiovascular tissues across vertebrate species, and work from our lab demonstrated that CASZ1 is required for

Xenopus cardiogenesis by regulating cardiomyocyte differentiation.(Charpentier et al., 2013a; Christine and Conlon, 2008; Liu et al., 2011d; Liu et al., 2006) Furthermore, genome-wide association studies have revealed a genetic link between the human *Casz1* locus and high blood pressure implicating CASZ1 in cardiovascular disease as well as development.(Takeuchi et al., 2010) Through the use of *Xenopus* and human endothelial cell models, we have recently demonstrated that CASZ1 is also required for blood vessel development.(Charpentier et al., 2013a) In the absence of CASZ1, *Xenopus* embryos failed to generate a well-branched vascular network and lumen formation was impaired. At the cellular level, CASZ1-depletion by short hairpin RNA in human umbilical vein endothelial cells (HUVECs) resulted in the inability of cells to maintain adhesion to the underlying substrate and failure of cells to undergo the G1/S cell cycle transition; likely due to defective control of cell contractility. To elucidate the mechanism by which CASZ1 regulates these processes, we performed a cloning chromatin immunoprecipitation screen to identify direct transcriptional targets of CASZ1. One of the identified targets was EGFL7, a secreted ECM protein implicated in vessel morphogenesis.(Fitch et al., 2004; Parker et al., 2004) We subsequently confirmed that CASZ1 is bound to *Egfl7* and that *Egfl7* was a *bona fide* CASZ1 target gene. Indeed, *Egfl7* levels were downregulated in the absence of CASZ1, and depletion of EGFL7 in embryos and cells phenocopied the defects associated with loss of CASZ1 providing evidence that CASZ1 was required for transcriptional activation of *Egfl7* for subsequent vessel development.

Although EGFL7 was previously implicated in vessel morphogenesis, the mechanism(s) by which EGFL7 regulated endothelial cell behaviors was unclear prior to these studies. Upon closer examination of CASZ1 and EGFL7-depleted HUVECs which displayed a thin, elongated

morphology associated with impaired contractility of the trailing edge, we hypothesized that Rho GTPase signaling was dysregulated. In fact, staining with focal adhesion and F-actin markers revealed an absence of adhesions from sites of substrate contact and reduced, diffuse stress fibers reminiscent of RhoA inhibition.(Katoh et al., 2011; Narumiya et al., 2000) Accordantly, levels of active RhoA and phosphorylation of non-muscle myosin II light chain were significantly diminished in CASZ1 and EGFL7-depleted cells. Somewhat surprisingly, total protein levels as well as transcript levels of RhoA were also greatly reduced under both conditions indicating that impairment of the RhoA pathway occurred at the level of gene transcription. Upon re-expression of *Egfl7* in CASZ1-depleted cells, endothelial cells no longer detached from the substrate and proper localization of focal adhesions and stress fibers was restored indicating that RhoA lies downstream of CASZ1/*Egfl7* (Figure A2.1).

As described above, regulation of RhoA activity by GEFs, GAPs, and RhoGDI is well-characterized and is presumed to be the main means by which RhoA activity is tightly controlled. However, it is well-documented that increased RhoA mRNA and protein expression are correlated with cancer progression, indicating that additional transcriptional layers of control likely exist and that it will be of importance to define the underlying mechanisms.(Bellizzi et al., 2008; Faried et al., 2007; Fritz et al., 1999; Horiuchi et al., 2003; Pille et al., 2005) The only transcriptional pathway that has been shown to regulate the *RhoA* gene is the oncogenic Myc-Skp2-Miz-p300 complex which promotes *RhoA* expression in breast carcinoma cell lines.(Chan et al., 2010) Since Myc is required for proper embryonic development (Davis et al., 1993), and abnormal development of blood vessels has been attributed to both overexpression and depletion of Myc (Baudino et al., 2002; Kokai et al., 2009; Rodrigues et al., 2008), it will be of future

interest to examine whether the Myc/*RhoA* transcriptional network is also operative in endothelial cells and (if so) how this pathway might contribute to the EGFL7-dependent regulation of RhoA during vascular assembly and morphogenesis.

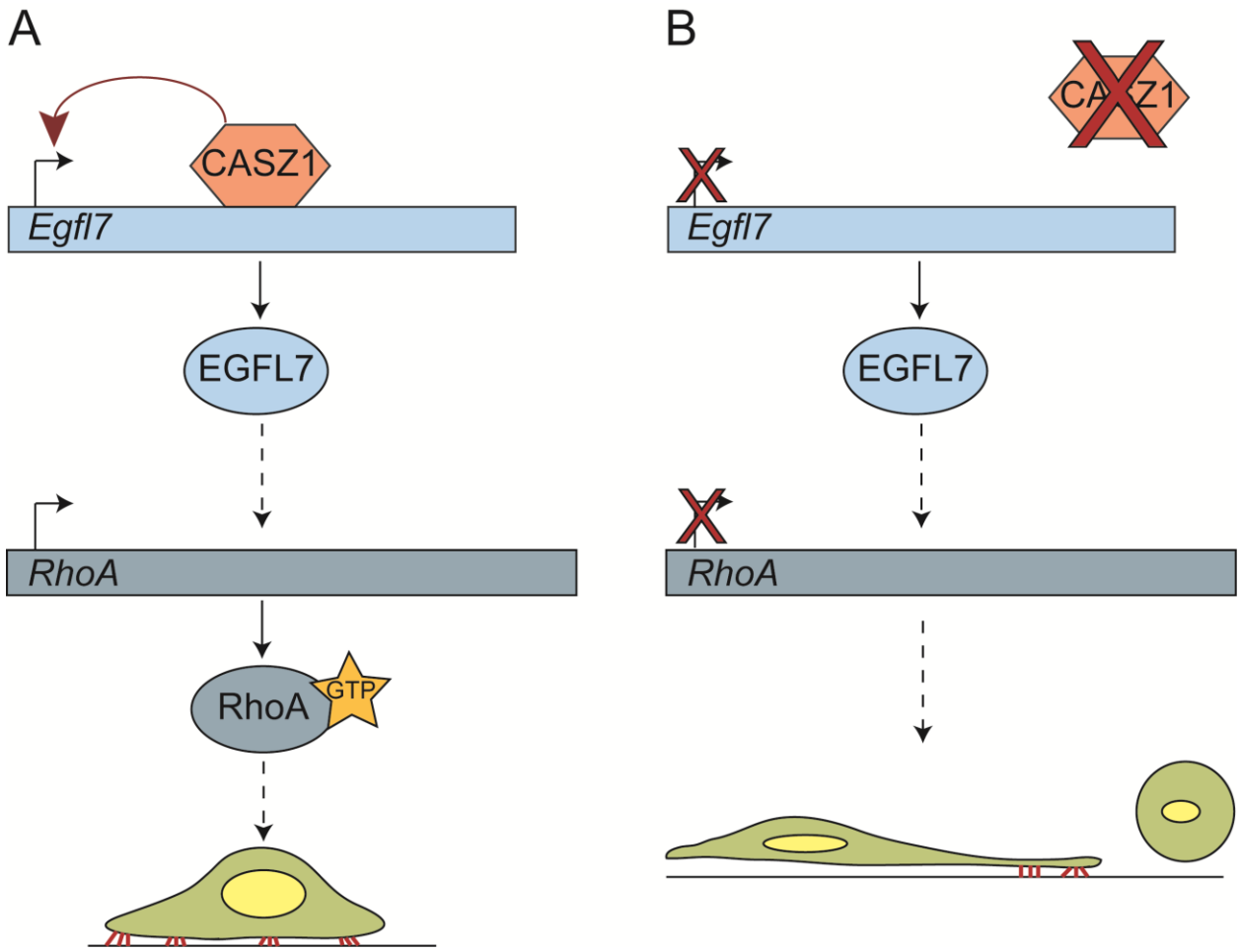
MicroRNAs have also been implicated in regulating RhoA transcript and protein abundance via direct binding to the 3' UTR of the *RhoA* gene. Again, regulation of RhoA expression by these means has been highlighted in a number of cancer cell models wherein miRs 185, 31, and 155 have each been shown to impart tight control of RhoA mRNA and protein levels(Kong et al., 2008; Liu et al., 2011b; Valastyan et al., 2009a; Valastyan et al., 2009b). Importantly, miR-31 was also shown to target RhoA in cardiomyocytes, wherein it limited hypertrophic remodeling, indicating that regulation of RhoA by microRNAs is not restricted to oncogenesis and likely represents a common regulatory mechanism.(Care et al., 2007) Therefore, it would be interesting to screen microRNA expression in CASZ1 or EGFL7-depleted cells as it is formally possible that aberrant microRNA activity downstream of CASZ1/*Egfl7* may be responsible for the diminished RhoA mRNA and/or protein levels.

While the mechanism by which EGFL7 promotes RhoA transcription is currently unclear, recent studies have begun to shed light on how EGFL7 might initiate cell signaling. EGFL7 was shown to interact with lysyl oxidase (LOX) enzymes and to inhibit their ability to crosslink elastic fibers within the ECM, a process that imparts elasticity and resiliency to vessels.(Lelievre et al., 2008) Thus it is possible that changes in the rigidity of the ECM alone could initiate mechanosensitive signals, or EGFL7-induced ECM remodeling could alter the accessibility/ binding affinity of cell-surface receptors responsible for mediating intracellular

signaling cascades. Alternatively, EGFL7 may activate or antagonize signaling events by serving as a ligand for transmembrane receptors. For example, EGFL7 was recently shown to bind to and activate the integrin αvβ3 and this association was shown to be important for proper vessel morphogenesis and angiogenic sprouting.(Nikolic et al., 2013) While integrins have been intimately associated with mediating extracellular signals through activation of Rho GTPases(Huveneers and Danen, 2009; Shen et al., 2012), less is understood about how modulation of integrins may affect their gene expression, though it remains a possibility that RhoA gene expression may be facilitated by positive feedback in response to increased RhoA activity.(Thompson et al., 2002) EGFL7 has also been demonstrated to antagonize Notch signaling in endothelial cells as well as neural stem cells.(Nichol et al., 2010; Schmidt et al., 2009) Again, however, it remains to be determined if RhoA is a direct or indirect downstream target of this pathway.

Although many questions still remain, our findings have uncovered a novel transcriptional pathway that may directly or indirectly promote *RhoA* gene expression. The depletion of CASZ1 or its direct target EGFL7 results in decreased RhoA mRNA, protein, and activity subsequently leading to improper endothelial cell behaviors associated with aberrant vascular development. These studies open up new models in which to probe the effects of dysregulated *RhoA* gene expression on cell behavior and to potentially provide insight into mechanisms directly controlling either RhoA gene expression or protein synthesis.

Figure A2.1. Model of CASZ1/Egfl7 function in endothelial cells. (A) The transcription factor CASZ1 directly binds to and activates expression of *Egfl7* in endothelial cells. By a yet unknown mechanism, this transcriptional pathway promotes expression of the *RhoA* gene and subsequent GTPase activity which leads to proper adhesion of endothelial cells to the underlying substrate (as indicated by red lines). **(B)** In the absence of CASZ1 (or EGFL7), *RhoA* expression is diminished resulting in thin, elongated cells that cannot stably form adhesions resulting in their detachment from the underlying substrate.



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