ALK1, a TGF-β type I receptor serine/threonine kinase, is critical for proper vascular development. Heterozygous loss of ALK1 results in the vascular disorder, hereditary hemorrhagic telangiectasia type 2 (HHT2), which is characterized by the development of arteriovenous malformations (AVMs) and affects 1:8000 people worldwide. \(alk1^{-/-}\) zebrafish develop embryonic lethal AVMs which form via a two-step mechanism. First, loss of \(alk1\) results in an increase in endothelial cell number in cranial arteries, which results in increased vessel caliber. In the second step, normally transient connections between arteries and veins are maintained as an adaptive mechanism to cope with an increased hemodynamic load. Using zebrafish as a tool to study the AVM formation due to loss of Alk1 signaling, I have found that Alk1 is required for directed arterial endothelial cell migration in opposition to blood flow. Embryos lacking \(alk1\) experience a redistribution of cells, with endothelial cells failing to efficiently migrate against the direction of blood flow and accumulating in more distal regions of \(alk1\)-dependent arteries. This altered cellular distribution causes an increase in arterial caliber and consequent retention of downstream arteriovenous connections, resulting in fatal AVMs.
Notch and ALK1 have been implicated in arterial specification and loss of function of either pathway causes AVMs. Furthermore, ALK1 can cooperate with Notch to upregulate expression of Notch target genes in cultured endothelial cells. These findings have led to the hypothesis that Notch and ALK1 collaboratively program arterial identity and prevent AVMs. I modulated Notch and Alk1 activities in zebrafish embryos and examined effects on Notch target gene expression and vascular morphology. Results demonstrate that control of Notch targets is context-dependent, with gene-specific and region-specific requirements for Notch and Alk1. Although loss of alk1 increases expression of dll4, which encodes a Notch ligand, and enhanced Notch signaling causes AVMs, AVMs in alk1 mutants could neither be phenocopied by Notch activation nor rescued by Notch inhibition. In conclusion, Alk1 is dispensable for acquisition and maintenance of arterial identity, and perturbations in Notch signaling cannot account or AVM development in alk1 mutants.
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LIST OF COMMONLY USED ABBREVIATIONS

AA1: first aortic arch
Alk1: activin receptor-like kinase
ALPM: anterior lateral plate mesoderm
AVM: arteriovenous malformation
BA: basilar artery
BCA: basal communicating artery
BMP: bone morphogenetic protein
CaDI: caudal division of the internal carotid artery
CCV: common cardinal vein
Coup-TFII: chicken ovalbumin upstream promoter transcription factor II
CrDI: cranial division of the internal carotid artery
CtA: central arteries
CV: caudal vein
DA: dorsal aorta
DLL4: delta-like ligand 4
dpf: days post fertilization
ECM: extracellular matrix
Edn1: endothelin-1
Efnb2: ephrinb2
ENG: endoglin
Fli1a: friend leukemia integration 1a
Gof: gain of function
HES1: hairy and enhancer of split
HHT: hereditary hemorrhagic telangiectasia
HIF-1α: hypoxia-inducible factor-1α
Hpfc: hours post fertilization
HUVECS: human umbilical vein endothelial cells
ICA: internal carotid artery
KLF: krüppel-like factor
LDA: lateral dorsal aorta
Lof: loss-of-function
MO: morpholino
MOC: midbrain organizing center
MtA: metencephalic artery
MTOC: microtubule organizing center
NF-κB: nuclear factor kappa B
NICD: notch intracellular domain
NO: nitric oxide
OA: optic artery
PCS: posterior communicating segments
PECAM-1: platelet endothelial cell adhesion molecule-1

PHBC: primordial hindbrain channel

PLPM: posterior lateral plate mesoderm

PMBC: primordial midbrain channel

RBPJ: recombination signal binding protein for immunoglobulin kappa J

ROC: rostral organizing center

Shh: sonic hedgehog

Sih: silent heart

Tg: transgenic

TGFβ: transforming growth factor β

VECAD: vascular endothelial cadherin

VEGF: vascular endothelial growth factor

vSMC: vascular smooth muscle cell.
1.0 INTRODUCTION

1.1 VERTEBRATE VASCULAR DEVELOPMENT

1.1.1 Origins of the primitive vasculature

The circulatory system is one of the first organ systems to form in development and is responsible for the transport of gases, nutrients, hormones and metabolites throughout the embryo. In all vertebrate embryos, the first blood vessels arise via the process of vasculogenesis, the de novo formation of blood vessels from endothelial precursor cells known as angioblasts. The molecular mechanisms involved in angioblast differentiation are highly conserved among vertebrate species: members of the ETS and FOX transcription factor families are important for specifying endothelial cell lineages within mesoderm in fish, mice and humans [1].

Extraembryonically, blood islands arise within the yolk sac mesoderm of developing mammals and avians: the outer cells differentiate into endothelial cells, cells that line the vascular lumen, whereas the inner cells differentiate into red blood cells [2, 3].

In the embryo proper, cells within the posterior lateral plate mesoderm (PLPM) give rise to angioblasts that arrange into clusters by embryonic day (E)7.0 in mice [2], by the 10-somite stage in zebrafish [4], and by the 1-somite stage in quail [5]. These cells migrate individually or as groups towards the midline, where they coalesce into cords and lumenize to form the trunk axial blood vessels [2, 4, 5].
Cranial vessels develop from anterior lateral plate mesoderm (ALPM) via a hybrid vasculogenic and angiogenic process. Instead of migrating to the midline, angioblasts migrate as discrete groups of cells to form large clusters; cells within these clusters proliferate and migrate via an angiogenesis-like sprouting mechanism to shape the cranial vessels. Specifically, within the developing zebrafish embryo, two sets of bilateral angioblast clusters form adjacent to the midbrain and the most rostral point of the ALPM. Sprouting from these clusters gives rise to the majority of cranial vessels [6]. A similar hybrid vasculogenic/angiogenic mechanism is involved in the formation of the pharyngeal arch arteries of mice and zebrafish: individual angiogenic clusters (one per arch artery) differentiate in the pharyngeal mesoderm and sprout dorsally to connect to the already patent dorsal aorta(e), and ventrally to give rise to the ventral aorta [7-9]. The ventral aorta connects directly to the outflow tract of the heart, thereby completing the initial primitive vascular loop.

1.1.2 Lumen Formation

Once a cord of endothelial cells has formed, it must hollow out to carry blood flow. While there is some evidence suggesting that cell hollowing (intracellular vacuole formation and fusion) may be involved in the formation of vessel lumens in vivo [10], a majority of the evidence now indicates that cord hollowing is the predominant mechanism by which vessels lumenize [11-13]. In the cord hollowing mechanism, apicobasal polarity is established via a Par3-mediated redistribution of junctional complexes away from the future apical surface and towards the periphery of endothelial cells within a cord [14]. Once polarity has been established, podocalyxin (PODXL) and CD34 are recruited to the apical surface [13]. These proteins, which
are members of the CD34-sialomucin family of proteins, are cell surface transmembrane proteins that mediate de-adhesion [15]. Additionally, PODXL/CD34 recruit moesin, which binds to F-actin, creating a network of actin filaments along the apical surface of the vessel and allowing further separation of apical surfaces via myosin-mediated contraction. Fluid passively enters the luminal space through paracellular gaps that are later resolved, resulting in an enclosed lumen [13].

1.1.3 Elaboration and remodeling of the primitive vasculature

Angiogenesis is the process by which the basic vessel architecture laid down during vasculogenesis is remodeled and expanded. Angiogenesis includes remodeling of capillary plexuses into hierarchical structures (Figure 1); sprouting of new vessels from existing vessels; and splitting of single vessels into multiple vessels (intussusception) [16].

During the activation phase of angiogenesis, basal lamina is degraded, mural cells detach and there is an increase in endothelial cell proliferation and migration. Vascular endothelial growth factor (VEGF) signaling is a critical regulator of angiogenesis and is required for nearly all aspects of vascular development. Signaling induces endothelial cell proliferation, migration and supports endothelial cell survival [17, 18]. There are five ligands in the VEGF family, VEGFA, VEGFB, VEGFC, VEGFD and placenta growth factor (PlGF), and three VEGF receptors, VEGFR1 (also known as FLT1), VEGFR2 (also known as FLK1 and KDR), and VEGFR3 (FLT4). VEGFR2 and VEGFR3 are tyrosine kinases and ligand binding results in receptor dimerization and phosphorylation. VEGFA binding to VEGFR2 acts as the primary positive regulator of angiogenesis. Phosphorylation of VEGFR2 activates a complex network of
intracellular signal transduction pathways including phospholipase C gamma (PLCγ), mitogen-activated protein kinase (MAPK), protein kinase B (PKB or AKT), focal adhesion kinase (FAK), and nitric oxide (NO) signaling [17, 19, 20]. In mice, heterozygous loss of either Vegfa or Vegfr2 is embryonic lethal due to decreased vascular density [21]. VEGFC and D interact with VEGFR3, and signaling through this receptor is critically important for lymphatic development and is involved in early venous specification [22, 23]. VEGFR1 has limited kinase activity [24] and acts primarily as a competitive inhibitor of VEGFR2 signaling by binding to VEGFA and VEGFB and acting as a ligand sink [25]. Vegfr1−/− mice die embryonically due to a hypervascular phenotype [26].

VEGF ligands and receptors are regulated by a wide variety of mechanisms. Hypoxia-inducible factor-1α (HIF-1α) upregulates VEGF signaling in response to tissue hypoxia, resulting in increased angiogenesis during tissue growth, development and wound repair [27]. ETS transcription factors (involved in angioblast specification as discussed above) can induce the expression of VEGF ligands and receptors [28]. Also Notch signaling is critical for regulating and restricting expression of specific VEGF receptors during sprouting angiogenesis [29-34].

An angiogenic sprout is composed of two different cell types, the tip cell and the stalk cell (Figure 2). Tip cells extend their filopodia towards an angiogenic stimulus and lead the sprout, while the stalk cells trail the tip cell and maintain a connection to the parent vessel and establish a vessel lumen [35, 36]. Microarray analysis of individual tip and stalk cells isolated by laser capture microdissection has revealed cohorts of genes that are differentially expressed in the two cell types [37]. VEGFR2 is highly expressed in tip cells and permits cells to migrate towards VEGFA, which is expressed in nearby tissues in a morphogenetic gradient. Delta-like ligand 4 (DLL4), a membrane-bound ligand of the Notch pathway, is also preferentially
expressed in the tip cell and binds to Notch receptors in the neighboring stalk cell to activate Notch signaling. Notch signaling represses expression of VEGFR2 in stalk cells, thereby decreasing responsiveness to VEGFA [34, 38-41]. Cells with higher levels of VEGF signaling and lower levels of Notch signaling are found in the tip position [29]. However, tip and stalk cells do not have a fixed identity in an angiogenic sprout. Additional genetic interactions between Notch and VEGF signaling result in a transient induction of DLL4 mRNA by VEGF [29, 42]. This tight regulation of DLL4 results in fluctuating levels of VEGFR2 expression. This results in a dynamic competition between overlapping cells within sprouts for the tip cell position [29, 43].
Figure 1: Arterial and venous differentiation and the formation of AVMs

Arteries (in red) are specified by Vegf-induced activation of Notch signaling, resulting in the expression of hey1, hey2, hes1 and ephrinb2. Notch/Dll4 signaling also represses the expression of ephb4 (a venous marker) in the arterial endothelium. Veins (in blue) express Couot-TFII, which represses Notch signaling in the venous endothelium and vice versa. High flow arteries have thick walls lined with vascular smooth muscle cells (brown cells) and flow into smaller caliber arterioles and finally into capillaries (purple), the location of oxygen and nutrient exchange (blue dots). Venules and then veins receive low magnitude flow and have valves to prevent backflow. AVMs are direct connections between arteries and veins, lacking an intervening capillary bed. These high flow shunts decrease oxygen and nutrient exchange. Also, veins that are downstream of these high flow shunts are enlarged. Impaired arterial venous specification has been implicated in AVM formation.
1.1.4 Vessel stabilization

During the resolution phase of angiogenesis, endothelial cells cease to migrate and proliferate and vessels are stabilized through the deposition of basal lamina and the recruitment of vascular smooth muscle cells (vSMCs) and pericytes, a type of vascular support cell [44]. Vascular mural cells function to support the endothelial cell layer and are able to contract and regulate vascular tone in response to environmental cues [45]. A number of signaling pathways are important for the resolution phase of angiogenesis. Platelet derived growth factor (PDGF) produced by endothelial cells attracts PDGFR-expressing pericytes and induces mural cell proliferation [46]. Additionally, sphingosine-1 phosphate (S1P)/S1P receptor 1 (S1PR1), angiopoetin1/Tie2 and Transforming growth factor β (TGFβ) signaling all contribute to vascular maturation through pericyte recruitment and/or extracellular matrix deposition [45].

1.1.5 Arterial and Venous Specification

Arteries and veins are genetically distinct prior to the onset of blood flow. Shortly after angioblasts are specified as endothelial cells they take on an arterial or venous (A/V) identity. In the trunk, sonic hedgehog (shh) expressed in the notochord induces the expression of vegf in the somites [47-49]. Vegf signaling induces dll4 expression and Notch activation in the endothelium (Figure 1) [49]. It is thought that venous identity is the default identity, while Notch signaling confers an arterial identity through the regulation of hey1, hey2 and hes genes [30, 50]. Additionally, Notch has been shown to regulate ephrinB2 [30]. EphrinB2 is a ligand for the EphB4 receptor. EphrinB2/EphB4 are transmembrane proteins that signal through cell-cell
contact and label arterial and venous cells, respectively [51-53]. While Notch signaling induces ephrinB2 expression in arterial endothelial cells, loss of Notch signaling results in the ectopic expression of ephB4 in these same cells [30].

**Figure 2: Angiogenic sprouts are composed of tip and stalk cells**

Cells leading the way in a sprouting vessel extend filopodia as they migrate toward an angiogenic environmental cue (purple dots). Tips cells (cool colored cells) express high levels of dll4 and vegfr2 in relation to stalk cells (warm colored cells), which express notch and vegfr1. Stalk cells trail the tip cells, maintaining a connection to the parent vessel and forming a lumen. Interactions between Notch and Vegf pathways result in a dynamic competition between overlapping tip cells.
In addition to *ephB4* expression in venous endothelial cells, chicken ovalbumin upstream promoter transcription factor II (Coup-TFII), also known as nuclear receptor subfamily 2, group F, member 2 (Nr2f2) is an orphan nuclear receptor that is cell autonomously involved in determining venous identity [54]. Coup-TFII functions to downregulate Notch signaling in veins, and ectopic overexpression of coup-TFII in endothelial cells results in the fusion of arteries and veins [55, 56]. While it is thought that Coup-TFII functions to inhibit Notch signaling and repress arterial specification, Notch signaling has also been shown to block Coup-TFII expression during arterial differentiation (Figure 1), underscoring the complicated interactions involved in determining A/V identity that are not yet fully understood [57, 58].

While the identity of endothelial cells is genetically determined before the onset of blood flow, arterial and venous identity is reversible and highly plastic. In quail/chick grafting experiments, donor arterial or venous cells populate either type of vessel in a host embryo and take on the genetic identity of the host vessel [59, 60]. Additionally, time-lapse imaging of chick vessel development nicely demonstrates the plastic nature of vessel identity. As the vascular network remodels and arterial sprouts let go of a parent artery and reconnect to the venous plexus, they take on a distinct venous identity [61]. Hemodynamic forces also influence vessel identity. Arterial ligation in a chick embryo results in an arterial-to-venous identity change [61]. These results suggest that vessel identity is first determined through genetic mechanisms, but can be altered after the onset of circulation in response to blood flow as the vascular network remodels.
1.2 BLOOD FLOW AND MECHANOSENSING

1.2.1 Hemodynamic forces in blood vessels

Prior to the onset of blood flow, vessel development is governed by local paracrine factors. Upon the onset of circulation, mechanical forces and endocrine factors influence vessel morphology, endothelial cell behavior and maintenance of arterial-venous identity.

There are three mechanical forces sensed by vessels: shear stress, cyclic strain, and hydrostatic pressure. Shear stress is a frictional force that acts directly on luminal surface of endothelial cells, parallel to the surface of the vessel. It is proportional to the flow rate and viscosity of the blood and inversely proportional to the vessel radius. Cyclic strain results in circumferential stretching of the vessel wall, whereas hydrostatic pressure acts perpendicular to and pushes outward on the vessel wall. Whereas shear stress is directly sensed only by endothelial cells, cyclic strain and hydrostatic pressure can be sensed by all cells in the vessel [62]. Of these forces, shear stress is the best studied. Vessels attempt to maintain normalized forces. In the face of increased blood flow rate (increased cardiac output) or viscosity, vessels will increase their radius to decrease the total shear forces experienced by the endothelial cells. Likewise, decreased shear forces result in decreased vessel caliber and in some cases, vessel regression [63-67].

Different types of shear stress have been shown to result in different biochemical responses. Straight vessels experience pulsatile laminar shear stress associated with high magnitude shear forces. Branched and curved vessels experience disturbed flow and low shear stress [62]. These different types of flow have different effects on gene expression and
endothelial cell behavior [68]. High pulsatile laminar shear stress activates Krüppel-like factor 2 (KLF2), a flow regulated transcription factor that favors vessel quiescence [69-71], while disturbed flow activates nuclear factor kappa B (NF-κB), resulting in an activated inflammatory response [72]. KLF2 inhibits the expression of NF-κB responsive genes, suggesting a mechanism by which an inflammatory response is silenced as the endothelium establishes a laminar shear flow pattern and the vessels become quiescent [73]. To accomplish these changes, the vessels need to be able to sense these different flow patterns and mechanical forces and then translate them into a biochemical response.

1.2.2 Mechanosensation of shear stress

It is not yet fully understood how shear stress is sensed nor how sensation is transduced to changes in gene expression and cell behavior. However, the primary cilium, cell-cell adhesion dynamics, the glycocalyx and nuclear hydrodynamic drag have all been implicated in vascular mechanosensation of shear stress (Figure 3) [62, 74].

1.2.2.1 Primary cilia

Primary cilia (Figure 3) are composed of microtubules arranged in a 9+0 pattern. They are nonmotile and reside on the apical surface of a cell. In endothelial cells, cilial bending results in a transient calcium influx that ultimately results in the production of NO, a potent vasodilator [75]. However it is unlikely that this is the primary means of mechanosensation. In regions of disturbed flow, some endothelial cells have a primary cilium [76, 77], whereas cells experiencing high physiological levels of shear stress dismantle their primary cilium [78-80]. Additionally,
while this mechanism would relay flow magnitude to the cell, it is unclear how the calcium influx could translate directional information [74].

1.2.2.2 Glycocalyx

The endothelial glycocalyx (Figure 3) consists of sulfated proteoglycans, hyaluronan and glycoproteins creating a gel-like layer that covers the apical membrane of endothelial cells [81]. The glycocalyx serves many functions including regulating vascular permeability and the formation of docking sites for plasma-derived molecules, creating microenvironments of growth factors and atheroprotective proteins [82]. In relation to mechanotransduction, it is thought that the glycocalyx is displaced in the direction of flow and transduces mechanical forces to the actin cytoskeleton via adherens junctions [83].

1.2.2.3 Adherens complex

Platelet endothelial cell adhesion molecule-1 (PECAM-1) and vascular endothelial cadherin (VECAD) are endothelial-specific proteins that localize to adherens junctions. Along with VEGFR2, these cadherins have been implicated in a flow-sensing complex that is critical in transducing shear stress into a biochemical response (Figure 3). PECAM-1 is thought to act as a direct mechanosensor because cultured endothelial cells incubated with PECAM-1 antibody-coated magnetic beads exhibit rapid PECAM-1 phosphorylation upon application of magnetic force, similar to that seen upon application of fluid shear stress. [84]. Through a mechanism that is not yet fully understood, shear stress leads to an accumulation of VEGFR2 at adherens junctions, and shear stress-induced phosphorylation of PECAM-1 results in ligand-independent phosphorylation of VEGFR2. With VECAD acting as a scaffolding protein, VEGFR2 initiates a
series of molecular events beginning with the activation of phosphatidylinositol-3-kinase (PI3K) and Akt [85]. Akt activation enhances NO production [86, 87], and increases activation of integrins [85], resulting in a cascade of molecular pathways that result in an endothelial cell response to flow.

1.2.2.4 Hemodynamic drag

In response to shear stress, endothelial cells become planar polarized, with the golgi apparatus and the microtubule organizing center positioned upstream of the nucleus with respect to the direction of flow (Figure 3). This arrangement has been shown to be dependent on PECAM-1/VEGFR2/VECAD mechanosensing complex [85]; however a direct mechanical push on the nucleus may also be a major factor in this polarization [74]. The bulge of the nucleus slightly protrudes into the lumen of the vessel and hydrodynamic drag pushes the nucleus downstream. The nuclear envelope, which is attached to the actin cytoskeleton, gradually rearranges as the location of the nucleus shifts. If the actin cytoskeleton is weakened by latrunculin treatment, there is less resistance to the downstream nuclear shift, and the endothelial cells polarize faster in the presence of flow [74]. In support of this model, a Nesprin-mediated link between the actin cytoskeleton and the nuclear envelope is necessary for shear stress induced endothelial cell polarization [88].
Figure 3: Mechanosensation of shear stress, four possible mechanisms

1: Primary cilia are displaced by blood flow resulting in a calcium influx, resulting in the production of NO. 2: The glycocalyx on the surface of endothelial cells bends in the direction of flow, transducing mechanical forces to the actin cytoskeleton via adherens junctions. 3: Shear stress activated PECAM-1 phosphorylates VEGFR2 and together with VECAD initiates signaling cascades that alter gene expression and cell behavior. 4: The nucleus bulges into the lumen of the vessel, experiencing hemodynamic drag, and orients itself downstream of the golgi apparatus and the microtubule organizing center, relaying both direction and strength of force to the actin cytoskeleton.
1.3 ARTERIOVENOUS MALFORMATIONS

1.3.1 Anatomical and functional differences between different types of blood vessels

Arteries and veins (Figure 1) have developed structural features that reflect differences in the hemodynamic factors that each vessel type encounters. Arteries carry blood away from the heart and are designed to cope with high hemodynamic forces (pressure, shear stress, stretch): they have thick walls composed of several layers of smooth muscle and elastic fibers [16, 62]. Arteries lead to smaller caliber arterioles, which then ramify into a complex network of thin-walled capillaries [16]. Capillaries are sparsely supported by pericytes and serve as the site of nutrient and oxygen exchange [45]. As blood flows through the highly branched capillary bed, velocity decreases dramatically, and thus veins experience much lower magnitudes of hemodynamic force [89]. Accordingly, veins have thin walls and valves, which function to prevent back flow of blood.

1.3.2 Anatomy of AVMS

Arteriovenous malformations (AVMs) are direct, high flow connections between thick walled arteries and thin walled veins, lacking an intervening capillary bed. Over time, these connections become increasingly complex and tortuous, forming a tangled web of enlarged vessels, or nidus, which leads to a grossly enlarged draining vein. These malformations acquire a thick smooth muscle coat, thereby barring gas exchange (Figure 1) [90, 91]. Although the etiology of AVMs is
unclear, there is evidence to suggest that in some cases they arise due to failed regression of normally transient arterial/venous connections, or failed repulsion between arteries and veins due to improper arterial/venous (A/V) specification [53, 92].

1.3.3 Clinical consequences of AVMs

The clinical consequences of an AVM will depend on the location and size of the lesion. Generally, AVMs decrease gas exchange and cause localized ischemia, and these malformations may rupture due to the inability of veins to handle high magnitudes of mechanical forces [91]. Specifically, cerebral AVMs may cause localized ischemia or hemorrhagic stroke; pulmonary AVMs rarely rupture but can lead to cyanosis, brain abscess, transient ischemic attacks, and embolic stroke; and very high flow hepatic AVMs can lead to high output cardiac failure [93]. AVMs connecting small, mucocutaneous vessels are known as telangiectasias. Dermal telangiectasias may bleed but are primarily a cosmetic issue, whereas bleeding from GI and nasal telangiectasias can cause anemia and severe hemorrhage [94].

1.3.4 Genetic basis for AVMs

A majority of AVMs are sporadic, however, a subset of these vascular lesions is caused by genetic mutations. Capillary malformation-arteriovenous malformation (CM-AVM) is caused by heterozygous mutations in *Rasa1*, which encodes RAS p21 protein activator 1, and is characterized by multiple small capillary malformations [95]. Hypotrichosis-lymphedema-telangiectasia syndrome (HLTS) results from mutations in *Sox18*, a known regulator of *Dll4*, and
results in telangiectasias, lymphatic defects and renal failure [96]. Ataxia-Telangiectasia is an autosomal recessive mutation in *Ataxia-telangiectasia mutated* (*ATM*) gene. *ATM* is a serine/threonine protein kinase and is critical for normal repair of double stranded DNA breaks. As such, patients harboring mutations in this gene suffer from a wide array of symptoms including compromised immune systems, gonadal dysgenesis and telangiectasias [97]. Mutations in genes involved in the TGFβ signaling pathway are linked to the vascular dysplasia hereditary hemorrhagic telangiectasia (HHT) [98], the disease that is the main focus of my research.

1.3.5 **Hereditary Hemorrhagic Telangiectasia**

HHT, also known as Osler-Rendu-Weber syndrome, is a genetic disorder characterized by a predisposition to telangiectasias and AVMs. HHT is estimated to affect approximately 1:8000 individuals. However, due to the nonspecific symptoms (e.g. frequent nosebleeds) and variability in expressivity and age of onset of this disease, HHT is thought to be significantly underdiagnosed [98].

1.3.6 **Genotype/phenotype correlations in HHT**

Heterozygous mutations in members of the TGF-β signaling pathway are causally related to HHT. Mutations in Endoglin (*ENG*), a co-receptor in the pathway, result in HHT1 [99]. Mutations in *ACVRL1*, which encodes the TGF-β type I receptor, ALK1, result in HHT2 [100]. Additionally, mutations in *SMAD4*, a critical intracellular signal mediator within this pathway,
result in a combined juvenile polyposis-HHT syndrome [101]. Two additional loci on chromosomes 5q31.3-32 and 7p14 have been linked to HHT; however, the responsible genes within these loci have not yet been identified [102, 103]. HHT1 and HHT2 present with different phenotypic severity and location. HHT1 patients tend to experience more severe symptoms with an earlier age of onset. 49-75% of HHT1 patients present with pulmonary AVMs (PAVMs), 15-20% with cerebral AVMs (CAVMs) and 2-8% with hepatic AVMs (HAVMs). Between 60-72% of HHT1 patients have GI telangiectasias with approximately 18% of these patients experiencing bleeding [104-106]. HHT2 patients are typically diagnosed around the age of 40 and, compared to HHT1 patients, present with similar incidences of GI (gastrointestinal) telangiectasias/bleeding and lower incidences of PAVMS and CAVMs (5-44% and 0-2%). However, the incidence of HAVMs is between 28-84% in these patients [104-106]. The reason for the differences in the phenotypic severity and presentation between the two HHT sub-groups is unknown but may reflect differential tissue distribution or function of Endoglin and Alk1 [107].

1.3.6.1 ALK1 signaling

**Overview of TGF-β family signaling**

In TGF-β signaling, dimeric ligand binds to a heterotetrameric complex of type I and type II receptors. Upon ligand binding, the type II receptor phosphorylates the type I receptor. The type I receptor then phosphorylates Smad transcription factors. Once activated, Smad proteins bind to the common partner Smad, Smad4, and translocate into the nucleus to regulate the transcription of target genes (Figure 4). TGF-β superfamily signaling involves seven type I receptors (ALK1-
ALK7) and five type II receptors (ActRIIA, ActRIIB, BMPRII, TGFβII and AMHRII), all of which are serine/threonine kinases. Ligand binding to a heterotetrameric complex of type I and type II receptors can be facilitated by a type III receptor, ENG, which does not have enzymatic activity [108]. The family of TGF-β ligands is large and can be divided into multiple subfamilies. Ligands in the TGF-β and activin subfamilies bind to receptor complexes that phosphorylate Smad2 and Smad3. Bone morphogenetic protein (BMP) ligands bind to different receptor complexes that phosphorylate Smad1, Smad5 and Smad9 [109].

**Alk1 in vascular development**

ALK1 is a transmembrane protein containing an extracellular N-terminal domain that binds ligand, a short, single pass transmembrane domain and a large intracellular domain. The intracellular domain contains three main motifs: a GS domain, a serine/threonine kinase domain and a cytoplasmic tail. The GS domain is phosphorylated by the type II receptor and contains a highly conserved TTSGSGSG motif [110, 111]. To date, 434 mutants in Alk1 have been identified, of which 50% have been found to be pathogenic (http://www.arup.utah.edu/database/hht/). Of these, 46% are missense mutations. Limited in vitro analysis of HHT2-associated ALK1 mutations suggests that the majority of mutant proteins are localized to the cell surface and are able to bind to BMP9 (except for mutants in the extracellular domain), and that mutant protein does not affect activity of wild type protein [112, 113]. These data indicate that these mutations do not act as a dominant negative and suggest instead that phenotypes result from a haploinsufficiency [112].
Circulating Bmp9 or Bmp10 bind to a heterotetrameric complex consisting of two type I receptors (Alk1), two type II receptors (ActRIIA, ActRIIB or BMPRII) and the type III accessory receptor, Endoglin. Activated Alk1 phosphorylates Smad1/5/9, releasing it from an auto-inhibitory fold and allowing it to complex with Smad4, the common partner Smad. The activated Smad complex translocates to the nucleus where it binds DNA and affects the transcription of target genes.
*Alk1* null mice are embryonic lethal at E11.5 due to enlarged vessels, impaired vascular remodeling, decreased vascular support cell coverage and AVMs [114, 115]. Heterozygous adults exhibit age-related dilated vessels, hemorrhage and bleeding within the GI tract [116]. Endothelial-specific deletion of *Alk1* during embryogenesis results in AVMs and is lethal by postnatal day 5 [117, 118]. Endothelial-specific deletion in adulthood is also lethal within 10-14 days of deletion due to vascular defects, mostly within the GI tract. However, development of dermal telangiectasias in these mice requires wounding. These data suggest that active angiogenesis is required for AVM and telangiectasia development in the absence of Alk1. [119, 120].

Zebrafish lacking *alk1* develop cranial AVMs in 100% of embryos by 36 hours post fertilization (hpf) and die by 5 days post fertilization (5 dpf). Heterozygous embryos have no phenotype and adults appear to be indistinguishable from their wild type siblings [92, 121]. While no apparent vascular phenotype has been observed in *alk1* heterozygous adults, the condition has not been studied thoroughly.

**Endoglin in vascular development**

ENG is an integral membrane protein that functions as a homodimer in conjunction with type I and type II TGFβ receptors to facilitate ligand binding [122]. BMP9 and BMP10 have been shown to bind directly to ENG [123, 124]. Homozygous mutant *Eng* mice die between E10.5-11.5 due to cardiac and vascular defects including enlarged vessels, impaired vascular remodeling and a decrease in vascular support cells [125-128]. However, these mice do not develop severe AVMs, as would be expected based on HHT1 patients. This is most likely due to cardiac defects resulting in early lethality. In support of this idea, neonatal endothelial-specific
deletion of *Eng* results in AVMs in a high percentage of mice [129], and adult mice harboring a single *Eng* mutation tend to develop age-related HHT-like phenotypes including nosebleeds, enlarged vessels and telangiectasias [126, 130, 131].

**ALK1 Ligands, BMP9 and BMP10**

BMP9 and BMP10 have recently been identified as the physiologically relevant ligands for ALK1 signaling and vascular development [112, 132-135]. BMP9 and BMP10 are highly related proteins, sharing 65% sequence identity at the protein level. Both proteins undergo very similar biosynthesis [112, 136]. Pre-pro-proteins are cleaved by convertase enzymes such as furin into a prodomain and mature peptide. After secretion, the prodomain remains associated with the mature peptide through non-covalent interactions. BMP9 is active when associated with the prodomain and able to bind to ALK1 and induce Smad1/5 phosphorylation [136]. However, BMP10 is latent until the prodomain is removed [137]. Although the metalloproteinase BMP-1 (unrelated to BMP ligands) can cleave the BMP10 prodomain in vitro [137], the mechanism by which BMP10 is activated in vivo is not understood. Perhaps mechanical forces, interactions with the extracellular matrix or accessory receptors are required to dissociate the prodomain from the BMP10 mature peptide in a physiologic setting.

In humans, *BMP9* is expressed in the liver (hepatocytes, biliary epithelial cells) and circulates in its biologically active form at 110 pg/ml in serum [133, 138, 139]. In mice, *Bmp9* is also expressed in liver as early as E9.75 and is simultaneously detectable in serum. *Bmp9* null mice have no vascular defect and are viable [140]. BMP10 is produced by the heart [141], specifically within the ventricular cardiomyocytes as early as E8.5 then restricted to the atrial cardiomyocytes by E16.5 in mice [140], and is also detectable in mouse and human serum [112,
142], though in an inactive prodomain-bound complex [112]. BMP10 null mice die at E10.0 from failed trabeculation and AVMs. Interestingly, vascular but not cardiac defects in these mice could be rescued by the insertion of Bmp9 into the Bmp10 locus [140]. Together, these data suggest that BMP9 and BMP10 are endocrine ALK1 ligands, and that BMP9 can functionally compensate for BMP10 in the vasculature if expressed in a BMP10-like spatiotemporal pattern.

In zebrafish, morpholino oligonucleotide mediated knockdown of bmp9 is not lethal but results in a failure of the caudal vein to properly remodel [143]. These embryos do not develop enlarged cranial shunts similar to those observed in alk1 mutants [92, 121]. Like in mice, bmp10 is expressed earlier than bmp9 and concomitant knockdown of bmp10 and bmp10-like (a zebrafish bmp10 paralog) results in large cranial shunts and is embryonic lethal [135]. Together with the data from the mouse models, these results suggest that in early development, BMP10 is necessary for embryonic vascular development and BMP9 and BMP10 ultimately function redundantly to maintain normal vasculature.

**Type II receptors that complex with ALK1**

Ligand binds to a heterotetrameric complex of type I and type II receptors. Type II receptors are thought to be constitutively active and phosphorylate the type I receptors when they are brought into a complex together by ligand binding. BMPs have been shown to preferentially interact with ActRIIA, ActRIIB or BMPRII [109].
1.4 ZEBRAFISH AS A MODEL SYSTEM FOR STUDYING HHT-ASSOCIATED AVMS AND ALK1 SIGNALING

1.4.1 General attributes of the zebrafish model

Zebrafish are an excellent model system for the study of vertebrate development. External fertilization, small size, and optical clarity allow for the observation of development from the 1-cell stage. Development occurs rapidly, with gastrulation occurring at 6 hpf, heartbeat beginning at 24 hpf and circulation through the head and tail of the embryo by 27 hpf [144]. Furthermore, using confocal or two-photon microscopy, development or particular organ systems can be monitored with high spatiotemporal resolution in live transgenic embryos expressing fluorescent proteins under the control of cell type-specific promoters.

Zebrafish are also amenable to genetic manipulation. DNA and RNA can be injected easily into 1-cell stage zebrafish embryos to ectopically express genes in a tissue-specific manner or globally, respectively. In addition, genes of interest can be knocked down transiently using morpholino-modified antisense oligonucleotides. These short (~25 bases) oligos are highly stable and contain a morpholine ring in place of the deoxyribose ring and non-ionic phosphorodiamidate group in place of the anionic phosphodiester linkages between bases [145]. They are designed to target specific mRNA sequences and through steric inhibition block either translation of the message or proper mRNA splicing [146, 147].

Forward genetic screens using N-ethyl-N-nitrosourea (ENU) mutagenesis, viral insertion mutagenesis or transposon-mediated gene disruption have generated thousands of genetic mutants that are used to study gene functions and signaling cascades important for embryonic
and larval development [148]. More recent advances in reverse genetics have allowed for the targeted disruption of genes using TALEN and CRISPR/Cas9 technology [149, 150].

In addition to genetic approaches, zebrafish are easily manipulated using pharmacological approaches. Addition of soluble small molecules to the water in which the embryos are reared allows researchers to perturb specific biochemical pathways using previously characterized drugs, and to perform large scale, medium throughput chemical screens to identify novel small molecules that perturb particular signaling pathways or developmental processes [148]. Together, these attributes make zebrafish an extremely powerful system for the study of vertebrate vascular development in a physiologically relevant in vivo setting.

1.4.2 Zebrafish alk1 mutants develop cranial AVMs

1.4.2.1 Zebrafish cranial blood vessel development:
Embryonic zebrafish vascular morphology has been studied in great detail, first through the use of confocal microangiography and then through live time-lapse imaging of transgenic embryos expressing fluorescent proteins under the control of endothelial-specific promoters [6, 144]. Microangiography specifically highlights vessels that are lumenized and carrying plasma/blood flow, while imaging of transgenics allows assessment of development both prior to and subsequent to lumen formation. By utilizing both techniques, a complete understanding of vessel formation and maturation can be gained.
1.4.2.2 *alk1⁻/⁻* AVMs arise via a two-step process

The spatial and temporal predictability of shunt formation in *alk1⁻/⁻* zebrafish embryos make them an ideal model for studying the molecular and cellular missteps that lead to AVM formation. In the absence of Alk1 signaling, zebrafish embryos develop grossly enlarged cranial arteries that contain supernumerary endothelial cells: *alk1⁻/⁻* embryos have a 1.2-fold increase in the number of endothelial cells in the BCA/PCS beginning at 32 hpf and a 1.8-fold increase by 48 hpf [92, 121, 135]. By 40 hpf, AVMs form downstream of these arteries, between the BCA and PMBC (anterior shunt) and/or the BA and PHBC (posterior shunt) [92]. Thus, arterial enlargement precedes shunt formation. Time-lapse confocal microscopy has revealed that AVMs are the result of failed regression of one or more transient BCA/PMBC or BA/PHBC connections. These connections are retained in *alk1* mutants only in the presence of blood flow, suggesting that the increased shear stress caused by cranial arterial enlargement triggers an adaptive response aimed at normalizing hemodynamic force [92]. In sum, these data support the idea that AVM development in *alk1* mutants is not genetically determined but instead represents a two-step process that involves genetically programmed arterial enlargement followed by an Alk1-independent response to changes in the hemodynamic environment [92].

1.4.3 *alk1* expression is regulated by blood flow

*Alk1* is expressed in arteries that are proximal to the heart in zebrafish embryos. Beginning at 26 hpf, *alk1* expression can be observed in the AA1 and the LDA, vessels that are a part of the initial circulatory loop. Shortly thereafter, expression within the ICA can be detected followed by CaDI and BCA expression at 30 hpf. It is interesting to note that *alk1* is not expressed in the
PCS or BA, and is restricted in the cranial arterial endothelium to arteries that are proximal to the heart and experience the highest magnitude of hemodynamic forces [92, 121]. In silent heart (sih) embryos, which lack heartbeat and therefore blood flow, overall vascular patterning is normal; however, alk1 is not expressed, indicating that flow is required for alk1 expression. Additionally, pharmacological inhibition of heartbeat also inhibits alk1 expression [92]. Maintenance of alk1 expression is also highly dependent on flow. In experiments where flow was stopped after alk1 expression had been initiated, expression quickly faded in a distal-to-proximal pattern. Upon restoration of heartbeat, alk1 expression was detectable within an hour in the AA1 and completely restored by 8 hours (Jim Donovan, unpublished data). In gata1 mutant embryos, which lack erythrocytes, alk1 expression is unaffected, indicating that expression is not dependent on endothelial-cell/red blood cell interaction [92].

1.4.3.1 Alk1 lies downstream of blood flow in phosphorylation of Smad1/5/9

Alk1 activity within the vascular endothelium can be assayed by assessing pSmad1/5 [110, 133, 134], which is absent in alk1 mutants and in sih morphant embryos lacking blood flow (and therefore also lacking alk1 expression) [135]. Stable transgenic expression of alk1 driven by a flow-independent vascular endothelial promoter is able to rescue pSmad1/5 in alk1 mutants but not sih morphants, suggesting that flow is necessary for Alk1 activity as well as expression [135]. Accordingly, injection of recombinant human BMP10 protein directly into the base of the CaDI of flow-deprived embryos ectopically expressing alk1 was able to restore pSmad1/5, suggesting that blood flow is required to activate Alk1 signaling by circulating Bmp10, which is produced in the heart [135] (Figure 5).
Figure 5: Blood Flow/BMP10/Alk1 Schematic

BMP10 is a circulating angiogenic factor and is delivered to the Alk1 receptor upon the onset of flow. This results in the activation of Alk1, Alk1-dependent phosphorylation of Smads 1/5/9 in the arterial endothelium, and increased expression of \( \text{alk1} \) and \( \text{edn1} \) and the repression of \( \text{cxcr4a} \).

1.4.3.2 Alk1 lies downstream of blood flow in expression of some shear stress responsive genes

A subset of genes that is known to be regulated by shear stress is dysregulated in \( \text{alk1}^{-/-} \) embryos. Endothelin-1 (Edn1), a vasoactive peptide expressed within the vascular endothelium and/or vascular smooth muscle, has been shown to be negatively regulated by laminar shear stress and positively regulated by cyclic strain [151-153]. Interestingly, vascular \( \text{edn1} \) expression is restricted to \( \text{alk1} \)-positive arteries in the developing zebrafish. In the absence of Alk1 signaling
or blood flow, \textit{edn1} expression is lost, indicating that \textit{alk1} is either directly or indirectly required for \textit{edn1} expression. Cxcr4a is a promigratory chemokine receptor and has been shown to be negatively regulated by laminar shear stress [154]. \textit{cxcr4a} expression is increased in \textit{alk1}-positive arteries in the absence of Alk1 or blood flow (Figure 5).

Not all flow responsive genes are dysregulated in \textit{alk1}^{-/-} embryos. \textit{klf2a}, a transcription factor known to integrate shear stress-responsive pathways, is positively regulated by shear stress [70, 151] and expression is completely lost in zebrafish embryos lacking blood flow [92]. However, \textit{klf2a} expression is unaffected in \textit{alk1}^{-/-} embryos [92]. These results suggest that Alk1 signaling may act upstream of \textit{edn1} and \textit{cxcr4a} in a \textit{klf2a}-independent flow responsive pathway required to limit endothelial caliber upon the onset of flow. In support of this hypothesis, BMP10 protein injection coupled with transgenic, flow-independent \textit{alk1} expression restored \textit{cxcr4a} and \textit{edn1} expression and endothelial cell number to wild type levels in the absence of blood flow [135] (Figure 5).

\section*{1.5 NOTCH SIGNALING AND AVMS}

With its membrane bound ligand and receptors, Notch signaling allows communication between neighboring cells. It is a fundamentally conserved signaling pathway that is used reiteratively in the development of many different tissues and has been shown to regulate proliferation, apoptosis, self-renewal and differentiation. Notch signaling is highly context specific and therefore it is unwise to make generalized predictions on how Notch signaling will influence the development of one cell type based on results of another [155]. Throughout much of the
introduction, I have touched on Notch signaling and its key roles in angiogenesis, specifically in tip and stalk cell determination, regulation of VEGF signaling and arterial-venous identity. Notch signaling is implicated in the formation of AVMs, and there is also a large amount of evidence suggesting that a Notch and Alk1 interaction is important for proper vascular development.

1.5.1 Notch pathway summary

Notch receptors are single-pass transmembrane proteins that contain an extracellular ligand binding domain and an intracellular domain critical for Notch signal transduction. Notch ligands are transmembrane proteins from the Delta-like ligand (Dll) and Jagged families. Of these, Dll4 has the most prominent role in vascular development. The notch intracellular domain (NICD) is processed upon ligand binding by ADAM metalloproteases [156], which activate the receptor, and then further cleaved by γ-secretase enzymes that release the activated NICD into the cell [157]. The intracellular domain of the Notch receptor binds to CSL (Cp-binding factor 1 (CBF-1), Suppressor of hairless [Su(h)], and Lag-1), also known as recombination signal sequence-binding protein-J kappa (RBPJκ) [158]. In the absence of NICD, RBPJκ acts as a transcriptional repressor. Binding of NICD to RBPJκ converts the complex to a co-activator and results in the transcription of Notch target genes, including the hairy and enhancer of split-1 (HESI) family [155, 159].
1.5.2 Perturbation of Notch signaling results in AVMs

Arterial/venous specification and its role in AVM prevention are evidenced by Notch perturbations resulting in these vascular malformations in zebrafish and mice. In zebrafish, mutations in the mindbomb (mib), a regulator of Notch ligand endocytosis, results in the formation of AVMs as well as impaired arterial/venous specification [30, 160]. While mutations in hey2 results in defects in dorsal aorta due to altered arterial/venous identity [160, 161]. Similarly in mice, decreased expression of the Notch ligand, dll4 or endothelial specific deletion of RBPJκ results in loss of arterial specification and AVMs that are small in caliber and appear atretic [162, 163]. Ectopic activation of Notch signaling also results in improper A/V specification and vascular abnormalities. Zebrafish ectopically overexpressing the NICD in the vascular endothelium have enlarged vessels and decreased expression of venous ephB4 [164, 165], and expression of a constitutively active Notch4 (Notch4CA) in the mouse endothelium results in venous expression of arterial markers and AVMs in the liver, uterus, skin, brain and lung of adult mice [166-169]. Brain AVMs that result from inducible transient expression of Notch4CA are reversible: when the transgene is turned off, vessels shrink back to normal size and A/V marker expression is restored [170]. While AVMs resulting from decreased Notch signaling are small in caliber, Notch overexpression AVMs are large and contain an increased number of endothelial cells [31, 166, 168, 171, 172].
1.5.3 Evidence for Notch/Alk1 interactions

Evidence from cultured endothelial cells demonstrates that Smad1/5 can bind to the promoters of the canonical notch target genes, hey1, hey2 and hes1 and enhance expression [173], and Alk1 activation increases expression of some Notch targets [32, 174, 175]. In addition, Notch target gene expression is synergistically increased by simultaneous Notch and Alk1 pathway activation [175]. BMP9/ALK1 effects were found to be independent of RBPJκ suggesting that Alk1 signaling through Smad1/5 may function to reinforce arterial identity independent of NICD/RBPJκ [175]. Phenotypic evidence of an interaction between Notch and Alk1 signaling is less convincing. While inhibition of both pathways separately has been shown to enhance VEGF-stimulated angiogenesis both in cell culture and in vivo, combined inhibition does not appear to have an additive effect [175]. Similarly, constitutively active Notch or Alk1 has been shown to inhibit sprouting with no increased effects of combined pathway activation [174]. And lastly, the inhibition of either pathway does not alter phenotypic effects of activation of the other [174]. A better understanding of how each of these pathways function and interact is required to understand why an apparent genetic synergy does not translate into a phenotypic synergy.

1.6 SUMMARY AND DISSERTATION AIMS

Patients with HHT2 develop AVMs due to a mutation in ALK1 [91, 112, 113], but the function of ALK1 in endothelial cells and the natural history of AVMs is unknown. Zebrafish alk1 mutant embryos develop enlarged arteries containing supernumerary endothelial cells, causing a change
in hemodynamic environment that leads to AVMs [92, 121, 135]. The source of the additional endothelial cells has been thought to be a result of increased proliferation and/or migration, supporting the hypothesis that Alk1 signaling is antiangiogenic and contributes to vessel stabilization [132, 176]. Here, I show that the increase in endothelial cell number in the cranial arterial endothelium is due to an improper distribution of cells. Arteries proximal to the heart experience a decrease in cell number due to an accumulation of cells in the more distal arteries. These data suggest that Alk1 is required for directed endothelial cell migration towards the heart and in opposition to blood flow.

In addition, an interaction between Alk1 and Notch signaling pathways has been thought to be important for proper vascular development and AVM prevention [175, 177]. Here I demonstrate that Alk1 and Notch signaling have context specific interactions in the regulation of the expression of some Notch target genes, but there are only weak phenotypic interactions between the two pathways in vivo.
ALK1, a TGF-β type I receptor serine/threonine kinase, is critical for proper vascular development. Heterozygous loss of ALK1 results in the vascular disorder, hereditary hemorrhagic telangiectasia type 2 (HHT2), which is characterized by the development of arteriovenous malformations (AVMs) and affects 1:8000 people worldwide. $alk1^{-/-}$ zebrafish develop embryonic lethal AVMs which form via a two-step mechanism. First, loss of alk1 results in an increase in endothelial cell number in cranial arteries, which results in increased vessel caliber. In the second step, normally transient connections between arteries and veins are maintained as an adaptive mechanism to cope with an increased hemodynamic load. Using zebrafish as a tool to study the AVM formation due to loss of Alk1 signaling, I have found that Alk1 is required for directed arterial endothelial cell migration in opposition to blood flow. Embryos lacking alk1 experience a redistribution of cells, with endothelial cells failing to efficiently migrate against the direction of blood flow and accumulating in more distal regions of alk1-dependent arteries. This altered cellular distribution causes an increase in arterial caliber and consequent retention of downstream arteriovenous connections, resulting in fatal AVMs.
2.1 INTRODUCTION

Hereditary hemorrhagic telangiectasia (HHT) is a haploinsufficiency characterized by a predisposition to development of arteriovenous malformations (AVMs). These fragile, direct connections between arteries and veins can lead to hemorrhage or stroke. HHT is caused by defects in transforming growth factor-beta (TGF-β) superfamily signaling. Specifically, mutations in the type III accessory receptor, endoglin (ENG), cause HHT1; mutations in the type I receptor serine threonine kinase, activin receptor-like kinase 1 (ACVRL1, or ALK1), cause HHT2; and mutations in the signaling mediator, SMAD4, cause a combined syndrome of juvenile polyposis with HHT [99-101]. Together, mutations in these three genes account for approximately 85% of HHT. Despite the fact that these gene products all participate in TGF-β signaling, whether mutations affect one or more discrete pathways and how these pathways function to prevent AVMs remain poorly understood.

Based on histological observation of cutaneous AVMs (telangiectasias) from HHT patients, it has been postulated that the first step in AVM development is focal dilation of a postcapillary venule, followed by arteriole dilation and subsequent loss of intervening capillaries [90]. However, these conclusions were reached from static observations of independent lesions and not from longitudinal analysis. In Alk1- and Eng-deleted adult mice, wound-induced subdermal AVMs develop via angiogenic elongation of both arteries and veins, with de novo arterial-venous connections developing prior to vessel dilation [117, 120]. Although these findings represent a longitudinal analysis, imaging of vascular growth was performed only once per day and was not at cellular resolution. Therefore, the aberrant cell behaviors that lead to AVMs could not be elucidated.
Zebrafish are an excellent model for the study of both normal and pathological vascular development because signaling pathways that control endothelial cell differentiation and vessel patterning are conserved from fish to mammals, and because optically transparent transgenic zebrafish embryos allow real-time imaging of vessel development at cellular resolution. Zebrafish \textit{alk1} mutants develop AVMs at a predictable time (approximately 40 hours post-fertilization, hpf) in a predictable location (beneath the midbrain or hindbrain) and therefore serve as an excellent model for exploring the cellular basis of HHT-associated AVM development [92, 121, 135].

In zebrafish, \textit{alk1} is expressed after the onset of blood flow in cranial arterial endothelial cells closest to the heart, including (in ordered series) the outflow tract and first aortic arch (AA1), internal carotid artery (ICA), caudal division of the internal carotid artery (CaDI), and basal communicating artery (BCA). We previously reported increases in arterial endothelial cell number in and diameter of the contiguous CaDI, BCA, and posterior communicating segments (PCS) in \textit{alk1} loss-of-function mutants as early as 32 hpf [92, 135]. Between 32-40 hpf, BCA/PCS endothelial cell number increases similarly in the absence of \textit{alk1} function or in the absence of blood flow, and blood flow is required for \textit{alk1} expression [92]. These data suggest that Alk1 transmits a flow-based signal that limits arterial caliber. In \textit{alk1} mutants, high-flow shunts develop by 40 hpf downstream of enlarged arteries, connecting either the BCA to the primordial midbrain channel (PMBC) or the downstream \textit{alk1}-negative basilar artery (BA) to the primordial hindbrain channel (PHBC). These shunts represent aberrant retention of normally transient arteriovenous connections that initiate development of and serve as early drainage for the nascent arterial system [92, 121]. In \textit{alk1} mutants, late increases in endothelial cell number (40-48 hpf) and AVM development require blood flow [92], suggesting that these effects are
secondary to enlargement of \textit{alk1}-positive cranial arteries closest to the heart and represent an adaptive response of downstream vessels to altered hemodynamics. Therefore, I focused this study on defining the primary role of Alk1 in arterial endothelium in limiting arterial caliber.

Results demonstrate a defect in arterial endothelial cell distribution within lumenized vessels as the primary effect of loss of Alk1 function. With the onset of blood flow, wild type cranial arterial endothelial cells in the lumenized AA1, ICA, and CaDI migrate in a distal-to-proximal fashion towards the heart, against the direction of blood flow. Some cells originally located in AA1 or the ICA enter the heart and incorporate into ventricular endocardium. In contrast, cells distal to the ICA/CaDI junction generally remain in place after the onset of blood flow, and there is little to no mixing of arterial cells derived from different sprouts or angioblast pools. In \textit{alk1} mutants, endothelial cell distribution is altered, with decreased cranial arterial endothelial cell contribution to endocardium, increased distal migration of endothelial cells, and increased mixing of arterial endothelial cells derived from different sprouts or angioblast pools. Together, these data suggest that loss of \textit{alk1} results in enhanced movement of arterial endothelial cells in the direction of blood flow, resulting in accumulation of cells in and enlargement of cranial arteries distal to the heart.
2.2 ORIGIN AND PATTERNING OF *ALK1*-POSITIVE ZEBRAFISH CRANIAL ARTERIES

Basic development of the cranial vascular system has been described previously [6]. I focus here on a more detailed analysis of the development of the *alk1*-positive cranial arteries. Angioblasts differentiate in the anterior lateral plate mesoderm at the 1-somite stage (~10.5 hpf) and coalesce into two pairs of bilateral clusters by the 7-somite stage (~12.5 hpf). Between the 14 and 18-somite stage (~16 hpf), a pair of ventral, caudally-directed sprouts emerge from the paired rostral clusters (rostral organizing centers, ROCs) and meet with rostrally-directed sprouts from the paired caudal clusters (midbrain organizing centers, MOCs) to form the ICAs. Both ROC- and MOC-derived ICA sprouts dive medially and form a transient left-right connection directly below the forming CaDI. The ROCs also launch dorso-posteriorly directed sprouts around this time navigate around the hypothalamus to form the bilateral CaDIs, which meet at the midline at ~23 hpf. Finally, cells from the MOCs migrate medially around the 20-somite stage (~19 hpf) to form the first aortic arches (AA1), which connect the outflow tract of the heart to the lateral dorsal aortae and ICA. Although this cranial arterial system is in place by 24 hpf, flow does not commence in these vessels until around 26 hpf, when primordial midbrain channel (PMBC)-derived sprouts connect to the apex of the CaDI and allow drainage (see Chapter 3). At this point, the apex of the CaDI compacts along the anterior-posterior axis and elongates along the left-right axis to become the BCA. The outflow tract, AA1, ICA, CaDI, and BCA become *alk1*-positive with the onset of blood flow [92], and there are no patterning defects in this cranial arterial system in *alk1* mutants.
Figure 6: Zebrafish cranial blood vessel development

By 12 somites, 2 bilateral clusters of angioblasts referred to as the midbrain organizing center (MOC) and rostral organizing center (ROC) have formed. By 18 somites, the MOC has begun to sprout anteriorly and posteriorly, with the more dorsal cells giving rise to the cranial veins and the more ventral cells contributing to the first aortic arch (AA1), lateral dorsal aortae (LDA) and the internal carotid arteries (ICA). The ROC has also begun to sprout and form the cranial division of the internal carotid artery (CrDI), the optic artery (OA), the caudal division of the internal carotid artery (CaDI) and the ICA. By 24 hpf, the heart has begun to beat and the final cranial connections are being made in anticipation of circulation. At 30 hpf, transient connections between the basal communicating artery (BCA) and the primordial midbrain channel (PMBC) are carrying flow and providing the only circulatory outlet until the connections between the metencephalic arteries (MtA), posterior communicating segments (PCS) and the basilar artery (BA) become patent at ~36 hpf.
2.3 PROXIMAL AND DISTAL ARTERIAL ENDOTHELIAL CELL NUMBERS ARE DIFFERENTIALLY AFFECTED IN ALK1 MUTANTS

Our laboratory previously reported an increase in endothelial cell number in alk1 mutant embryos compared to wild type embryos in the combined BCA/PCS [92, 121, 135] and CaDI [135]. Increases in BCA/PCS cell number were significant from 32-48 hpf, whereas CaDI cell number was examined only at 36 hpf. To better understand the origin of these increases in cell number, I investigated the development of and endothelial cell number in (from proximal to distal, with respect to the heart) AA1, ICA, CaDI, and BCA between 24 and 36 hpf. Because these vessels form a contiguous arterial system, I defined the boundaries of each based on their parent vessels, according to the diagrams in Figure 7 and Figure 11.

In wild type embryos, the number of endothelial cells in the proximal regions of AA1 (shaded in gray in Figure 6) decreased steadily between 24 hpf and 36 hpf (15.3± .6 to 12.0± 1.2 cells, mean ± SEM, Students T test, p<0.05 Figure 7A, C). Over this same period of time, AA1 diameters increased slightly, by approximately 5 μm (Figure 7B). Endothelial cell number in the ICA also decreased over this time period in wild type embryos (35.8± 1.3 to 22± .91 cells, mean ± SEM, Students T test, p<0.05 Figure 7A, C)

In alk1 mutant embryos, AA1 endothelial cell number also decreased over time and was indistinguishable from wild type siblings at 24-28 hpf, but was decreased compared to wild type by 30-36 hpf (16± .46 to 9± .55 cells, mean ± SEM, Students T test, p<0.05 Figure 7A,C). The morphology of AA1 was dynamic and variable in alk1 mutant embryos: the paired vessels often
developed asymmetrically, with one side dramatically decreasing in diameter (Figure 7A) and in rare cases seemingly disconnecting from the heart outflow tract. In contrast to wild type embryos, in which mean AA1 diameters increased slightly over time, the mean AA1 diameter did not change between 24 and 36 hpf in alk1 mutants, though variability was very high (Figure 7B). In the ICA, endothelial cell number was not different from wild type at 24-26 hpf but failed to decrease, as in wild type, at later times, resulting in a significant increase in cell number compared to wild type between 28 and 36 hpf (31.9± 1.7 to 34± 1.4 cells, mean ± SEM, Students T test, p<0.05 Figure 7A,C).

In contrast to the steady decrease in endothelial cell number in AA1 and the ICA, endothelial cell number in the more distal CaDI and BCA increased steadily over time in wild type embryos (CaDI: 14.5± .5 to 25.4± .6 cells, PCS: 0± 0 to 7.9± .7 cells mean ± SEM, Students T test, p<0.05 Figure 8). Endothelial cell number in the CaDI and BCA was significantly increased in alk1 mutants compared to wild type siblings from 30-36 hpf (CaDI: 15.3± .7 to 36.6± .5 cells, PCS: 0± 0 to 7± .5 cells mean ± SEM, Students T test, p<0.05 Figure 8). These data demonstrate that endothelial cell number in alk1 mutants is decreased in AA1, the artery most proximal to the heart, but increased in more distal arteries, including ICA, CaDI and BCA.
Figure 7: Proximal arteries have altered endothelial cell distribution and vessel morphology in alk1 mutants

Endothelial cell (EC) number decreases in AA1 between 24-36 hpf and this decrease is significantly higher in alk$^{1^{Y6}}$ embryos beginning at 30 hpf. The number of ECs decreases in the ICA over time in wt embryos and fails to decrease compared to wt siblings beginning at 28 hpf in alk$^{1^{Y6}}$ embryos. A, wire diagrams of the dorso-frontal view of the ventral cranial arterial system between 24-36 hpf of wt and an alk$^{1^{Y6}}$ sibling. The boundaries for each vessel are shaded in gray (AA1) and maroon (ICA) and correlate with data presented in C. Colored numbers in A represent the average number of cells ± SEM in the AA1 or ICA at each time point. B, The change in AA1 diameter between 24 and 36 hpf is increased by 5 μm on average in wt embryos and is highly variable in alk$^{1^{Y6}}$ embryos. C, Values are mean ± SEM, significance was determined by Students T test, *p<0.05 for individual comparisons.
2.4 ARTERIAL ENDOTHELIAL CELL NUMBER CHANGES IN ALK1 MUTANTS DO NOT RESULT FROM CHANGES IN PROLIFERATION OR APOPTOSIS

alk1 mutants have fewer arterial endothelial cells in AA1 but more in the ICA and CaDI. Because all of these endothelial cells are alk1 positive, it seems unlikely that these changes could be caused by enhanced apoptosis in AA1 and increased proliferation or decreased apoptosis in the ICA and CaDI. In support of this reasoning, no differences in apoptosis or proliferation were identified in these vessels in alk1 mutants versus wild type siblings by time-lapse (having analyzed 8 wt/control morpholino time-lapse movies and 12 alk1\(^{y6}/alk1\) morpholino time-lapse movies). In fact, we detected almost no proliferation or apoptosis in this vessel system regardless of genotype. These data suggest that a fixed number of differentiated endothelial cells distribute themselves over time in a stereotypical way within these contiguous arteries, and that this distribution is altered in alk1 mutants.
Figure 8: Distal cranial arteries have increased endothelial cell number in alk1 mutants

The CaDI (red) and the BCA (blue) have an increased number of endothelial cells beginning at 28 hpf in the BCA and 30 hpf in the CaDI in alk\textsuperscript{135} embryos compared to wt siblings. Values are mean ± SEM, significance was determined by Student’s T-test, *p<0.05 for individual comparisons. Boundaries for the CaDI and BCA are shaded in red and blue, respectively, in the wire diagrams (frontal views, anterior bottom), 24 to 36 hpf. Colored numbers in the wire diagrams represent the mean number of cells SEM at each time point. Opaque colors (first bar in pair), wild type; transparent colors (second bar in pair), alk1 mutants.
2.5 ARTERIAL ENDOTHELIAL CELLS PROXIMAL TO THE OUTFLOW TRACT MIGRATE TOWARD THE HEART, AGAINST THE DIRECTION OF BLOOD FLOW

The lack of proliferation or apoptosis in the developing cranial arterial system suggested that cell number changes in wild type cranial arteries from 24-36 hpf (decreased cell number in AA1 and ICA, increased cell number in CaDi) resulted from a redistribution of endothelial cells. To better appreciate the cell movements that generate the cranial arterial system, I performed time-lapse two-photon microscopy of Tg(fli1a:negfp)y7;Tg(fli1a.ep:mRFP-CAAX)yps504 embryos, which express GFP in endothelial cell nuclei and mRFP in endothelial cell membranes. Imaging between 23 and 33 hpf revealed a striking net movement of AA1 endothelial cells toward the heart beginning at approximately 24-25 hpf, just after the onset of heartbeat and blood flow (Figure 9A-B). Tracking of individual endothelial cells (Figure 9B-B’) demonstrated that on average 6-10 cells entered the heart from AA1 (red arrows), 3-6 cells entered AA1 from the ICA/LDA (blue arrows) and the rest of the cells remained in AA1 while migrating towards the heart (black arrows) during this window of development.

To confirm this observation, I performed fate mapping using Tg(fli1a:GAL4FF;UAS:kaede) embryos which expresses a photoconvertible fluorescent protein in the vascular endothelium. All cells in either the left or right AA1 were photoconverted from green to red at 24 hpf using a 405 nm laser, and photoconverted cell locations were recorded at 48 hpf (Figure 10). Nearly all embryos showed photoconverted cells in the outflow tract and heart, with approximately 60% of embryos having photoconverted cells within the proximal portion of the ventricle, adjacent to the atrioventricular canal. Approximately 20% of embryos had photoconverted cells remaining in AA1 but no embryos had photoconverted cells more distal
Figure 9: AA1 EC migration towards the heart is impaired in *alk1* morphants.

Time-lapse analysis of *tg(fli1a.ep:neGFP)*, *tg(fli1a.ep:mRFP-CAAX)* embryos injected with either a control MO or *Alk1* MO. The AA1 and where it intersects with the LDA/ICA and the heart is outlined in white dashed lines. Control MO: A-A”, *alk1* MO 1: C-C” and *alk1* MO 2: E-E”. Individual cells were manually tracked over a 12 hour time period, with the cell paths labeled every 2 hours (B, D, and F). The net migration, representing the distance between the initial and final position of each cell is charted in B’, D’ and F’. In control MO embryos (A), cells steadily march towards the heart, with cells that were originally proximal to the heart entering over the 12 hours. Cells from the ICA/LDA enter the AA1 as the vessel decreases in length, maintaining normal vessel caliber and morphology. In *alk1* MO embryos, the migration patterns are highly variable and result in abnormal AA1 morphology. In *alk1* MO1 (C), cells demonstrate a less steady migration towards the heart. No new cells enter the vessel over the 12 hour time period and the vessel caliber decreases significantly. In *alk1* MO 2 (E), cells on one side of the AA1 quickly migrate either towards the heart or the ICA/LDA and no new cells enter the vessel. On the other side, cells demonstrate a slower migration towards the heart, and one cell enters from the ICA/LDA region. This results in the thinning of one side of the vessel and an enlargement of the caliber of the other side of the vessel.
Figure 10: Photoconverted cells in AA1 enter the heart and this migration is altered in alk1 mutants and embryos lacking blood flow.

One side of AA1 (A), ICA or base of the CaDI was converted from green to red at 24 hpf in $tg(fli1:gal4FF)^{abl4};(uas:Kaede)^{abl4}$ embryos injected with either a control, alk1 MO or tnnt2a MO using the 405 laser. The location of the photoconverted cells was imaged at 48 hpf, with the 488 laser exciting the green Kaede cells and the 516 laser exciting the red Kaede cells. In control embryos, the majority of photoconverted cells were located in the heart. In alk1 MO embryos, photoconverted cells were often observed in the AA1 (white arrow head) and the
Images at 24 hpf are single z-stacks, dorsal, anterior down. 48 hpf are two-dimensional confocal projections, frontal with dorsal up. B. The cranial vasculature was divided into regions demarcated on the wire diagram. Cells were converted on one side of AA1, ICA or CaDI at 24 hpf and the location of the converted cells was scored at 48 hpf and the percentage of embryos with cells in each vessel region was calculated and graphed (C). The distribution of cells is shifted away from the heart in alk1 and tnt2a morphants. Numbers in the top right hand corner of each graph represents the number of embryos assayed for each condition.

than the middle (loop) region of AA1 (Figure 10). Additional photoconversion experiments revealed that cells in the ICA at 24 hpf either remain in the ICA or have moved toward the heart, against the direction of blood flow, by 48 hpf. However, ICA cells reach only distal regions of the ventricle (22% of embryos). Endothelial cells at the base of the CaDI also move toward the heart and contribute to the ICA in nearly all cases (90% of embryos) but only rarely to more proximal arteries (10-18% of embryos) and never to the heart. Together, these data demonstrate that endothelial cells initially residing in lumenized arteries most proximal to the heart migrate towards the heart, against the direction of blood flow, in wild type embryos.

In tnt2a morphants, which lack a heartbeat and blood flow, converted cells within one half of AA1 at 24 hpf did not migrate efficiently into the heart by 48 hpf (Figure 10). The location of these converted cells were shifted more distally when compared to the control morpholino embryos, with only 30% of embryos having cells located in the most distal region of the ventricle (versus 90%), ~90% in the outflow tract and 10% of embryos with cells located as distally as the CaDI. Photoconversion of the ICA and base of the CaDI revealed similar trends, with embryos having photoconverted cells present in more distal vessels at 48 hpf when
compared to control morpholino siblings. These results indicate that blood flow triggers the migration of endothelial cells towards the heart between 24 and 48 hpf.

In \textit{alk1} morphants, which completely phenocopy \textit{alk1} mutants, there was high variability in the migratory behavior of AA1 endothelial cells. In the Alk1 MO1 example (Figure 9C-D), 3 cells entered the heart from AA1, only 1 cell entered AA1 from distal vessels, and in general the migration was less directed. In the Alk1 MO2 example (Figure 9E-F), 6 cells entered the heart, 2 cells exited AA1 toward distal vessels, (migrating in the direction of blood flow) and no new cells entered AA1 from the LDA/ICA. These trends are also observed in additional experiments (n=6 for control morpholino and 8 for \textit{alk1} morpholino). Additionally, in kaede conversion experiments, the distribution of arterial endothelial cells was shifted distally compared to control siblings: a lower percentage of embryos showed AA1-derived photoconverted cells in the proximal ventricle (10\% versus 60\%), and a higher percentage showed photoconverted cells remaining in proximal (62\% versus 21\%) and distal (30\% versus 0\%) regions of AA1. Arterial endothelial cells residing at 24 hpf in the ICA and CaDI were also shifted distally in \textit{alk1} morphants compared to control siblings at 48 hpf (Figure 10). Together, with proliferation and apoptosis data, these data demonstrate that 1) the loss of arterial endothelial cells from wild type AA1 and ICA over time is due to proximal migration of these cells into the heart; and 2) the changes in arterial endothelial cell number in \textit{alk1} mutants (decreased in AA1, increased in ICA and CaDI) are likely due to decreased proximal migration (against the direction of blood flow) and/or increased distal migration (with the direction of blood flow).

To directly determine whether increased distal migration contributes to increased endothelial cell number in the CaDI in \textit{alk1} morphants, I performed time-lapse two-photon microscopy to image endothelial cell contributions to this vessel in \textit{Tg(fli1a:negfp)} embryos, 24-
36 hpf. In 24 hpf control embryos, the ROC-derived bilateral CaDIs (red nuclei) have surrounded the hypothalamus and connected dorsally, and the PMBC-derived sprouts (dark blue nuclei) are connecting to the apex of the CaDI (Figure 11A). The arterial CaDI-derived cells become the anterior portion of the BCA, whereas the venous PMBC-derived cells become the posterior portion of the BCA. By 27-28 hpf, bilateral metencephalic artery-derived sprouts (pink nuclei) have connected to the PMBC-derived cells, and by 31-32 hpf, these cells migrate posteriorly to form the posterior communicating segments (PCS). The PCSs meet at the midline and drain into the developing primordial hindbrain channel-derived basilar artery (BA) [178] by approximately 36 hpf. Thus, this elegant cranial arterial system is derived from two arterial sources (ROC, MtA) and two venous sources (PMBC, PHBC) and there is little to no mixing of these cells once connections have been made. Between 24 and 36 hpf, an average of 3 cells entered the CaDI from the ICA but remain at the base of these vessels, most likely reflective of the changing morphology of the vessels and supporting the idea of limited net migration in the direction of blood flow (Figure 11A, B).

In alk1 morphants, timing of development and basic patterning of this cranial arterial system is unchanged, but more cells enter the CaDI from the ICA and more distally, supporting the idea that increased distal migration is responsible for CaDI enlargement in these embryos (Figure 11). Furthermore, although the number of cells contributing to the developing arterial system from the PMBC and MtA is not different in alk1 morphants compared to controls, there is aberrant mixing of arterial- and venous-derived cells, with CaDI-derived cells reaching into territory normally occupied by PMBC-derived cells, and PMBC-derived cells reaching into territory normally occupied by MtA-derived cells. In summary, enhanced distal migration of alk1-dependent cells results in increased endothelial cell number in and caliber of the CaDI and
disrupts the “boundaries” between venous-derived and arterial-derived endothelial cells. The increased arterial caliber alters the hemodynamic load within the vasculature and precipitates flow-dependent shunt formation.

**Figure 11: Endothelial cell migration in the distal cranial vasculature**

A. Time-lapse analysis of $\text{Tg(fli1a.ep:neGFP)}^{y7}$ injected with either control or $\text{alk1 MO}$ between 24-36 hpf. Red cells originate from the ICA (originally the ROC), blue cells from the PMBC, pink cells from the MtA and light blue cells from the PHBC. Maroon cells are cells that have entered the CaDI from the ICA after the start of the movie. Wire diagrams were created from two dimensional confocal projections and pseudo-colored in Photoshop. B. Quantification of the number of cells that have entered the cranial arterial system from the ICA, PMBC and the MtA over the course of the movie. The increase in cell number in $\text{alk1 MO}$ embryos can be attributed to an increase in the number of cells entering from the ICA.
2.6 ARTERIAL ENDOTHELIAL CELLS REPRESENT A NOVEL SOURCE OF ENDOCARDIAL CELLS

The contribution of arterial endothelial cells to the heart has not previously been reported. To determine which cardiac cell type these AA1-derived endothelial cells become, I performed double immunofluorescence on Tg(alk1e5:egfp) embryos for EGFP and MF20. EGFP expression in Tg(alk1e5:egfp) embryos marks alk1-positive endothelial cells in AA1 at 24 hpf but is not detectable in the heart at 48 hpf. MF20 marks sarcomeric myosin heavy chain and only labels the myocardium [179]. Preliminary analysis indicates that these cells do not contribute to myocardium and are likely therefore endocardial, as would be expected by their endothelial origin (Figure 12). Future work is required to clearly identify the fate of these cells.

Figure 12: Endothelial cells migrating into the heart appear to become a part of the endocardium

Tg(alk1e5:egfp)$_{pt517}$ marks alk1-positive endothelial cells with gfp, but is too faint to visualize without antibody staining before ~32 hpf. MF20 labels sarcomeric myosin heavy chain and will specifically label myocardium. EGFP expressing (green) cells do not colocalize with MF20 (red) but are present inside the myocardium, indicating these cells are in the endocardium. Images are 2D confocal projections of 30 µM cryosections, dorsal view, anterior downward. Scale bar, 50 µm.
Alk1 signaling functions to transmit a flow-based signal that is required to limit arterial endothelial cell number and vessel caliber [92]. Here I demonstrate that upon the onset of flow, endothelial cells within the first aortic arch (AA1) migrate in opposition to flow and enter the heart, likely contributing to the endocardium. The cranial arterial system is derived from cells of both arterial and venous origins. Due to the carefully coordinated timing of the migration of these cells to their final destination, there is rarely any cell mixing and these cells generally cease to migrate after the onset of flow. In the absence of alk1, endothelial cell distribution is altered, with fewer cells in AA1 and more cells in the more distal ICA, CaDI and BCA. Because there was no observed endothelial cell apoptosis or differences in proliferation, these data suggest that the primary defect in alk1 mutants is due to aberrant migration and allocation of a fixed number of arterial endothelial cells. Arterial endothelial cells proximal to the heart fail to migrate in opposition to flow and/or migrate with flow, resulting in an accumulation of cells in distal cranial arteries.

We have previously reported that alk1 mutant zebrafish embryos develop AVMs via a two-step mechanism. The first step occurs independent of blood flow and results in an increased number of endothelial cells in the CaDI, resulting in an increased vessel caliber. In the second flow-dependent step, transient connections between arteries and veins are maintained as an adaptive response to the increased flow through the system due to the increased CaDI caliber [92, 135]. In this work, I have demonstrated that the increase in endothelial cell number that is central to the first step in AVM development occurs due to aberrant endothelial cell migration in what are normally alk1-positive arteries. Furthermore, I show through time-lapse analysis that
Alk1 signaling does not influence endothelial cell proliferation and increased proliferation does not account for the increase in arterial cell number in alk1 mutants (data not shown), as has been previously speculated [176, 180]. However, EDU and TUNEL staining are required to confirm proliferation and apoptosis time-lapse observations.
3.0 CONTEXT-SPECIFIC INTERACTIONS BETWEEN NOTCH AND ALK1 CANNOT EXPLAIN ALK1-ASSOCIATED ARTERIOVENOUS MALFORMATIONS

Notch and activin receptor-like kinase 1 (ALK1) have been implicated in arterial specification, angiogenic tip/stalk cell differentiation, and development of arteriovenous malformations (AVMs), and ALK1 can cooperate with Notch to upregulate expression of Notch target genes in cultured endothelial cells. These findings suggest that Notch and ALK1 might collaboratively program arterial identity and prevent AVMs. I therefore sought to investigate the interaction between Notch and Alk1 signaling in the developing vertebrate vasculature. I modulated Notch and Alk1 activities in zebrafish embryos and examined effects on Notch target gene expression and vascular morphology. Although Alk1 is not necessary for expression of Notch target genes in arterial endothelium, loss of Notch signaling unmasks a role for Alk1 in supporting hey2 and ephrinb2a expression in the dorsal aorta. In contrast, Notch and Alk1 play opposing roles in hey2 expression in cranial arteries and dll4 expression in all arterial endothelium, with Notch inducing and Alk1 repressing these genes. Although alk1 loss increases expression of dll4, AVMs in alk1 mutants could neither be phenocopied by Notch activation nor rescued by Dll4/Notch inhibition. Control of Notch targets in arterial endothelium is context-dependent, with gene-specific and region-specific requirements for Notch and Alk1. Alk1 is not required for arterial identity, and perturbations in Notch signaling cannot account for alk1 mutant-associated AVMs. These data
suggest that AVMs in HHT patients are not caused by defective arterial specification or altered Notch signaling.

### 3.1 INTRODUCTION

Notch signaling is critical for cell fate determination in many tissues. When activated by transmembrane ligands of the Delta and Jagged families, the Notch intracellular domain (NICD) is cleaved, translocates to the nucleus, binds to the DNA binding protein, recombination signal binding protein for immunoglobulin kappa J (RBPJ), and induces target gene expression [181]. In the vasculature, delta-like ligand 4 (Dll4)/Notch signaling controls arterial specification and angiogenic tip/stalk cell selection [30, 33, 38, 182], and in mouse and zebrafish models, decreased Dll4/Notch function leads to direct connections between arteries and veins, or arteriovenous malformations (AVMs) [30, 162, 166, 172, 183]. Because Dll4/Notch signaling transcriptionally upregulates the arterial endothelial marker, ephrinb2 (*Efnb2*) [184], and decreased Dll4/Notch signaling results in loss of *Efnb2* and ectopic arterial expression of the venous marker, *Ephb4* [30, 31, 162, 166, 171, 174, 183, 184], AVMs resulting from decreased Dll4/Notch signaling are generally attributed to disruption of arterial-venous identity. Notch loss-of-function (Notch*lof*) generates small caliber AVMs that are associated with thin, nearly atretic arteries [162, 183, 185]. Notch gain-of-function (Notch*gof*), which enhances *Efnb2* and represses *Ephb4*, also results in AVMs in mice [30, 31, 162, 166, 171, 174, 183, 184], and human brain AVMs exhibit increased Notch signaling [168, 186]. AVMs associated with Notch*gof* involve enlarged arteries containing supernumerary endothelial cells [31, 166, 167, 171, 172], suggesting
that failed repulsion mediated by EfnB2/EphB4, which is required for segregation of venous and arterial cells in developing vessels [187, 188], may be responsible for these AVMs. Thus, both Notch\textsuperscript{lof} and Notch\textsuperscript{gof} result in AVMs associated with disrupted arterial-venous identity, but the morphological characteristics of these AVMs are distinct, with low flow, small caliber shunts associated with Notch\textsuperscript{lof} and high flow, large caliber shunts associated with Notch\textsuperscript{gof}.

Similar to Notch signaling, bone morphogenetic protein (BMP) signaling has also been implicated in AVM prevention. BMP ligands bind to a heterotetrameric complex of type I and type II serine/threonine kinase receptors; non-signaling type III receptors facilitate ligand binding. Upon complex formation, type II receptors phosphorylate type I receptors, which in turn phosphorylate Smad1, Smad5, and/or Smad9. Phosphorylated Smads bind to Smad4, translocate to the nucleus, and bind to DNA to regulate gene expression [189]. In humans, heterozygous loss of endoglin (\textit{ENG}, encoding a type III receptor), activin receptor-like kinase 1 (\textit{ACVRL1} or \textit{ALK1}, encoding a type I receptor), or \textit{SMAD4} results in hereditary hemorrhagic telangiectasia (HHT), a disease characterized by a predisposition to development of telangiectasias and AVMs [98, 101, 190, 191]. \textit{Alk1} mutant mice exhibit decreased \textit{Efnb2} expression in the dorsal aorta (DA) [192], and BMP9/ALK1 transcriptionally induces \textit{EFNB2} in cultured human umbilical artery endothelial cells [174], suggesting that ALK1, similar to Notch, is required for arterial identity. Also like Notch, ALK1 has been implicated in maintenance of stalk cell identity [175]. Because both Notch and ALK1 are required for arterial differentiation, stalk cell fate determination, and prevention of AVMs, ALK1 and Notch might function in a common pathway to control arterial and/or stalk cell identity and prevent AVMs.

Several lines of evidence suggest that Notch and ALK1 interact in endothelial cells. Activation of either DLL4/Notch or BMP9/ALK1 in cultured endothelial cells enhances
expression of canonical Notch targets \textit{HEY1}, \textit{HEY2}, and \textit{HES1},\cite{32, 174, 175} and simultaneous activation of these pathways synergistically increases expression of \textit{HEY1} and \textit{HEY2} \cite{175}. A less dramatic effect on \textit{HEY2} expression is observed in response to combined stimulation by constitutively active forms of Notch (\textit{NICD}) and \textit{ALK1 (ALK1}\textsuperscript{CA} \cite{184}). Induction of \textit{HEY1} and \textit{HEY2} by BMP9/\textit{ALK1} requires \textit{SMAD4} but not \textit{RBPJ} \cite{175}, and BMP9 induces SMAD1/5 binding to \textit{HEY1}, \textit{HEY2}, and \textit{HES1} promoters in cultured human umbilical vein endothelial cells (HUVECs) \cite{193}. These findings suggest that BMP9/\textit{ALK1} directly stimulates canonical Notch targets by a SMAD-dependent and NICD/RBPJ-independent pathway. Notch and \textit{ALK1} additively induce \textit{VEGFR1}, a stalk cell marker, and inhibit \textit{APELIN}, a tip cell marker, and mosaic sprouting assays demonstrate that endothelial cells lacking \textit{ALK1}, \textit{SMAD4}, or \textit{HEY2} are more likely to be in the tip position \cite{175}. Together, these results suggest that BMP9/\textit{ALK1}/SMAD signaling induces canonical Notch targets independently of NICD/RBPJ and reinforces Notch-mediated acquisition of arterial identity and maintenance of stalk cell fate.

Although Notch and \textit{ALK1}\ exhibit synergistic interactions with respect to Notch target gene expression in cultured endothelial cells, evidence for synergy is less compelling for phenotypic endpoints. Both \textit{NICD} and \textit{ALK1}\textsuperscript{CA} dampen endothelial cell sprouting, but simultaneous pathway activation shows no further effects, and inhibition of one pathway does not inhibit effects of activation of the other pathway \cite{174}. Similarly, both \textit{ALK1} and Notch inhibition enhance VEGF-stimulated tube formation in HUVECs and increase vascular area in the postnatal retina; however, combined inhibition of these pathways shows less than additive effects \cite{175}. Thus, the synergy between Notch and \textit{ALK1} in controlling gene expression may not directly translate to synergistic effects on endothelial cell behavior.
To better understand the interaction between Notch and Alk1 in arterial endothelium, I assayed expression of Notch target genes and vascular phenotype in zebrafish embryos with altered Notch and/or Alk1 activity. Abrogation of Alk1 signaling did not decrease arterial endothelial expression of Notch targets, demonstrating that Alk1 is not necessary for maintenance of Notch target gene expression or arterial identity in vivo. However, concomitant inhibition of Notch and Alk1 revealed context-dependent interactions, with these two pathways cooperatively maintaining hey2 and efnb2a in the DA, yet exhibiting opposing roles in controlling hey2 expression and no role in controlling efnb2a expression in cranial arteries. In addition, I observed increased dll4 expression in both trunk and cranial arterial endothelium in the absence of Alk1 signaling, in contrast to the dramatic loss of expression observed with Notch inhibition. These molecular data suggested that AVMs in alk1 mutants might arise due to Notch gain-of-function; however, ectopic Notch activation failed to phenocopy and Notch inhibition failed to rescue AVMs associated with loss of alk1. Taken together, these data demonstrate that Notch and Alk1 exhibit context-specific and target-specific interactions in controlling Notch target gene expression in vivo, and that AVMs associated with Alk1 deficiency do not result from perturbations in Notch activity.

3.2 NOTCH IS ACTIVE CONCOMITANT WITH ALK1 IN CRANIAL ARTERIAL ENDOTHELIUM

Because my goal was to determine whether Alk1 and Notch signaling interact during vascular development, and because alk1 plays a critical role in zebrafish cranial arterial development [92,
I first assessed Notch activity in cranial endothelium using a double transgenic line, $Tg(tp1:egfp)^{um14};Tg(fli1a.ep:mRFP-CAAX)^{pt504}$. These fish report Notch activity, as visualized by EGFP expression [194], on a background of mRFP-labeled endothelial cells. Embryos were imaged at 36 hpf, a time point when Alk1 is active in cranial arterial endothelium [135]. Notch activity was weak to moderate in cranial arteries, including the first aortic arch (AA1), internal carotid artery (ICA), caudal division of the internal carotid artery (CaDI), optic artery (OA), and basal communicating artery (BCA) [Figure 13A]. All of these arteries are $alk1$-positive at 36 hpf [92]. Notch activity was also detected in the $alk1$-negative posterior communicating segments (PCS) and metencephalic arteries (MtA), but was absent in cranial veins (Figure 13A). These data demonstrate that cranial vascular Notch activity is arterial-specific, and that all $alk1$-positive arteries have active Notch signaling.

Next, I assayed cranial vessel expression of endogenous Notch targets. Hairy and enhancer of split (HES)-related proteins are transcriptional repressors that are induced by NICD/RBPJ [195], and the HES-related genes $HES1$, $HEY1$, and $HEY2$ are upregulated by BMP9/ALK1 in cultured endothelial cells [175, 184, 193]. The zebrafish genome contains two $hes1$ paralogs, $her6$ and $her9$ [196]. However, neither these genes nor $hey1$ were detectable in endothelium at 24-36 hpf (data not shown). In contrast, $hey2$ was expressed in all $alk1$-positive cranial arteries at 36 hpf (Figure 13B). $efnb2a$, another Notch target [184], as well as $dll4$, which encodes a Notch ligand that is positively regulated by Notch signaling [42, 197, 198], were also expressed in all $alk1$-positive cranial arteries at 36 hpf (Figure 13B). $dll4$ was additionally expressed in the $alk1$-negative PCS and MtA. These data demonstrate that Notch targets $hey2$, $efnb2a$, and $dll4$ are expressed in cranial arterial endothelium concomitant with active Alk1 signaling and are good candidates for cooperative regulation by Notch and Alk1.
Figure 13: Notch is active concomitant with Alk1 in cranial arterial endothelium

A, Notch activity is detectable in cranial arterial but not venous endothelium (“v”) at 36 hpf. Two-dimensional confocal projections of $Tg(tp1:egfp^{um14})$ (green in merge) and $Tg(fli1a.ep:mRFP-CAAX)^{p504}$ (magenta in merge). alk1-positive arteries: AA1, aortic arch 1; ICA, internal carotid artery; LDA, lateral dorsal aorta; CaDI, caudal division of internal carotid artery; OA, optic artery; BCA, basal communicating artery. alk1-negative arteries: PCS, posterior communicating segment; MtA, metencephalic artery. Images represent N = 20 embryos. Lateral and dorsal views, anterior leftward; frontal view, anterior up. Scale bar, 50 μm. B, Wiring diagrams (arteries black, veins gray) and representative whole mount in situ hybridization for alk1, hey2, efnb2a, and dll4 at 36 hpf. Expression of Notch...
targets is detected in the alk1-positive AA1 (white arrow), ICA (blue arrowhead), LDA (white asterisk), CaDI (white arrowhead), OA (blue asterisk), and BCA (blue arrow). dll4 is also expressed in the alk1-negative PCS (black asterisk) and MtA (black arrowhead). Plane of focus of dll4, frontal view, is deeper than other frontal images because of interfering dll4 brain expression. Images represent N > 63 embryos. Lateral and dorsal views, anterior leftward; frontal view, anterior up. Scale bar, 100 μm.

3.3 NOTCH AND ALK1 COOPERATIVELY REGULATE HEY2 AND EFNB2A BUT OPPOSITELY REGULATE DLL4 IN THE DORSAL AORTA

To investigate the interaction between Notch and Alk1 in the regulation of Notch targets in vivo, I assayed tp1:egfp, hey2, efnb2a, and dll4 in embryos with impaired Notch and/or Alk1 signaling. To inhibit Notch signaling, I treated embryos with 10 μmol/L LY411575, a gamma-secretase inhibitor, between 23 and 36 hpf. This time period brackets the critical time period of Alk1 function: alk1 is first detectable around 26 hpf, and alk1 mutation results in enlargement of cranial arteries by 32 hpf [92]. LY411575 treatment had no effect on heartbeat or blood flow but resulted in severe trunk curvature (data not shown) as expected in Notch-inhibited embryos [30, 33, 199].

Notch signaling is active in the zebrafish DA throughout embryonic development [30, 161, 182], and despite no obvious requirement for Alk1, alk1 is indeed expressed in the DA as early as 26 hpf [92]. LY411575 treatment resulted in complete loss of DA tp1:egfp expression (Figure 14A), demonstrating effective abrogation of NICD/RBPJ-mediated transcription. However, the effect of Notch inhibition on expression of endogenous Notch targets was variable.
LY411575 treatment had no effect on hey2, moderately decreased efnb2a, and completely abolished dll4 (Figure 14A). These observations agree with published data [30, 34] and suggest that among these DA genes, dll4 is most sensitive to perturbation of Notch signaling, whereas other pathways maintain hey2 and efnb2a in the absence of Notch.

Next, I examined Notch target gene expression in the DA in alk1f09e mutants (Figure 14A). alk1 mutation had no effect on expression of tp1:egfp, hey2, or efnb2a in the DA (Figure 14A), demonstrating that Alk1 is not necessary for expression of these arterial-specific Notch targets or for acquisition of arterial identity. In contrast, dll4 expression was upregulated in the DA in alk1f09e mutants (Figure 14A). This observation was confirmed in alk1v6 and alk1s407 mutants and in alk1 morphants (Figure 15). Furthermore, endothelial-specific expression of alk1CA (fli1a.ebs:alk1CA-mCherry) dramatically repressed dll4 (Figure 15). These data suggest that Alk1 opposes Notch in dll4 regulation in the DA.

Compared to Notch inhibition alone, combined alk1f09e mutation and Notch inhibition had no effect on tp1:egfp or dll4 expression (Figure 14A), supporting the idea that Notch is required for expression of these genes. In contrast, concomitant abrogation of Alk1 and Notch signaling decreased hey2 and nearly eliminated efnb2a (Figure 14A). hey2 results, originally obtained in LY411575-treated alk1f09e embryos, were recapitulated in DAPT-treated alk1v6 mutants and LY411575-treated alk1 morphants (data not shown). These findings suggest cooperative support of hey2 and efnb2a expression by Notch and Alk1 in the DA and agree with published data suggesting that both genes are regulated independently via NICD/RBPJ and ALK1/Smad1, 5 [30, 174, 175, 193].
Figure 14: Notch- and Alk1-mediated control of Notch target gene expression is gene-specific and context-dependent

Whole mount in situ hybridization for *tp1:egfp, hey2, efnb2a, dll4, and cdh5* in 36 hpf wild type (wt) and *alk1<sup>100e</sup>* embryos treated with 1% DMSO or 10 μmol/L LY411575, 23-36 hpf. **A**, Trunk. Lateral view, anterior leftwards. **B**, Head. AA1, white arrow; ICA, blue arrowhead; LDA, white asterisk; CaDI, white arrowheads; OA, blue asterisks; MtA, black arrowheads. *tp1:egfp, efnb2a, cdh5*: frontal views, anterior up. *hey2, dll4*: lateral views, anterior left. Numbers in upper right corners indicate number of embryos with similar phenotype/total number of embryos assayed. Scale bars, 100 μm.
Figure 15: dll4 is negatively regulated by Alk1 signaling

Whole mount in situ hybridization for dll4 in 
alk1^+/6 (36 hpf), alk1^+/407 (32 hpf) and alk1 morphants (MO) (36 hpf) in the trunk (A) and head (C). In the absence of alk1, dll4 expression is increased in the dorsal aorta (trunk), aortic arch 1 (white arrow), internal carotid artery (blue arrowhead), lateral dorsal aorta (white asterisks), and metencephalic artery (black arrowhead) when compared to wild type (wt) or control MO siblings. Tg(fli1a.ebs:alk1^{CA-mcherry}) embryos show no vascular dll4 expression at 36 hpf in the trunk (B) or head (D). Expression of collagen type IV alpha1 (col4a1), a pan-endothelial marker, demonstrates the presence of vessels in Tg(fli1a.ebs:alk1^{CA-mcherry}) embryos. Numbers in lower right corners indicate number of embryos with similar phenotype/total number of embryos assayed. Lateral views, anterior left. Scale bar, 200 μm.
3.4 NOTCH AND ALK1 EXHIBIT GENE-SPECIFIC ANTAGONISTIC INTERACTIONS IN REGULATION OF CRANIAL ARTERIAL ENDOTHELIAL GENE EXPRESSION

I next examined regulation of Notch target genes in cranial arteries, which enlarge upstream of AVMs in alk1 mutants. LY411575 treatment abrogated tp1:egfp expression in cranial arterial endothelium and neural domains (Figure 14B and Figure 16), as expected. However, whereas Notch inhibition dramatically decreased hey2 in cranial arterial endothelium, efnb2a was refractory to this treatment (Figure 14B and Figure 16). These effects were different from those observed in the DA (Figure 14A), suggesting context-specific gene regulation. LY411575 treatment eliminated arterial dll4 expression in cranial arteries, similar to the DA, but increased dll4 in neural tissues (Figure 14B and Figure 16). These observations support the idea of a positive feedback loop specific to arterial endothelial cells in which Notch signaling directly regulates DLL4 expression [34, 42, 197, 198]. As in the DA, in cranial arteries tp1:egfp, hey2, and efnb2a were unaffected by alk1 mutation or knockdown, whereas dll4 was markedly upregulated, and a fltl1a.ebs:alk1CA-mCherry transgene dramatically repressed dll4 (Figure 14B, Figure 15C-D, and Figure 16). Also similar to the DA, loss of alk1 failed to rescue abrogation of tp1:egfp or dll4 expression induced by LY411575 treatment (Figure 14B and Figure 16). In contrast, hey2 and efnb2a behaved differently in the absence of both Notch and Alk1 signaling in trunk versus cranial arterial endothelial domains. In cranial arteries, efnb2a expression proved refractory to combined loss of Notch and Alk1 signaling, whereas this same treatment increased hey2 expression compared to Notch inhibition alone (Figure 14B and Figure 16). hey2 results, originally obtained in LY411575-treated alk1<sup>f109e</sup> embryos, were recapitulated in DAPT-treated
alk1<sup>16</sup> mutants and LY411575-treated alk1 morphants (Figure 17). These results suggest that neither Notch nor Alk1 is necessary for cranial arterial efnb2a expression, whereas Notch activates and Alk1 dampens hey2 expression in cranial arteries, with Alk1 acting either downstream of NICD or independently of Notch. These data support the notion that region-specific regulatory networks control arterial expression of Notch target genes. Effects of Notch and Alk1 manipulation on Notch target gene expression are summarized in Table 1.

**Table 1: Qualitative changes in arterial gene expression in response to altered Notch and/or Alk1 signaling**

* Embryos were treated with 1% DMSO or 10 μmol/L LY411575, 23-36 hpf, and analyzed by in situ hybridization at 36 hpf. Table indicates increased (up arrows), decreased (down arrows; number of arrows indicates qualitative strength of response), or no change (NC) in expression compared to DMSO-treated wild type embryos.

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<td>wild type</td>
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<tr>
<td>tp1:egfp</td>
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<td>hey2</td>
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Figure 16: Notch targets are differentially regulated by Alk1 and Notch signaling in cranial arterial endothelium

Transverse cranial vibratome sections (50 μm) of 36 hpf control morpholino- and alk1 morpholino-injected embryos treated with 10 μmol/L LY411575 or 1% DMSO, 23-36 hpf. Embryos were subjected to whole mount in situ hybridization for Notch-regulated egfp, hey2, efnb2a, dll4 and cdh5 prior to sectioning. Arrowheads, caudal divisions of the internal carotid artery. N = 12-20 embryos per treatment. Frontal views, dorsal up. Scale bar, 40 μm.
Figure 17: Opposing roles of Alk1 and Notch signaling in cranial arterial *hey2* expression

Whole mount in situ hybridization for *hey2* in 48 hpf wild type (wt) and *alk1* mutant embryos treated with 50 μmol/L DAPT, 23-48 hpf (A), and 36 hpf control morphant and *alk1* morphant embryos treated with 10 μmol/L LY411575, 23-36 hpf (B). *hey2* expression in the first aortic arch (white arrow), internal carotid artery (blue arrowhead), and lateral dorsal aorta (white asterisk) is decreased with Notch inhibition, unaffected by *alk1* loss-of-function, but returned to control levels by concomitant Notch inhibition and *alk1* mutation or knockdown. Numbers in lower right corners indicate number of embryos with similar phenotype/total number of embryos assayed. Lateral views, anterior left. Scale bar, 200 μm.
3.5  **NOTCH**\(^{GOF}\) AND **ALK1**\(^{LOF}\) GENERATE VASCULAR MORPHOLOGIES WITH SOME PHENOTYPIC OVERLAP BUT WITH INDEPENDENT ETIOLOGIES

My in vivo gene expression studies demonstrated that loss of *alk1* is associated with increased endothelial *dll4* expression. Therefore, I reasoned that enhanced Dll4/Notch signaling might phenocopy cranial AVMs in *alk1* mutants. To investigate this possibility, I compared cranial vascular development in wild type embryos, *Tg(fli1a:GAL4FF)ubs3;Tg(5xUAS-E1b:6xMYC-notch1a)kca3* embryos [which ectopically express Notch1a ICD in all endothelial cells; hereafter referred to as *Tg(endo:N1ICD)*], and *alk1* morphant embryos.

In wild type embryos, single sprouts emerged from the most posterior aspect of each bilateral venous primordial midbrain channel (PMBC) ~22 hpf and migrated medially to connect to a sprout emanating from the apex of the paired CaDIs by ~ 26 hpf, forming the BCA. These transient BCA/PMBC connections serve as the primary drainage for the CaDI/BCA between 26-36 hpf but regress thereafter as downstream arteries develop [92].

In *Tg(endo:N1ICD)* embryos, the CaDIs developed and lumenized normally, but sprouting from the PMBC was impaired, with BCA/PMBC connections delayed up to 8 hours compared to wild type [Figure 18, compare A-E, WT to F-J, *Tg(endo:N1ICD)*]. Vascular morphology was variable in 36 hpf *Tg(endo:N1ICD)* embryos, with establishment of early alternative drainage connections (for example, BCA to PCS/MtA, Figure 18G-H) associated with relatively normal caliber vessels, and establishment of late connections associated with CaDI/BCA engorgement and enlargement (Figure 18P,Q). Regardless of 36 hpf phenotype, BCA area was not significantly increased compared to control at 48 hpf (Figure 18S), but all
*Tg(endo:N1ICD)* embryos maintained at least one BCA/PMBC connection, resulting in a small caliber AVM (Figure 18P’,Q’,T).

Although the *Tg(endo:N1ICD)* phenotype bears some resemblance to the *alk1* mutant phenotype, these phenotypes originate and progress differently. In *Tg(endo:N1ICD)* embryos, delayed venous sprouting compromises early cranial arterial drainage, resulting in variable changes in CaDI/BCA calibers and small AVMs stemming from persistent BCA/PMBC connections (Figure 18F-J, Q, Q’). In *alk1* mutants, venous-derived angiogenic sprouting is not delayed, and all vessel connections develop normally (Figure 18K-O). However, increased endothelial cell number in the CaDI leads to increased caliber and altered hemodynamics, causing downstream vessels to adapt by maintaining normally transient arterial-venous connections (Figure 18T), most often between the BCA and PMBC [92, 121, 135]. Although the *Tg(endo:N1ICD)* phenotype decreases in severity over time (Figure 18Q, Q’), the *alk1* mutant phenotype exacerbates over time (Figure 18R, R’), with progressive increases in vessel caliber both upstream and downstream of the AVM.
Figure 18: notch\textsuperscript{of} and alk1\textsuperscript{of} cranial AVMs have independent etiologies

(A-J) Time-lapse analysis of cranial arterial development in wild type (A-E), Tg(endo:N1ICD) (F-J), and alk1 morphant (K-O) embryos, 28-36 hpf. See also Supplemental material online, Movies S4, 5, 6. In Tg(endo:N1ICD) embryos (F-J), PMBC-derived sprouts (yellow arrowheads) are delayed, compromising CaDI/BCA drainage. In
alkl morphant embryos (K-O), all connections form normally, but BCA enlargement is evident as early as 30 hpf. Two-dimensional projections of Z-stacks from two photon/confocal time-lapse imaging, frontal views, anterior up. Images represent N = 8 WT, 6 Tg(endo:N1ICD), 10 alkl MO. Endothelial transgenes imaged: WT and Tg(endo:N1ICD), Tg(fli1a:GAL4FF;UAS:kaede); alkl MO, Tg(fli1a:mrfp-caax)ps504. (P-R’) Two-photon imaging of WT (P, P’), Tg(endo:N1ICD) (Q, Q’), and alkl y6 mutant (R, R’) embryos at 36 and 48 hpf. Tg(endo:N1ICD) embryos show phenotypic overlap with alkl mutants, with variable enlargement of the CaDI at 36 hpf and consistent retention of BCA/PMBC connections at 48 hpf. Two-dimensional projections of Z-stacks, dorsal views, anterior up. Images represent N = 10 WT, 8 Tg(endo:N1ICD), and 8 alkl y6 mutants. Imaged transgene is Tg(kdrl:gfp)a116. (A-R) Pseudocoloring: PMBC, blue; arteries, red; BCA/PMBC connection, purple. Scale bars, 50 μm. (S) BCA area is significantly increased in alkl mutants but not Tg(endo:N1ICD) at 48 hpf. N = 6-9 for each condition; lines represent mean ± SEM. One-way ANOVA followed by Tukey’s post-hoc test, ***P<0.001, ****P < 0.0001. (T) Presence of shunts at 48 hpf.
Figure 19: dll4 expression is not required for AVM development in alk1 mutants

Embryos from an alk1<sup>y6/+;Tg(kdrl:gfp)<sup>la116</sup> incross were injected at the 1 to 4-cell stage with 15 ng dll4 MO or 5-bp mismatch control MO, imaged at 36 (A-D) or 48 (E-H) hpf, and genotyped. Two-dimensional projections of two-photon Z-stacks. Pseudo-coloring: PMBC (blue), arteries (red), and BCA/PMBC connection (purple). Dorsal views, anterior up. Images represent N = 7-41 embryos per condition. Scale bars, 50 μm. (I) dll4 knockdown failed to rescue increased BCA area in alk1<sup>y6</sup> mutants at 36 hpf. Lines represent mean ± SEM, N = 6-21 embryos per condition. One-way ANOVA followed by Tukey’s post-hoc test, * P<0.05; ****P<0.0001. (J) dll4 knockdown failed to rescue cranial shunt formation in alk1<sup>y6</sup> mutants at 48 hpf.
(A) RT-PCR following injection of dll4 MO or 5-bp mismatch control MO (15 ng) using primers flanking the MO binding site. In 3 dpf control morphant cDNA, a single 345 bp band was detected. In 3 dpf dll4 MO cDNA, dll4 wild type product was decreased to 37% of control and an additional exon 3-deleted product (287 bp) was detected. Band intensities were normalized to β-actin and identities were confirmed by sequencing of gel-extracted bands.

(B) Injection of dll4 MO but not 5-bp mismatch control MO (15 ng) caused hypersprouting (arrowheads) in trunk intersegmental vessels, 3 dpf, in wild type and alk1 y6 y6 mutant embryos. Two-dimensional confocal projections, Tg(fli1a:mrfp-caax)pt504, anterior left. Scale bar, 50 µm

(C) Hypersprouting was present in 63% of dll4 MO-injected embryos, with similar representation in wild type and alk1 mutant embryos.
3.6 NOTCH ACTIVITY IS NOT REQUIRED FOR AVM DEVELOPMENT IN ALKI MUTANTS

To further explore the role of enhanced Dll4/Notch in AVM development in the absence of Alk1 function, I inhibited Notch activity in alkl mutants and assessed cranial vascular phenotype. First, I injected a splice-blocking dll4 morpholino[33] into alkl mutants. The morpholino generated an aberrant splice product that eliminated exon 3, decreased wild type transcript to approximately 37% of control, and resulted in intersegmental vessel hypersprouting [33, 34] in 60% of embryos (Figure 20). Wild type dll4 morphants exhibited a small but significant increase in BCA area at 36 hpf (Figure 19A, B, I) but normal cranial vessel morphology, with no AVMs, at 48 hpf (Figure 19E, F, J). dll4 knockdown failed to rescue increased BCA area (36 hpf) or cranial AVMs (48 hpf) in alkl mutants (Figure 19C, D, G, H, I, J).

Because I could not achieve complete knockdown of dll4, I treated alkl mutants with LY411575 (beginning at 23 hpf) to eliminate Notch activity and assessed cranial vascular phenotype at 36 and 48 hpf (Figure 21). In DMSO-treated wild type embryos, the anterior central arteries (CtAs) [200] sprouted as the BCA/PMBC connection regressed: one or two sprouts emerged from more anterior aspects of each PMBC and migrated medially, with sprouts interacting ipsilaterally but not contralaterally and ultimately connecting to the BCA by 36 hpf (Figure 21A). Notch inhibition in wild type embryos had no effect on the CaDI/BCA but caused hypersprouting in the PMBC-derived CtAs at 36 hpf, with significant increases in number of PMBC-derived sprouts, connections to the BCA, branch points, and contralateral sprout connections (Figure 21B, Figure 22A-B’, E-H).
In contrast to Notch-inhibited embryos, DMSO-treated alk1 mutants exhibited enlarged CaDIs/BCAs at 36 hpf, as previously reported [92, 121, 135], but PMBC-derived CtAs were unaffected (Figure 21C, Figure 22C-D’, E-H). Notch-inhibited alk1 mutants were indistinguishable from DMSO-treated alk1 mutants in terms of increased BCA caliber at 36 hpf (Figure 21C, D, I). At 48 hpf, LY411575 treatment decreased PCS caliber in both wild type and alk1 mutant embryos but failed to rescue alk1 mutant AVMs (Figure 21E-H, J). Furthermore, despite enhanced dll4 expression, Notch signaling, as quantified by mean BCA EGFP fluorescence intensity in Tg(tp1:egfp)\textsuperscript{um14} embryos, was unchanged in alk1 morphants compared to control (Figure 23). These data support dll4 morphant data and suggest that enhanced Dll4/Notch signaling does not underlie AVM development in the absence of alk1.

Although Notch inhibition failed to rescue the alk1 mutant phenotype, alk1 mutation dampened Notch inhibitor-induced effects on CtAs, decreasing number of PMBC-derived sprouts, number of connections to the BCA, and CtA branch points to levels between wild type DMSO-treated and wild type LY411575-treated embryos (Figure 21A-D and Figure 22). However, effects did not achieve statistical significance. Further studies are required to determine whether this result reflects a genetic interaction between Alk1 and Notch pathways or an effect of altered vascular hemodynamics.
Figure 21: Notch activity is not required for AVM development in alk1 mutants

Embryos from an alk1^106/+;Tg(kdrl:gfpl116 incross were treated with 1% DMSO or 10 μmol/L LY411575 at 23 hpf and cranial vasculature imaged at 36 (A-D) and 48 (E-H) hpf. Two-dimensional projections of two-photon Z-stacks; in (E-H), dorsal planes were removed to highlight BCA/PCS. Pseudo-coloring: PMBC (blue), CtA (cyan); CaDI/BCA/PCS/BA (red), and BCA/PMBC shunt (purple). Dorsal views, anterior up. Scale bars, 50 μm (36 hpf) and 100 μm (48 hpf). (I) Notch inhibition failed to rescue increased BCA area in alk1^106 mutants at 36 hpf. Lines represent mean ± SEM, N = 4-7 embryos per condition. One-way ANOVA followed by Tukey’s post-hoc test, *P<0.05; ***P<0.001. (J) Notch inhibition failed to rescue cranial shunt formation in alk1^106 mutants at 48 hpf.
Figure 22: The Notch\textsuperscript{huf} hypersprouting phenotype in midbrain and forebrain central arteries is partially rescued by alk\textsuperscript{1} mutation

Embryos from an alk\textsuperscript{1} y6/+; Tg(kdrl:gfp)\textsuperscript{la116} incross were treated with 1% DMSO or 10 \textmu mol/L LY411575, 23-36 hpf. Two-dimensional two-photon projections of the 36 hpf vasculature (A-D) were traced to generate wiring diagrams (A'-D') of the midbrain and forebrain central arteries (CtA). Blue dots: connections to the primordial midbrain channel (PMBC); red dots: connections to the basal communicating artery (BCA); green dots: branch points. Frontal views, dorso-anterior up. Scale bar, 50 \textmu m. Graphs quantify the number of PMBC-derived sprouts (E), CtA/BCA connections (F), CtA branch points (G), and CtA contralateral connections (H). N= 4-7 for each condition; lines represent mean ± SEM. One-way ANOVA followed by Tukey’s post-hoc test, *P<0.05, ***P<0.001.
Figure 23: Notch signaling output is unaffected by alk1 knockdown

(A) A single Z-plane through the center of the BCA of 36 hpf Tg(tp1:egfp)$^{um14}$;Tg(fli1a.ep:mRFP-CAAX)$^{p8504}$ control morphant (MO) and alk1 MO embryos. NICD/RBPJ-driven EGFP is green, endothelial cell membranes are magenta. Dorsal views, anterior up. Scale bar, 50 μm. (B) The mRFP channel was used to create a mask of the endothelial cell membrane, and the average EGFP intensity of the BCA was measured. N = 10-12 for each condition; lines represent mean ± SEM. Student’s t-test: not significant.
alk1 mutants develop enlarged cranial arteries (red) that drain through an aberrantly-retained connection (purple) to major primitive drainage veins (blue). Although this phenotype is associated with increased arterial dll4, notch^lof fails to phenocopy and notch^gof fails to rescue this defect. notch^gof causes impaired venous-derived sprouting (black), whereas notch^lof causes enhanced venous-derived sprouting. 48 hpf, dorsal views, anterior up.

3.7 DISCUSSION

My results in zebrafish embryos demonstrate context-specific effects of Notch signaling on arterial endothelial gene expression. Although Notch inhibition abrogated transcription of a synthetic Notch reporter and endogenous dll4 in both trunk and cranial arteries, other arterial Notch targets were less sensitive to Notch inhibition, suggesting that additional control elements
sustain expression of these genes in the absence of Notch. Furthermore, Notch sensitivity of particular genes showed regional variation, suggesting unique regulatory mechanisms in different vascular beds. These data suggest that context-specific regulation, which may be lost or dampened in cultured endothelial cells, plays an important role in the control of Notch target gene expression in vivo.

Based on published work demonstrating cooperative interactions between DLL4/Notch and BMP9/ALK1 in enhancing arterial Notch target gene expression in cultured endothelial cells [175], I had anticipated that combined Notch and Alk1 inhibition might additively if not synergistically decrease Notch target gene expression and impair arterial specification. However, abrogation of Alk1 signaling failed to decrease expression of Notch targets or disrupt arterial (hey2, efnb2a, dll4) or venous (data not shown) identity, and I uncovered only minor cooperative interactions between Alk1 and Notch, with both contributing to maintenance of efnb2a and hey2 expression in trunk but not cranial arteries. Furthermore, I uncovered opposing roles of Notch and Alk1 in expression of the Notch target and arterial marker, dll4. Although dll4 is upregulated in the zebrafish DA in the absence of blood flow [201], lack of blood flow cannot account for increased dll4 in alk1 mutants: blood flow remains strong in alk1 mutants at 36 hpf, with only a subtle redistribution of flow towards cranial vessels [92]. Together with previous data demonstrating increased cxcr4a and decreased edn1 in the absence of blood flow or alk1 expression [92], these data support the idea that Alk1 mediates a flow-based signal that controls expression of a subset of arterial genes. It is also possible that dll4 upregulation in the absence of alk1 might be attributed to the loss of the Smad1/5 target, id1. In the DA, id1 is downregulated in the absence of alk1 or blood flow (data not shown), and ID1 stabilizes HES1 (Her6 in zebrafish), which in turn represses DLL4 [177, 202]. Therefore, decreased Id1 might decrease Her6, thereby
resulting in increased dll4. However, I was unable to detect her6 in zebrafish arterial endothelial cells by in situ hybridization.

In addition to the unanticipated opposing effects of Notch and Alk1 on dll4 expression, I demonstrated a paradoxical interaction with respect to cranial arterial hey2 regulation, with loss of alk1 restoring hey2 to near control levels in Notch-inhibited embryos. This finding suggests that Alk1 may repress hey2 in cranial arterial endothelial cells either downstream of NICD cleavage or via an independent mechanism. Although it is possible that enhanced blood flow through enlarged, alk1-dependent vessels might increase hey2 expression, this seems unlikely given that hey2 expression is unchanged in alk1 mutants with intact Notch signaling (Table 1) or in the absence of blood flow (data not shown). Further studies are required to untangle the relationship between Notch and Alk1 in cranial arterial hey2 regulation.

Dll4 is a critical arterial endothelial Notch ligand [182, 184] and Notch gof and in particularDll4 overexpression results in large caliber arteries and AVMs similar in morphology to AVMs in Alk1 knockout mice [31, 166, 167, 171, 172]. Therefore, my finding that Alk1 inhibited dll4 expression initially suggested to us that increased Notch signaling might contribute to AVMs in alk1 mutants. However, multiple lines of evidence fail to support this hypothesis. First, although ectopic endothelial expression of N1ICD results in small cranial AVMs involving the same vessels as in alk1 mutants, the origin and progression of these AVMs differs dramatically. The primary defect leading to AVMs in N1ICD-expressing embryos is delayed venous-derived sprouting, whereas the primary defect in alk1 mutants is increased endothelial cell number in and caliber of upstream arteries [92]. Differences in the spatiotemporal expression of flila-driven N1ICD versus alk1 limit the utility of my approach; however, I would expect that earlier N1ICD expression would cause an even more pronounced arterial phenotype.
if enhanced arterial Notch signaling could serve as a proxy for arterial alk1 loss. Second, neither dll4 knockdown nor Notch inhibition rescues alk1 mutant AVMs. Third, despite increased dll4 expression, I failed to detect increased canonical Notch activity in alk1 mutants. Together, these results suggest that AVMs arise independently in Notch^{gof} and Alk1^{lof} embryos.

Although AVMs initiate via independent mechanisms in Notch^{gof} and Alk1^{lof} embryos, both represent retention of normally transient BCA/PMBC connections. In alk1 mutants, maintenance of BCA/PMBC connections occurs as an adaptive response to increased shear stress caused by enlargement of upstream arteries [92]. In Notch^{gof} embryos, impaired venous-derived sprouting delays CaDI/BCA drainage, which likely alters cranial vascular hemodynamics and affects remodeling. Thus, an adaptive response to altered hemodynamics, downstream of independent primary molecular and cellular defects, may be a unifying factor in development of Notch^{gof} and Alk1^{lof} AVMs.

Although I failed to rescue vascular defects in alk1 mutants via Notch inhibition, I partially rescued CtA hypersprouting defects in Notch-inhibited embryos via alk1 mutation, suggesting some interaction between Notch and Alk1 in the cranial vasculature. It is possible that restored hey2 expression in the absence of both Notch and Alk1 signaling might contribute to this phenomenon, as knockdown of HEY2 enhances sprouting in cultured HUVECs [175]. However, whether these observations represent a true genetic interaction or a response to changes in vascular hemodynamics remains to be determined.

In summary, my in vivo analysis of Notch and Alk1 signaling demonstrates gene-specific and context-specific interactions, with examples of both cooperative and antagonistic control of gene expression. However, results fail to support the idea that these pathways interact synergistically to control Notch target genes, program arterial identity, and prevent AVMs.
4.0 CONCLUSIONS AND FUTURE DIRECTIONS

The work presented in this thesis provides insight into the role of Alk1 signaling in endothelial cell migration and presents a model for AVM formation caused by pathway disruption. Previous work in the lab had demonstrated that AVMs form via a two-step mechanism upon loss of alk1. In the first step, endothelial cell number increases in cranial arteries and this increase is independent of blood flow [92, 121, 135]. As an adaptive response to increased flow, transient connections are maintained forming flow dependent arteriovenous shunts [92]. My work has focused on furthering the understanding of how loss of alk1 results in an increase in endothelial cell number in cranial arteries in the zebrafish embryo. I show that Alk1 is necessary for directed endothelial cell migration upon the onset of blood flow and that alk1 mutant AVMs do not form as a result of defects in arterial/venous identity. I have also contributed to the general knowledge of vascular development by describing in detail the formation of the cranial vascular system between 24 and 36 hpf, a key developmental period when cranial circulation begins. To this end, I have found that endothelial cells migrate in opposition to flow and contribute to the endocardium, described the sources of the endothelial cells that contribute to the cranial arterial system, and described how the timing of sprouting is key to forming a normal mature vascular system.
There are many unresolved questions. Whether Alk1 functions as a mechanosensor/transducer is not clear. Alk1-positive arteries do not appear to have primary cilia (B. Roman, unpublished), and the expression of vegfr and vecad is unaffected in alk1 mutants (data not shown). However, Alk1 may regulate vegf or vecad at the translational or post-translational level and experiments testing the role of Alk1 signaling on the localization of Vegfr2 and Vecad protein in the presence of flow would be informative. Based on the observation that endothelial cells are defective in directed endothelial cell migration, it is possible that Alk1 is involved in endothelial cell polarization. In the presence of high magnitude flow endothelial cells polarize with the nucleus downstream of the microtubule organizing center and the golgi apparatus [74]. In preliminary experiments, I was able to inject a GFP tagged centrin construct (centrin is a key protein of the centrosomes) into zebrafish embryos and visualize endothelial cell centrin in AA1 (data not shown). I hope to determine if endothelial cell polarization is affected in alk1 mutant embryos and in ALK1-depleted cultured endothelial cells exposed to shear stress.

Angiogenesis is often viewed as a binary process. A vessel is either actively undergoing angiogenesis (endothelial cell proliferation, migration and degradation of the ECM) or is quiescent (no proliferation or migration and vessels are stabilized through ECM deposition and smooth muscle recruitment). A long standing model in the field suggested that TGFβ1 activation of Alk1 and Alk5 induced activation or stabilization, respectively, and that the relative activation of the two pathways determined the activation state of the endothelium [176, 180, 203, 204]. AVMs were thought to arise due to a disruption in this balance causing aberrant angiogenesis [203, 205]. This model was shown to be inaccurate because in mice and zebrafish, Alk1 and Alk5 are not coexpressed in the vascular endothelium and Alk5 is not required in endothelial
cells for normal vascular development [118, 206, 207]. Subsequent work has postulated that AVMs form in HHT models due to defects in arterial venous identity (as evidenced by decreased ephrinB2 expression in the DA of mouse alk1 mutants [114, 121]) or due to focal capillary regression [90].

It is my hope that the work I present in this document provides a more nuanced understanding of angiogenesis and AVM development. ALK1 signaling is neither pro- nor anti-angiogenic, and AVM formation is not the result of “too much” or “too little” angiogenesis. Instead, AVM development involves defects in post-angiogenic endothelial cell behaviors and force-dependent remodeling. Alk1 is important upon the onset of flow to provide a means for endothelial cells to migrate towards the heart in the vessels that experience the highest hemodynamic load. Without this signal, arterial endothelial cells are not able to respond appropriately and while the immediate result is somewhat variable, the final result is a decrease in cells proximal to the heart and an accumulation of cells in downstream arteries. My work has also demonstrated that while Alk1 is not necessary for determining or maintaining arterial identity, there is still much to be understood regarding the relationship and interactions between Alk1, Notch and blood flow.

4.1 CURRENT HHT TREATMENTS

Current HHT treatments focus on treating symptoms. Large PAVMs or BAVMs may be coil-embolized or surgically resected, but these procedures are invasive and risky. The observation that wounding is necessary for adult endothelial-targeted Eng (Eng-iKO\(^5\)) [120] and inducible
knockout Alk1 (Alk1-iKO<sup>e</sup>) mice [117] to develop AVMs has led to the hypothesis that sites of active angiogenesis are more susceptible to AVM formation in HHT patients. To this end, anti-angiogenic treatments have been shown to greatly improve Eng-iKO<sup>e</sup> and Alk1-iKO<sup>e</sup> mouse vascular phenotypes. Specifically treatment with antibodies designed to block VEGF have reversed vascular abnormalities in these mice and restored levels of key angiogenic factors that had been dysregulated in affected tissues [208, 209]. In addition, HHT patients treated with similar therapies have experience significant improvement in nose bleeds, GI bleeding and liver complications [210-213]. Given that VEGF does not appear to be involved in Eng-iKO<sup>e</sup> and Alk1-iKO<sup>e</sup> mouse disease pathology, the mechanism by which anti-VEGF treatment improves HHT symptoms is not understood [107]. In light of my research, I suggest that AVMs develop in immature vessels because of aberrant distribution of arterial endothelial cells that causes arterial enlargement, which results in an altered hemodynamic load and consequent downstream AVM formation to normalize hemodynamic forces. Inhibiting angiogenesis prevents the initiation of a sequence of events that would lead to improper endothelial cell migration and distribution.
5.0 MATERIAL AND METHODS

5.1 ZEBRAFISH LINES AND MAINTENANCE

Adult zebrafish (Danio rerio) were maintained according to standard protocols [214]. Embryos were grown at 28.5°C in 30% Danieau [17 mM NaCl, 2 mM KCl, 0.12 mM MgSO₄, 1.8 mM Ca(NO₃)₂, 1.5 mM HEPES]. For imaging, embryo medium was supplemented with 0.003% phenylthiourea (Sigma, St. Louis, MO, USA) at ~8 hpf hours post-fertilization (hpf) to prevent melanin synthesis. Mutant lines alk1<sup>fo09e</sup> (p.Y88X), alk1<sup>y6</sup> (p.L240F), alk1<sup>s407</sup> (g.IVS8-2A>T), alk1 splice-blocking morpholino, and alk1<sup>y6</sup> genotyping assay have been described [92, 121, 215]. Line alk1<sup>fo09e</sup> was genotyped by dCAPs assay [216] using primers 5' -GTGCTACGTACCTGCTATTCCTGGAGTCTA-3' and 5' -CGAACAACCCAGAAACGAG-3'. The forward primer contains a single mismatch (underlined) that creates an XbaI site in the mutant allele. Line alk1<sup>s407</sup> was genotyped using PCR primers 5' -GACAATTTCCAGTCTCCTCCTC-3' and 5' -CGAATTCTTGGGCTGTGGCCGTC-3' followed by restriction digest with DdeI (cuts wild type only). Transgenic lines Tg(fli1a:GAL4FF)<sup>ubs3</sup>, Tg(UAS:Kaede)<sup>y88</sup> and Tg(kdr:GFP)<sup>la116</sup> have been described [217-219]. Two new transgenic lines were created by Gateway cloning (Invitrogen/Life Technologies, Carlsbad, CA, USA) into tol2 transposon arm-flanked vectors followed by injection into one-cell stage embryos [220-222]. Tg(fli1a.ep:mRFP-CAAX)<sup>p504</sup> has mRFP-labeled endothelial cell membranes.
*Tg(fli1a.ebs:alk1\(^{Ca}\)-mCherry)* expresses constitutively active *alk1* [121] fused to mCherry in all endothelial cells. This transgene is embryonic lethal; therefore, F1 embryos were analyzed from mosaic P0 founders. A previously described troponin T type 2a (*tnnt2a*) morpholino [223] was used to prevent heartbeat and blood flow. A full list and description of transgenic zebrafish used in this work can be found in Table 2.

**Table 2: Transgenic zebrafish used in this work**

<table>
<thead>
<tr>
<th>Transgenics</th>
<th>Allele</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>alk1e5:egfp</td>
<td>pt517</td>
<td>Expresses gfp under the control of an alk1 enhancer element</td>
</tr>
<tr>
<td>cmlc2:ndsred2</td>
<td>f2</td>
<td>Expresses dsred in myocardial nuclei</td>
</tr>
<tr>
<td>fli1ebs:alk1ca-mcherry</td>
<td>-</td>
<td>Ectopically expresses a constitutively active mcherry tagged alk1 in the vascular endothelium</td>
</tr>
<tr>
<td>fli1:gff</td>
<td>ubs4</td>
<td>A gal4 variant: drives uas expression in the vascular endothelium</td>
</tr>
<tr>
<td>fli1ep:mRFP-CAAX</td>
<td>pt504</td>
<td>Expresses mRFP in endothelial cell membranes</td>
</tr>
<tr>
<td>flk1:nEGFP</td>
<td>y7</td>
<td>Expresses gfp in endothelial cell nuclei</td>
</tr>
<tr>
<td>flk1:GFP</td>
<td>la116</td>
<td>Expresses gfp in endothelial cell cytoplasm</td>
</tr>
<tr>
<td>flk1:nls-mcherry</td>
<td>is4</td>
<td>Expresses mcherry in endothelial cell nuclei</td>
</tr>
<tr>
<td>gata1:dsred</td>
<td>sd2</td>
<td>Expresses dsred in erythrocytes</td>
</tr>
<tr>
<td>tp-1MmHb5:eGFP</td>
<td>um14</td>
<td>Expresses gfp under the control of a Notch enhancer element</td>
</tr>
<tr>
<td>uas:kaede</td>
<td>rkr8</td>
<td>Expresses a photoconvertible cytoplasmic protein in the presence of gal4</td>
</tr>
<tr>
<td>uas:myc-N1ICD</td>
<td>kca3</td>
<td>Expresses a myc tagged intracellular domain of Notch1 in the presence of gal4</td>
</tr>
</tbody>
</table>
5.2 MORPHOLINOS AND MORPHOLINO VALIDATION

Morpholinos were purchased from GeneTools, Philomath, OR, USA. Morpholinos used in this study are listed in Table 3. Splice blocking morpholinos are denoted as SB and translation blocking is denoted by TB. The control morpholino was injected at the same concentration as the experimental morpholino for each experiment. Dll4 MO was validated by RT-PCR using primers listed in Table 4 according to the published protocol [33].

<table>
<thead>
<tr>
<th>Morpholinos</th>
<th>Sequence</th>
<th>Concentration</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>alk1</td>
<td>5’-ATCGGTTTCACTCACCACACACTC-3’</td>
<td>2.5 ng</td>
<td>SB</td>
</tr>
<tr>
<td>tnnt2a</td>
<td>5’-CATGTTTCGCTGTATCTGACACGCA-3’</td>
<td>4 ng</td>
<td>TB</td>
</tr>
<tr>
<td>dll4</td>
<td>5’-CGAATCTTTACCTACAGGTAGATCCG-3’</td>
<td>15 ng</td>
<td>SB</td>
</tr>
<tr>
<td>dll4 5-mismatch</td>
<td>5’-CGAATgTTAgCTAgAGcTAcATCCG-3'</td>
<td>15 ng</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>5’-CCTCTTACCTCAACACCACACACTC-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.3 CONFOCAL AND TWO-PHOTON IMAGING

For live imaging, up to 12 embryos were anesthetized in 160 mg/ml tricaine (Sigma) and embedded in 0.5% low melting temperature NuSieve GTG Agarose (Lonza, Rockland, ME, USA)/30% Danieau. Z-series (1.48 mm steps) were collected using a TCS SP5 multiphoton/confocal microscope (Leica Microsystems, Wetzlar, Germany) outfitted with a custom motorized stage (Scientifica, Uckfield, East Sussex, UK), an APO L 20x/1.00 water
immersion objective or an HCX IRAPO L 25x/0.95 water immersion objective, non-descanned detectors, and spectral detectors, with a 1.7X zoom. EGFP was excited with a Mai Tai DeepSee Ti:Sapphire laser (Newport/Spectra Physics, Santa Clara, CA, USA) at 900 nm, whereas mCherry and dsRed were excited with a 561 nm diode. Sequential frame scanning was performed using a resonant scanner with unidirectional (8000 Hz) or bidirectional (1600 Hz) scanning and 16x or 32x line averaging. For time lapse experiments, (X,Y) coordinates were set using the LAS AF “Mark and Find” function, and images were collected every 18-23 minutes, with z-stack parameters redefined for each (X,Y) coordinate. Images were analyzed using LAS AF (version 3.0.0 build 834) and Adobe Photoshop CS6. Confocal time series were converted to QuickTime (.mov) files using LAS AF and annotated using Final Cut Pro and iMovie.

5.4 ENDOTHELIAL CELL TRACKING

Maximum projection z-stacks were compiled using the Leica ASF-AF software and each time point was saved as an individual tiff. Tiffs were imported into an Adobe Photoshop file, with each time point occupying a single layer. Individual cells were labeled in Photoshop using the paint tool and tracked over each layer. 3D projections were referenced in the Leica ASF-AF software when it became difficult to track an individual cell with certainty.
<table>
<thead>
<tr>
<th>Allele</th>
<th>Forward Primer 5'-3'</th>
<th>Reverse Primer 5'-3'</th>
<th>Digest</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>alk1&lt;sup&gt;y6&lt;/sup&gt;</td>
<td>cacggtccactaaggeatgaaacacctt</td>
<td>atggacagagaagtgaagtaagaat</td>
<td>BsaI1</td>
<td>dCAPS; Cuts wt</td>
</tr>
<tr>
<td>alk1&lt;sup&gt;f009e&lt;/sup&gt;</td>
<td>gtgctacgtacctgttatctggtgtaet</td>
<td>cgaacaacctcagaaacag</td>
<td>XbaI</td>
<td>dCAPS; cuts mutant</td>
</tr>
<tr>
<td>alk1&lt;sup&gt;x407&lt;/sup&gt;</td>
<td>gagatttctccagtctcttc</td>
<td>ctgggctgtgctgtgcttc</td>
<td>DdeI</td>
<td>RFLP; cuts WT</td>
</tr>
<tr>
<td>sih&lt;sup&gt;tc300b&lt;/sup&gt;</td>
<td>tattgctctttttaagttgctgtgtae</td>
<td>gaacataagactaaccctctgctgttc</td>
<td>XbaI</td>
<td>dCAPS; Cuts wt</td>
</tr>
</tbody>
</table>

**Transgenics**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Forward Primer 5'-3'</th>
<th>Reverse Primer 5'-3'</th>
<th>Digest</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>gfp</td>
<td>tgggtcccatctgtcagctgagctggt</td>
<td>aagtcgtgtgctctatgtg</td>
<td>n/a</td>
<td>1 band for +GFP</td>
</tr>
<tr>
<td>fli1ep:gffubs4</td>
<td>ctcgctgactagggcaactt</td>
<td>gacgcatcttttatccatttgc</td>
<td>n/a</td>
<td>1 band for +gff (200 bp)</td>
</tr>
<tr>
<td>mCherry</td>
<td>ccttcctccctctctgtt</td>
<td>cccatgtctctctctcat</td>
<td>n/a</td>
<td>1 band for +mcherry</td>
</tr>
<tr>
<td>uas:Kaede&lt;sup&gt;r8&lt;/sup&gt;</td>
<td>tgggagcaggaaggctgtgt</td>
<td>caccctcctctctctgtaag</td>
<td>n/a</td>
<td>1 band for +Kaede</td>
</tr>
<tr>
<td>uas:notch1ICD&lt;sup&gt;kaa3&lt;/sup&gt;</td>
<td>cgggtcgagctcagctggt</td>
<td>gttgaggtccagtcagtgt</td>
<td>n/a</td>
<td>1 band for NotchICD -350 bp</td>
</tr>
</tbody>
</table>

**Dll4 MO validation**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Forward Primer 5'-3'</th>
<th>Reverse Primer 5'-3'</th>
<th>Digest</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>dll4</td>
<td>cgtgtctccagtgactgtatcttt</td>
<td>gaacactgtcgcccagtagtaat</td>
<td>n/a</td>
<td>1 band at 345 bp in control, exon 3 excluded in MO injected resulting in 287 bp product</td>
</tr>
<tr>
<td>actinb2</td>
<td>cgtgctgtctcccctcatca</td>
<td>caccaccagactcttgttgttctg</td>
<td>n/a</td>
<td>1 band ~100 bp</td>
</tr>
</tbody>
</table>
5.5 KAEDE PHOTOCONVERSION

Up to 12 embryos were embedded in 0.5% low melt agarose at 24 hpf. The Leica “Frap Wizard” software was used for imaging and bleaching. An initial scan was captured with the argon laser set to 28% power, with the 488 nm line at 25%; the 561 nm diode at 25% and the 405 nm laser at 0%, 600Hz laser speed (3x line average) and a 2x zoom. Using this image, a region of interest (ROI) was drawn around the section of vessel that was to be photoconverted. For bleaching all parameters remained the same, with the exception of the 405 laser, which was set to 20%. The “set background to zero” and “use laser settings for all ROIs” options were selected. The bleaching time course consisted of 2 pre-bleach scans, 5 bleach scans, and 2 post-bleach scans. Embryos were removed from the agarose and placed in a 12-well plate and incubated at 28.5°C in the dark until 48 hpf. Embryos were then embedded again and imaged using the 488 nm and 516 nm wavelengths to determine the location of the converted cells.

To quantify the location of photoconverted cells, I divided the vessels into anatomical sections and scored whether or not an individual embryo had a converted cell in that specific region. The data represents the percentage of embryos that had at least one photoconverted cell in each vessel region.
5.6 CRYOSECTIONS AND IMMUNOFLUORESCENCE

Embryos were fixed overnight in 4% paraformaldehyde at 4°C. Embryos were then washed into a 15% and 30% sucrose/PBS solution before being embedded in Tissue Freezing Medium (TFM, Triangle Biomedical Sciences) and stored at -80°C. Cryosectioning was performed on a Leica CM 1850 and 30 µM sections were immediately placed on a Shandon Superfrost Plus positively charged slide (Thermo Scientific), dried at 37°C for 30 minutes and stored at -20°C overnight. Immunohistochemistry was performed using primary antibodies mouse anti-MF20 at 1:500 (sarcomeric myosin, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) or rabbit anti-GFP at 1:500 (Invitrogen, A-11122) and secondary antibodies goat-anti-rabbit Alexa Fluor 488 at 1:1000, and goat-anti-mouse Alexa Fluor 568 at 1:1000. Embryos were washed in a PBS/0.1% triton X-100/0.1% DMSO (PBDT) solution and blocked in 5% goat serum in PBDT. Sections were mounted with Vectashield Fluorescent mounting medium (Vector) and imaged with an Olympus Fluoview 1000 confocal microscope outfitted with a UPFLN 20x oil immersion objective. Two-dimensional projections were generated from Z-series (1 µm steps) using ImageJ 1.45s (National Institutes of Health, USA).

5.7 IN SITU HYBRIDIZATION

All embryos were collected at 36 hpf, fixed in 4% paraformaldehyde/PBS for approximately 36 hours at 4°C, dehydrated in methanol, and stored at -20°C for in situ hybridization. Digoxigenin-labeled riboprobes were generated according to the manufacturer’s protocol (Roche,
Indianapolis, IN, USA). cdh5 plasmid [92], dll4 plasmid [33], and efnb2a plasmid [30] have been described. hey2 was amplified from zebrafish cDNA and cloned into PCRII-TOPO (Invitrogen/Life Technologies). egfp was amplified from plasmid DNA and cloned into pCRII-TOPO. collagen type IV alpha 1 (col4a1) was amplified from zebrafish cDNA using primers appended with T3 (sense) and T7 (antisense) polymerase sites, and the PCR product was column purified (Qiaquick PCR Purification Kit) and used for riboprobe synthesis. Whole mount in situ hybridization was performed in an InSituPro VSi liquid handler (Intavis Inc, Chicago, IL, USA). Briefly, 36 hpf embryos were rehydrated to PBS/0.1% tween-20 (PBT), permeabilized with 50 mg/ml proteinase K for 15 minutes, and the permeabilization terminated with 0.2% glycine/PBT, 5 minutes. Embryos were then post-fixed for 20 minutes with 4% paraformaldehyde/PBS and hybridized at 65°C for 12 hr with riboprobe diluted 1:500 in hybridization buffer (50% formamide, 5x SSC, 5 mg/mL yeast tRNA, 50 mg/ml heparin, 0.1% tween-20). Embryos were then washed with 50% formamide/2x SSC/0.1% tween-20 (2 x 30 minutes, 65°C), 2x SSC/0.1% tween-20 (15 minutes, 65°C), and 0.2x SSC/0.1% tween-20 (2 x 30 mintues, room temperature). Specimens were then blocked with 5% sheep serum (Sigma)/PBT and incubated with embryo-adsorbed anti-digoxigenin-AP, Fab fragments (Roche), 1:5000 in 5% sheep serum/PBT. Embryos were then washed 6 x 15 minutes followed by overnight in PBT, transferred to 6-well plates containing NTMT (100 mmol/L NaCl, 100 mmol/L Tris-HCl, pH 9.5, 50 mmol/L MgCl₂, 1% tween-20), and incubated in NBT/BCIP (340 mg/ml nitro blue tetrazolium; 350 µg/ml 5-bromo-4-chloro-3-indolyly phosphate in NTMT) for color development. Embryos were photographed using an MVX-10 MacroView microscope and DP71 camera (Olympus America, Center Valley, PA, USA), scored for expression pattern and relative staining intensity, and then processed for genotyping (Table 4). Stained embryos were embedded in 4% NuSieve GTG
agarose (Lonza, Rockland, ME, USA), sectioned at 50 mm with a VT1000S vibratome (Leica Microsystems, Buffalo Grove, IL, USA), and photographed using a BX51 compound microscope with UPLFLN 20x/0.5 objective and DP71 camera (Olympus). All figures represent embryos that were simultaneously processed for fixation and staining.

5.8 CENTRAL ARtery SPROUT QUANTIFICATION

Manual tracing (Adobe Photoshop CS6) of maximum projections generated from confocal Z-stacks was used to generate simplified wiring diagrams of the forebrain and midbrain central arteries, which originate from the primordial midbrain channels. From these wiring diagrams, I counted: sprouts emerging from the primordial midbrain channel, sprout connections to the basal communicating artery, branch points, and contralateral connections (midline crossings). Each parameter was averaged within treatment groups and values are presented as mean ± SEM.

5.9 BCA AREA QUANTIFICATION

Approximate basal communicating area measurements were achieved by creating a region of interest on a two dimensional maximal projection around the basal communicating artery. Using the analyze tool in the LAS AF (version 3.0.0 build 8134) software, the area within the ROI was calculated. Areas were averaged within treatment groups and values are presented as mean ± SEM.
5.10 FLUORESCENCE INTENSITY MEASUREMENTS OF NOTCH REPORTER EMBRYOS

Fluorescence intensity of cranial arterial EGFP in Tg(tp1:egfp)$_{um14}$;Tg(fli1a.ep:mRFP-CAAX)$_{pt504}$ embryos was quantified using the LAS AF Version 3.0.0 build 8134 software. An ROI was created based on the mRFP channel (threshold, 55; background, 30), and GFP intensity within the masked ROI was measured and averaged over a single z plane, yielding a mean intensity of GFP fluorescence within the ROI. Mean intensities were averaged across samples (n=10 control morphants, 12 alk1 morphants), and values expressed as mean ± SEM. Results were verified by manually drawing ROIs over the basal communicating artery, using the mRFP channel as a guide, and averaging the colocalized GFP intensity across an entire stack for two embryos per treatment. This method gave similar results to the automated method.


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107


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