EXPRESSION OF *alk1* IS REGULATED BY A POSITIVE FEEDBACK MECHANISM INVOLVING BLOOD FLOW AND CIRCULATING LIGAND

by

Amy Biery Kunz

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This thesis was presented

by

Amy Biery Kunz

It was defended on

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and approved by

Zsolt Urban, PhD, Associate Professor, Department of Human Genetics,
Graduate School of Public Health, University of Pittsburgh

Neil Hukriede, Associate Professor and Vice Chair, Department of Developmental Biology,
School of Medicine, University of Pittsburgh

Andrea L. Durst, MS, DrPH, LCGC, Assistant Professor, Department of Human Genetics,
Graduate School of Public Health, University of Pittsburgh

Thesis Advisor: Beth L. Roman, PhD, Visiting Associate Professor, Department of Human
Genetics, Graduate School of Public Health, University of Pittsburgh

Beth L. Roman, PhD

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ABSTRACT

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 in 5,000 people worldwide. HHT is thought to be caused by a haploinsufficiency and therefore delineating how *ALK1* is regulated could provide avenues for targeted and effective clinical interventions. In the zebrafish model organism, *alk1* is expressed in arterial endothelial cells of vessels leading away from the heart. We have learned that *alk1* expression closely correlates with the presence of blood flow, but which component of blood flow is responsible for regulation is unknown. I propose that flow is required for activation of a positive feedback mechanism by which Bmp10 – a circulating ligand of Alk1 receptors that is produced by the heart and secreted into the bloodstream – through Alk1 activation maintains arterial *alk1* expression at the level of transcription. In this work, I define the spatiotemporal relationship between cessation and restoration of blood flow and *alk1* expression and show that flow-mediated *alk1* expression is regulated at the transcriptional level. Finally, I provide evidence that intact Bmp10/Alk1 signaling is required to maintain *alk1* mRNA expression. I hypothesize that this effect is driven by a gradient of Bmp10 ligand established by blood flow that triggers a positive feedback mechanism initiated by Bmp10/Alk1 signaling. The goal of my work is to understand the mechanism of *ALK1* regulation in order to progress toward

the goal of targets and effective clinical management for HHT patients. HHT is a relatively common genetic disease that often goes underdiagnosed due to its variable presentation even among members of the same family, hence studying this complex disease is significant for practice of public health.

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PREFACE

I would like to thank Beth for taking a chance on mentoring a genetic counseling student and exposing me to the world of vasculature genetics. I have already applied what I have learned about HHT to my graduate training in multiple clinical disciplines and I am sure will use my knowledge to better my practice throughout my career. I have worked on projects with many investigators in the past, and she is one of the most dedicated and creative scientists I have had the pleasure to work with.

I would like to thank members of the Roman Lab – past and present – that have contributed to my project. Special thanks goes to Teresa Capasso and Liz Rochon. They have offered hours upon hours of their time as well as their expertise in all things zebrafish, and my project would not have been successful without them. Not only have they been wonderful coworkers, but they have been amazing friends.

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A sincere thanks goes to my fellow genetic counseling classmates who have kept me sane throughout the two-year ordeal that is graduate school. I am so lucky to have found a group of

women who share the same geeky tendencies and sense of humor as me, and I am sad to see our time together come to a close.

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1.0   INTRODUCTION

1.1   VERTEBRATE VASCULAR DEVELOPMENT

1.1.1   Vasculogenesis

Vasculogenesis is the process by which the first blood vessels arise from angioblasts, the precursors to vascular endothelial cells, during vertebrate embryonic development. The mechanism by which angioblasts differentiate is highly conserved among vertebrates and implicates members of the ETS and FOX families of transcription factors [1]. Angioblasts migrate in large clusters into cord-like structures, forming the major trunk vessels and primitive cranial and yolk sac plexuses, and then hollow out to carry blood flow, a process called lumenization [2].

1.1.2   Angiogenesis

Angiogenesis is the process by which nascent vessels established by vasculogenesis become remodeled and expanded. For example, plexuses, which are honeycomb-like structures of similarly sized vessels, remodel into a hierarchical network of arteries, capillaries, and veins [3]. This remodeling of the vasculature occurs via segment pruning, vessel splitting (intussusception), and vessel fusion [3]. The activation phase of sprouting angiogenesis involves

extension of new vessels from existing vessels and requires endothelial cell proliferation and migration [3]. Eventually, endothelial cells cease to migrate and proliferate, signaling the resolution phase of angiogenesis. During this phase, new vessels are stabilized by deposition of extracellular matrix while vascular smooth muscle cells and other support cells called pericytes are recruited to these sites, a process that is enabled by angiopoietin, PDGFB and TGF-β signaling [4, 5].

1.2   HEREDITARY HEMORRHAGIC TELANGIECTASIA

1.2.1   Clinical disease in humans

Hereditary hemorrhagic telangiectasia (HHT) is a genetic syndrome that affects vascular development. It is categorized by the development of multiple arteriovenous malformations (AVMs), which are direct connections between arteries and veins in lieu of a capillary bed (Figure 1A). A loss of capillary structure results in compromised gas and nutrient exchange as well as susceptibility to rupture as higher than typical hemodynamic forces reach the veins [6]. AVMs in small vessels are referred to as telangiectases, which may be present in the skin, nasal mucosa, and gastrointestinal tract. A common symptom experienced by HHT patients is frequent nosebleeds, or epistaxis, due to rupture of nasal telangiectases; this can cause decreased quality of life and, if severe enough, life-threatening anemia requiring blood transfusions [6]. Large-vessel AVMs can form in virtually any area of the body but are commonly found in the lung, liver, and brain in HHT patients, putting them at risk for stroke, heart failure, brain abscess, and death [7].

1.2.2   Molecular genetics of HHT

1.2.2.1 TGF-β superfamily signaling

The exact disease mechanism of HHT is not well understood but stems from decreased transforming growth factor-β (TGF-β) superfamily signaling in vascular endothelial cells. In TGF-β family signaling, ligands dimerize and bind to a complex of type I and type II receptor serine/threonine kinases (Figure 1B). Ligand binding is aided by non-signaling type III receptors. Complex formation allows the phosphorylation of type I receptors by type II receptors. Type I receptors then go on to phosphorylate corresponding intracellular receptor-specific (R)-SMADs which pair with SMAD4 proteins to cross the nuclear membrane and regulate gene transcription. The family of TGF-β ligands is large and can be divided into multiple sub-families. 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 [8].

1.2.2.2 Known genes implicated in HHT

HHT is caused by genetic defects in at least three genes important for endothelial TGF-β family signaling. Heterozygous mutations in endoglin (*ENG)*, which encodes a type III receptor, cause HHT1 [9] while mutations in *ACVRL1*, which encodes the type I receptor activin receptor-like kinase 1 (ALK1), result in HHT2 [10] (Figure 1B). Mutations in these genes account for up to 96% of cases of HHT [11]. Another 2% of cases are caused by heterozygous mutations in *MADH4* [11] which encodes the common protein, SMAD4, required for most transcriptional responses involving the TGF-β protein superfamily. Mutations in *MADH4* cause combined HHT and juvenile polyposis syndrome (HHT-JPS) [12].
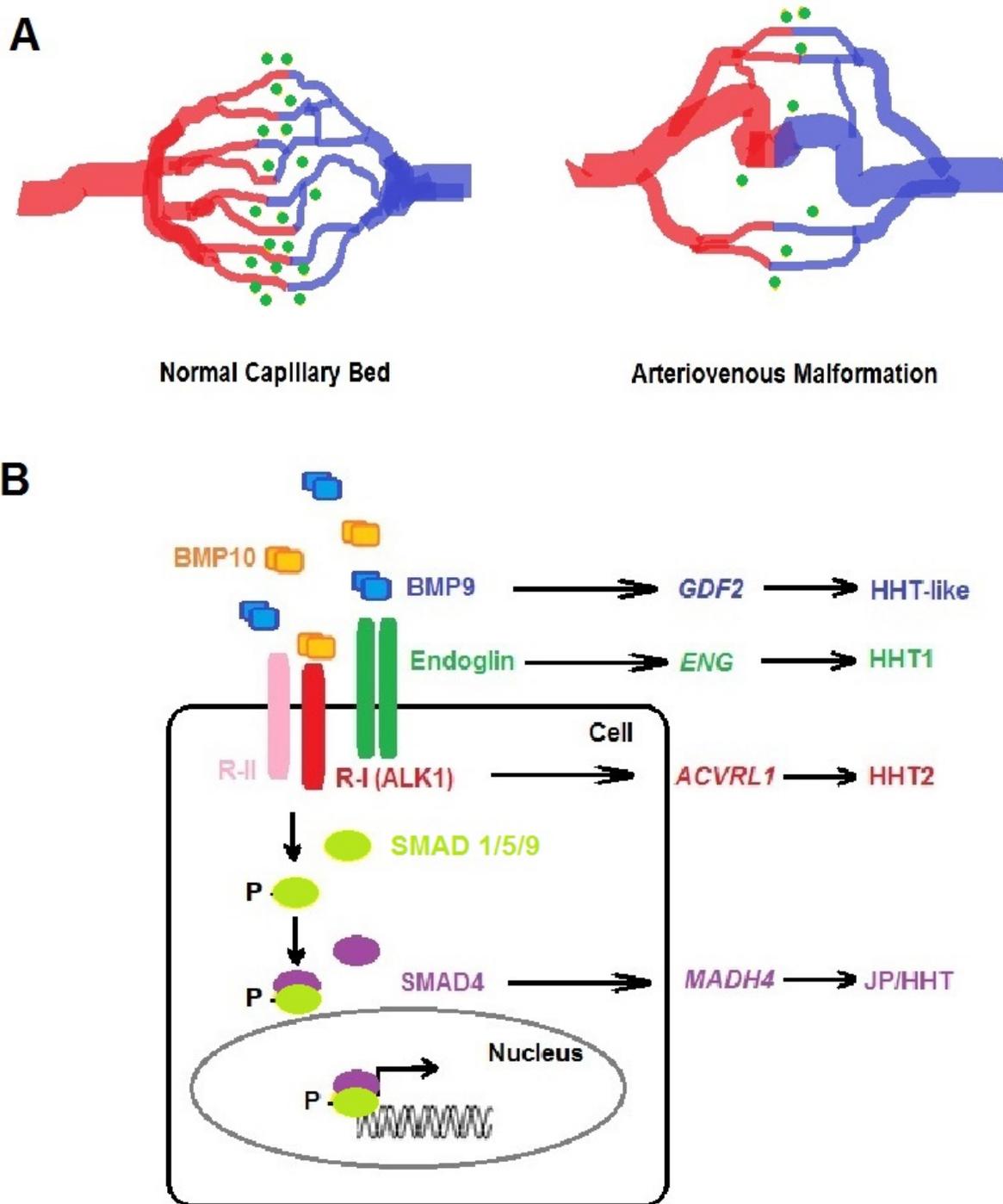
Figure 1. Arteriovenous malformations are a result of improper TGF- β signaling.
(**A**) Components of an AVM compared to a normal capillary bed. Arteries are red, veins are blue. Yellow circle signify oxygen molecules. (**B**) The TGF-β signaling pathway in arterial endothelial cells and its association with hereditary hemorrhagic telangiectasia.

1.2.3 Disease management

International guidelines for care of patients with HHT have been established [13]. Interventions for HHT patients suffering from epistaxis include laser ablation, septal dermoplasty (in which thicker skin is grafted onto the inside of the nose), and surgical nasal closure. Current pharmaceutical options non-specifically inhibit angiogenesis (i.e., bevacizumab) or enhance blood clotting (i.e. tranexamic acid) which produce unwanted side effects and only address disease symptoms and not underlying pathology [14]. Anemia and gastrointestinal bleeding are addressed with iron replacement therapy, blood transfusions, and avoidance of anti-coagulant and anti-inflammatory agents [13]. Treatment of AVMs is limited by their location. AVMs found in the lung can be reasonably managed via surgical embolization, in which a tiny metal coil or a small balloon is inserted via a catheter to block off the artery upstream of the AVM [15]. Brain AVMs are less likely to lead to complications, and surgical options including resection, ablation, and embolization are reserved for high-risk AVMs. Liver AVMs, if large or many in number, put a patient at risk for high-output cardiac failure due to decreased vascular resistance, necessitating liver transplant [7]. Overall, surgical interventions carry risks and are not completely effective in managing HHT. Surveillance for these patients includes annual evaluations for anemia, pulse oximetry testing to monitor for pulmonary AVMs (within the first decade of life), contrast echocardiogram every 5 years, baseline brain MRI in childhood, and periodic screening for GI polyps unless JP-HHT has been ruled out through molecular genetic testing [13].

1.2.4 Genetic Counseling for HHT

HHT is an autosomal dominant condition affecting as many as 1 in 5,000 individuals worldwide [14] and demonstrates age-related penetrance and highly variable expressivity even among family members [16, 17]. Individuals with HHT typically inherit their genetic mutation from a parent, but there is an estimated 3% rate of *de novo* mutations [18], and there are instances of somatic mosaicism [19]. Each child of a patient with HHT has a 50% chance of inheriting the mutation. The risk to siblings depends on the genetic status of the parents. Testing of at-risk asymptomatic individuals, including children and adolescents, may be warranted given the potential of early age of onset of symptoms but should include pre-test counseling to discuss the potential impact of positive or negative results. Genetic testing is not useful in providing information regarding age of onset, possible symptoms, or disease severity, and it should be offered to individuals whom meet a "suspected" or "definite" diagnosis of HHT using the Curaçao criteria [20]:

"1. Epistaxis: spontaneous, recurrent nosebleeds

2. Multiple (muco-)cutaneous telangiectases

3. Visceral lesions such as:

– gastrointestinal telangiectasia (with or without bleeding)

– pulmonary AVM (arteriovenous malformation)

– hepatic AVM

– cerebral AVM

4. Family history with an HHT-affected first-degree relative"

1.3   ACVRL1/ALK1

Because phenotype is thought to result from a haploinsufficiency [21], upregulation of *ENG* or *ALK1* expression is a potential avenue for the development of therapeutics that specifically target AVM development in HHT patients.

1.3.1   Ligands of ALK1 receptors

The ligands TGFβ1, TGFβ3, BMP9, and BMP10 can induce ALK1 phosphorylation of downstream SMAD1, 5 and 9 in cultured endothelial cells [22-24]. However, BMP9 and BMP10 are the only ligands that bind to ALK1 *in vitro* with high affinity [24–26]. BMP9 is produced mainly by hepatocytes and to a smaller degree by the lungs and brain [27, 28] while BMP10 is synthesized by the heart [29, 30]. Both ligands are generated as proproteins that are cleaved intracellularly into an N-terminal prodomain and a C-terminal growth factor domain [30]. These fragments remain noncovalently bound and are secreted as propeptide/growth factor complexes, or pro-complexes. Both proBMP9 and proBMP10 can activate ALK1 on endothelial cells and both circulate at physiological levels sufficient for ALK1 activation in mice and humans [24, 30, 31]. Mutations in *BMP9* have been associated with a vascular phenotype similar to but distinct from HHT [11], whereas there is not yet a genetic association between *BMP10* and vascular defects.

1.3.2 Functional studies in *Alk1* in mice

Numerous mouse models have been developed to better understand how *Alk1* functions to prevent AVMs. In an *Alk1^LacZ^* knockin model that expresses *LacZ* as a proxy for *Alk1*, β-galactosidase activity is predominantly in the endothelial cells of developing arteries. *Alk1* is reduced in adult arteries but increased in newly developing arteries secondary to wound healing and tumorigenesis [32]. These data suggest that *Alk1* function is required during angiogenesis but may be less important after vessels have stabilized.

As expected, *Alk1* is critical for proper arteriovenous connections in mice. Lethal AVMs form in embryonic mice with global or endothelial cell-specific loss of *Alk1*. These mice exhibit enlarged vessels with defective vascular smooth muscle coverage and upregulation of mitogens specific to endothelial cells [33, 34]. Global or endothelial-specific loss of *Alk1* expression in adult mice also results in the development of AVMs in vessels undergoing angiogenesis [33-35].

Knockout of *Bmp9* in mice does not produce embryonic AVMs but does result in a subtle postnatal vascular phenotype (patent ductus arteriosus) and minor defects in lymphatic structure and function [36, 37]. In contrast, *Bmp10* knockout produces cardiac and vascular defects in the early embryo, including AVMs similar to *Alk1* loss [33, 38–40]. These findings suggest that BMP10 is the key circulating ligand for ALK1 in endothelial cells, at least during embryonic development.

1.3.3 Functional studies of *alk1* in zebrafish

1.3.3.1 Zebrafish as a model organism for studying vasculature

Zebrafish (*Danio rerio*) are an ideal model organism because they are inexpensive to maintain in large numbers relative to mice. Zebrafish have a short generation time and reach sexual maturity within three months. Zebrafish clutches may contain hundreds of offspring that can be collected easily, and embryonic development is complete by 5 days post fertilization. Zebrafish are fitting model for studying HHT because embryos are optically clear, which allows for the study of heart rate, blood flow, and vascular development noninvasively and in real time.

1.3.3.2 Blood flow and vasculature in zebrafish

Blood flow begins in zebrafish around 26 hours post-fertilization (hpf), and the vascular network in zebrafish embryos at 36 hpf (Figure 2A) is as follows: blood leaves the heart through the first aortic arch (AA1) and flows cranially into the internal carotid artery (ICA), then caudal divisions of the internal carotid artery (CaDI), and the basal communicating artery (BCA). From the BCA, blood then flows through posterior communicating segments (PCS) and into the basilar artery (BA) and drains to neighboring veins (primoridal midbrain channel, PMBC; and primoridal hindbrain channel, PHBC) via transient vessel segments. Blood also flows caudally into the lateral dorsal aortae (LDA) and dorsal aorta (DA) before draining to the posterior cardinal vein. In these early embryos, *alk1* is exclusively expressed in arterial endothelial cells in vessels most proximal to the heart, including AA1 and the cranially-directed ICA, CADI, and BCA, as well as the caudally-directed LDA and DA (Figure 2A). Expression of *alk1* in these vessels initiates with the onset of blood flow [41] and is maintained at least through 5 days post-fertilization (dpf) (B.

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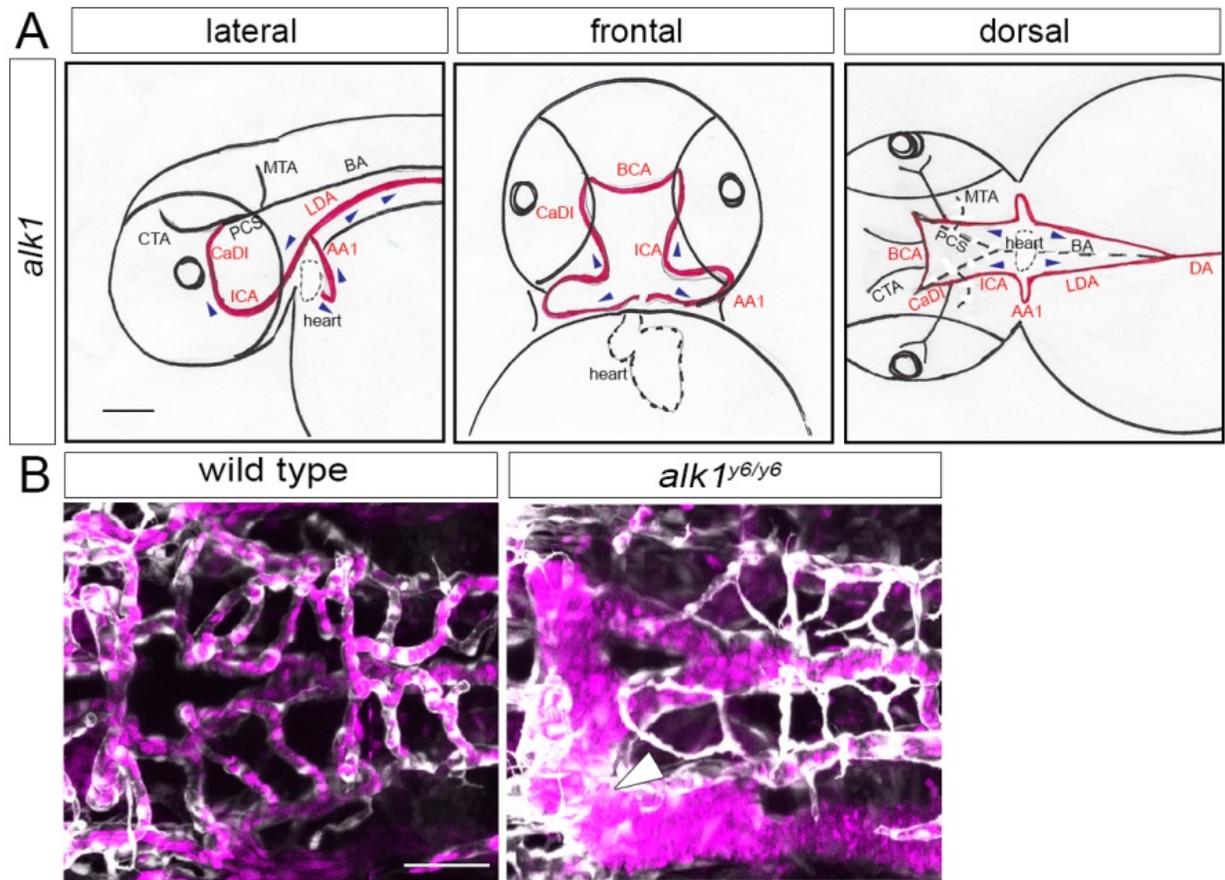
Figure 2. Zebrafish embryos require Alk1 for proper development of the cranial vessels.
(**A**) Cranial arties (red) that are known to express *alk1* starting at 26 hpf. Blue arrows show the direction of blood flow as it leaves the heart and travels towards the head or tail. Dashed lines indicate arteries positioned closer to the reader. Scale bar = 100 µm. AA1, first aortic arch; ICA, internal carotid artery; CaDI, caudal divisions of the internal carotid artery; BCA, basal communicating artery; LDA, lateral dorsal artery; DA, dorsal aorta; BA, basilar artery; PCS, posterior communicating segments; MTA, metencephalic artery; CTA, central artery. (**B**) Dorsal view of 52 hpf *Tg(flik1:gfp)la116;Tg(gata1:dsred)sd2* embryos showing loss of Alk1 results in enlargement of vessels and arteriovenous shunts (white arrowhead). Scale bar = 50 µm.

Roman, unpublished). In summary, the arterial segments closest to the heart, but not downstream cranial arteries or any veins, become *alk1* positive at the onset of blood flow [41].

1.4 **AVM DEVELOPMENT**

AVMs develop in zebrafish *alk1* mutants in a highly predictable time and location, making them an good model for studying the processes that result in AVM formation when Alk1 signaling is lost [42]. The development of AV shunts in *alk1* mutants is a two-step process. In the first step, *alk1*-dependent cranial arteries enlarge in part due to increased endothelial cell number [42]; specifically, there is a 1.2 fold increase in endothelial cells in the BCA/PCS at 32 hpf and a 1.8-fold increase by 48 hpf [41]. Careful analysis of cranial arterial endothelial cell number in wild type embryos, *alk1* mutant embryos and embryos lacking blood flow between 26 and 48 hpf revealed that there was a similar increase in cell number between 32 and 40 hpf in the *alk1* mutants and embryos lacking blood flow, suggesting that loss of blood flow and loss of *alk1* have the same effect on arterial endothelial cell number [41]. This observation suggests that Alk1 mediates a blood flow response. In support of this theory, a subset of shear stress-responsive genes is dysregulated in *alk1* mutants. Specifically, *cxcr4a*, a promigratory chemokine receptor is upregulated while endothelin-1, a vasoconstritive peptide is downregulated in both *alk1* mutants and embryos lacking flow [41]. Additionally, endothelial phosphorylation of Smad1/5/9 is lacking in embryos without blood flow or Alk1 receptors [40]. Gene expression and Smad1/5/9 phosphorylation can be restored in the absence of blood flow if the ligand, Bmp10, is injected into the vasculature of embryos transgenically expressing *alk1* in endothelial cells [40]. These results suggest that flow functions to deliver the heart-derived

ligand, Bmp10, in order to activate Alk1, regulate expression of a subset of shear stress-responsive genes, and restrict the number of endothelial cells in the cranial arterial endothelium.

The second step in AVM development in *alk1* mutants is failed regression of at least one of the transient BCA/PMBC or BA/PHBC connections downstream of the enlarged BCA. However, AVMs do not arise in *alk1* mutants in the absence of blood flow. Shear stress is the frictional force exerted by blood flow parallel to the direction of flow and is transduced by endothelial cells into biochemical signals [43]. The force of shear stress is directly proportional to the rate of flow and blood viscosity. Vessels adapt to accommodate increased shear stress by remodeling to bring this force back down to a particular set point. Therefore, the lack of AVMs in *alk1* mutants in the absence of flow suggests that AVMs are a compensatory response to altered shear stress. That is, increased vessel caliber in *alk1*-dependent arteries increases flow rate and shear stress in downstream arteries, resulting in an increased hemodynamic load. In response to this force, normally transient connections are maintained and even enlarge in an attempt to normalize shear stress, thus creating an AVM [41]. This hypothesis is supported by computational fluid dynamic modeling (P. Menon and B. Roman, unpublished).

1.5   *bmp10* AND *bmp10-like* IN ZEBRAFISH

Analysis of the zebrafish genome revealed two *bmp10* paralogs – *bmp10* and *bmp10-like* – which likely arose during a whole genome duplication event [44]. Both *bmp10* and *bmp10-like* are expressed exclusively in the heart. *bmp10* is expressed in endocardial cells of the ventricle by 28 hpf and expands to both chambers of the zebrafish heart by 36 hpf. *bmp10-like* is first detectable at 36 hpf and is restricted to distal ventricle and outflow tract myocardium [40]. Knockdown of

bmp10 is sufficient to produce AVMs similar to *alk1* mutants, but phenotype is not as penetrant or expressive as in *alk1* mutants. Knockdown of *bmp10-like* alone does not yield any effect on vasculature. However, knockdown of both *bmp10* and *bmp10-like* yield AVMs as robustly as *alk1* mutants [40]. Combinations of *bmp9*, *bmp10*, and/or *bmp10-like* knockdowns do not yield any additional phenotypic effect [40]. These data suggest that *bmp10-like* acts redundantly with *bmp10* and that Bmp10 – rather than Bmp9 – is the critical Alk1 ligand required for proper embryonic vascular development in zebrafish, as in mice.

1.6   REGULATION OF *ALK1* EXPRESSION

Because HHT is thought to be caused by *ALK1* or *ENG* haploinsufficiency, one approach to development of therapeutics is to enhance expression of the wild type copy of the disease gene. There is limited information regarding the regulatory elements that govern *ALK1* gene expression.

1.6.1   Regulatory elements

ChIP assays performed on arterial endothelial cells show that the 5' proximal promoter region of *ALK1* lacks TATA/CAAT boxes but has GC-rich Sp1 consensus sites [45]. Loss of these Sp1 sites results in lack of *ALK1* transcription, and increasing doses of Sp1 result in increased transcription [45]. In response to angiogenic stimuli, such as vascular injury, *ALK1* expression rapidly increases in endothelial cells [46]. This response is associated with nuclear translocation of Kruppel-like factor 6 (KLF6). Furthermore, *Klf6* knockout mice display a down-regulation of

Alk1 mRNA in their vasculature, and *Klf6*^{+/-} mice have lower transcriptional levels of *Alk1* than their wild type siblings [46]. KLF6 interacts with the *ALK1* promoter in endothelial cells during wound healing events – facilitated by Sp1. *ALK1* expression is also indirectly increased in response to soluble factors released by cellular damage, such as interleukin 6 [46].

An investigation into cis-acting elements necessary for artery specific *Alk1* expression in mice revealed a 9.2 kb genomic fragment, including a 2.7 kb promoter region through intron 2, is sufficient to drive expression [47]. It is suggested that mouse intron 2 (corresponding to human and zebrafish intron 1) may contain enhancer elements as a shortened 4.8 kb regulatory element containing the *Alk1* promoter and highly conserved regions within intron 2 showed specific expression in angiogenic arteries that was comparable to endogenous *Alk1* [48]. However, how this intronic fragment directs arterial expression is unknown.

1.6.2   Regulation by blood flow

Several lines of evidence suggest that *ALK1* expression is regulated by blood flow and/or shear stress. *Alk1* is upregulated in manipulated mouse arteries that are predicted to experience a relatively high magnitude of shear stress [49]. Furthermore, zebrafish embryos with no heartbeat or blood flow due to genetic or pharmacological manipulations have undetectable *alk1* expression but normal vessel morphology and normal expression of pan-endothelial markers such as vascular endothelial cadherin (*cdh5*) [41]. It is not yet known, however, if the sensitivity of *alk1* expression to blood flow is due to mechanical force acting upon endothelial cells or due to circulating endocrine factors.

1.7   HYPOTHESIS AND AIMS OF DISSERTATION

Understanding the mechanism by which *ALK1* expression is regulated will lend insight into development of drugs to overcome both *ALK1* and *ENG* haploinsufficiency and prevent and/or rescue AVMs in HHT patients. Based on mouse and zebrafish models, it is apparent that some component of blood flow—either biochemical or mechanical—is required for *ALK1* expression in arterial endothelial cells. BMP10 is a good candidate for this flow-based factor, as it is a critical ligand of ALK1 receptors and it is a circulating protein derived from the heart.

In this work I used zebrafish to test the hypothesis that blood flow is required for activation of a positive feedback mechanism by which Bmp10/Alk1 signaling maintains arterial *alk1* expression at the level of transcription. I pursued my hypothesis by first defining the spatiotemporal relationship between blood flow and *alk1* expression. It was previously demonstrated that stopping heartbeat for 8 hours caused loss of *alk1* expression [41]. In this work, I examined the spatiotemporal pattern of this effect and determined whether restoration of blood flow could restore *alk1* expression. I then go on to determine whether flow-mediated regulation of *alk1* occurs at the level of transcription. To test this hypothesis, we generated a zebrafish transgenic line that expresses *egfp* under the control of enhancer elements located in *alk1* intron 1. Zebrafish *alk1* intron 1 corresponds to mouse *Alk1* intron 2, which directs gene expression to arterial endothelial cells [32]. I manipulated blood flow in these embryos to determine whether *egfp* behaves similarly to *alk1*, suggestive of transcriptional control. Finally, I tested the hypothesis that intact Bmp10/Alk1 signaling is required to maintain *alk1* mRNA expression.

2.0   MATERIALS AND METHODS

2.1   ZEBRAFISH LINES AND MAINTENANCE

Adult zebrafish (*Danio rerio*) were maintained according to standard protocols and embryos were raised and staged using standard methods [50].

Transgenic line *Tg(fli1a:negfp)^{y7}* was made by expressing nuclear-localized EGFP under the control of a 15 kb fragment of the zebrafish *fli1a* promoter [42]. This *fli1a* promoter fragment directs transgene expression to migrating angioblasts, endothelial cells, and early blood cells.

The *ptol2-alk1e5:egfp* DNA construct contains a 1910 bp fragment of the first intron of the zebrafish *alk1* gene upstream of a basal promoter driving *egfp* expression and was generated using Gateway cloning (Invitrogen, Carlsbad, CA, USA). This construct was injected into one-cell embryos and F0 adults were screened for transgene transmission. We identified one founder that generated F1 offspring with EGFP expression in the same arteries of the head and tail that express endogenous *alk1*. We designated this line *Tg(alk1e5:egfp)^{pt517}*.

alk1^{y6} embryos were isolated from an ethylnitrosourea (ENU) mutagenesis screen on AB background. The *alk1^{y6}* allele contains a missense mutation (p.L249F) in *alk1* that is known to be a null allele with no resulting kinase activity [42]. Embryos that are heterozygous for the *alk1^{y6}* allele do not show any phenotype, while homozygous *alk1^{y6}* mutants develop embryonic

lethal AV shunts [42]. *alk1^{y6};Tg(alk1e5:egfp)^{pt517}* lines were established by crossing *alk1$^{y6/+}$* with hemizygous *Tg(alk1e5:egfp)^{pt517}*

bmp10^{pt527} mutants were generated via targeted TALEN-mediated mutagenesis [51] . In this line, an 8 bp deletion results in a frameshift that truncates Bmp10 prior to the growth factor domain at amino acid 48. *bmp10^{pt527};Tg(alk1e5:egfp)^{pt517}* fish were made by crossing *bmp10$^{pt527/+}$* to hemizygous *Tg(alk1e5:egfp)^{pt517}*.

2.2 MORPHOLINOS

Translation-blocking morpholino-modified antisense oligonucleotides (morpholinos; GeneTools, Philomath, OR, USA) were as follows:

bmp10-like, 5'-GCAGCAGAGAATCAGCCATGACTGC-3'

Control, 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

2 ng of control and *bmp10-like* morpholinos were injected into 1 to 8-cell embryos from a *bmp10$^{pt527/+}$:Tg(alk1e5:egfp)$^{pt517/+}$* incross and a subset were assessed for AVMs between 2-3 dpf and genotyped for *bmp10^{pt527}*. Injecting 2 ng of *bmp10-like* morpholino into *bmp10$^{pt527/pt527}$* generated a vascular phenotype identical to that seen in *alk1$^{-/-}$* with 100% penetrance. Remaining embryos were fixed for in situ hybridization at 36 hpf. Only 50% of fixed embryos carried the *alk1e5:egfp* transgene. To eliminate experimental bias, embryos were not sorted for EGFP before fixation but were instead genotyped for the *alk1e5:egfp* transgene post-processing.

2.3   DRUG EXPOSURE AND EMBRYO COLLECTION

To maintain optical clarity of the embryos, 0.003% phenylthiourea (PTU) was added to the embryo medium (Sigma, St. Louis, MO, USA) at 24 hpf. To stop heartbeat, dechorionated embryos were incubated 100 embryos per 100 mm dish in 800 μg/ml tricaine in 30% Danieau/0.003% PTU at 32 hours post-fertilization. Embryos were collected at designated time points and immediately placed in 4% paraformaldehyde and fixed at 4°C for 48-72 hours. After fixing, embryos were washed 5 times in 100% methanol and then stored at -20°C in 100% methanol.

2.4   WHOLE MOUNT IN SITU HYBRIDIZATION

Whole-mount in situ hybridization was performed as previously described [42]. Riboprobes were generated using a digoxigenin RNA labeling Kit (Roche, Indianapolis, IN, USA) (Table 1). For *alk1*, cDNA amplified using forward primer 5'-GGCCCTGGGTCTCGTCTT-3' and reverse primer 5'-ACCCCATCTTACCCTCACTTTAC-3' was cloned into pCRII-TOPO vector (Invitrogen). Complete *egfp* sequence was cloned into pCRII-TOPO vector. A 5' RACE *cdh5* product was cloned into a pCRII-TOPO vector. Pre-adsorbed sheep anti-dig-AP antibody was used at 1:5000 dilution.

Table 1. Plasmids and enzymes used to generate riboprobes.

Ribroprobe	Plasmid name	Number	Restriction enzyme	Polymerase
alk1	alk1 pr3 w1-1	12	Spe1	T7
Egfp	EGFP	329	Not1	Sp6
vecad/cdh5	Vecad	112	Not1	Sp6

2.5   IMAGING AND PHENOTYPIC SCORING

Bright-field images were captured using an MVX-10 MacroView macro zoom microscope equipped with an MV PLAPO 1×/0.25 NA objective, 2× magnification changer, and DP71 camera with DP controller software version 3.1 (Olympus America, Center Valley, PA, USA). Embryos were imaged and scored for gene expression in a blinded fashion, then digested for genotyping. Gene expression was scored as "positive" or "negative" based on whether staining (*alk1, gfp, cdh5*) was present in a particular vessel segment; namely, the first aortic arch (AA1), internal carotid artery (ICA), caudal division of the internal carotid artery (CaDI), basal communicating artery (BCA), and lateral dorsal aorta (LDA). Images were compiled with Adobe Photoshop CS2 version 9.0.2 (Adobe Systems, San Jose, CA, USA).

2.6   GENOTYPING

DNA was isolated from zebrafish fin clips and individual embryos for genotyping assays in DNA isolation buffer (10mM Tris HCl pH8.0, 50 mM KCl, 0.3% Tween-20, 0.3% Nonidet P-40 Substitute, 0.5 mg/mL Proteinase K) incubated at 50°C for 12-15 hours then at 98°C for 10 minutes. Assays consisted of PCR using primers designed for the specific allele with or without

restriction enzyme digestion following amplification (Table 2). PCRs were run for 32 cycles and

amplification products were visualized by 3% Metaphor agarose gel electrophoresis.

Table 2. List of genotyping assays.

Line	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Enzyme	Assay
bmp10 pt527	CTCCCCGGAGAGGCACCTCA	AGGTGGCTCTACACGAGTG	DdeI	dCAPS[1]; cuts M
vbg y6	CACGGTCCAACTAAGGCATGAAAACACCTT	ATGGACAGAGAAGTGTAAGTAAGAAAT	BsaJ1	dCAPS; cuts WT
tg(alk1e5:gfp)pt517	ACACTATCTGATTTGAGAAAC	AAGTCGTGCTGCTTCATGTG	No Digest	1 band alk1e5 + (459 bp)
egfp	TGGTGCCCATCCTGGTCGAGCTGG	AAGTCGTGCTGCTTCATGTG	No Digest	1 band (217 bp)

[1]dCAPS: derived cleaved amplified polymorphic sequence [52]

2.7   STATISTICS

Statistical comparisons were made by Fisher's Exact Test using online calculation tool [53] with

significance set at $p<0.05$. The number of experimental replicates and n values are detailed in

Table 3.

Table 3. Experimental replicates and n values (experimental repeats, n embryos total).

variable	*alk1*	*egfp*	*cdh5*
Flow Time course			
32 hpf control	3, 117	3, 86	3, 107
40 hpf control	3, 50	3, 67	3. 80
42 hpf control	3,103	3, 66	3, 81
48 hpf control	3, 87	3, 66	3, 103
32 hpf tricaine	3, 133	3, 68	3, 97
40 hpf tricaine	3. 70	3, 55	3, 72
42 hpf tricaine + washout	3, 106	3, 58	3, 99
48 hpf tricaine+washout	3, 144	3, 57	3, 100
Alk1 Loss of Function			
alk1+/+	3, 82	2, 23	2, 14
alk1 +/-	3, 130	2, 26	2, 31
alk1-/-	3, 119	2, 14	2, 12
Bmp10 Loss of Function			
bmp10pt527+/+ control MO	3, 19	3, 10	3, 15
Bmp10pt527+/- control MO	3, 24	3, 35	3, 18
Bmp10pt527-/- control MO	3, 7	3, 27	3, 3
Bmp10pt527+/+ Bmp10-like MO	3, 17	3, 24	3, 16
Bmp10pt527+/- Bmp10-like MO	3, 41	3, 24	3, 24
Bmp10pt527-/- Bmp0-like MO	3, 18	3, 7	3, 8
Tg(fli1a:negfp)^{y7} **without flow**			
36 hpf control	1, 30	1, 26	1, 31
36 hpf tricaine	1, 27	1, 27	1, 35
Tg(fli1a:negfp)^{y7} **+ Alk1 loss of function**			
alk1+/+	1, 7	1, 29	1, 8
alk1 +/-	1, 28	1, 74	1, 8
alk1-/-	1, 8	1, 23	1, 4

3.0   RESULTS

3.1   BLOOD FLOW ACUTELY REGULATES *alk1* mRNA EXPRESSION

It has previously been shown that *alk1* is not expressed in the absence of blood flow following a tricaine treatment at 32-40 hpf as well as in *tnnt2a* morphant embryos with noncontractile hearts [41]. In order to define the spatiotemporal relationship between blood flow and *alk1* expression over a timecourse, as well as to observe *alk1* response after flow restarts, I stopped the heartbeat of zebrafish between 32 hpf and 40 hpf using a 5x dose of the anesthetic, tricaine. I started tricaine treatment at 32 hpf because blood flow is robust and *alk1* expression in the arterial endothelium in the head (AA1, ICA, CaDI, BCA) and trunk/tail (LDA, DA) has been established by this time point. Furthermore, the embryo's demands for oxygen at this time can be met by diffusion; therefore, embryos without blood flow are not hypoxic [54].

I determined the presence of blood flow by visually examining the optically clear embryos for the movement of red blood cells through the head, trunk, and tail. Heartbeat was noticeably slowed within 30 minutes after tricaine treatment compared to untreated controls. By 60 minutes, about two-thirds of tricaine-treated embryos had no heartbeat or blood flow. For the other one-third of embryos there was still a steady but dramatically slowed rate of twitching of the heart at this time, but there was no blood flow. I collected tricaine-treated and untreated sibling embryos at 34 hpf and 40 hpf for in situ hybridization for *alk1* and *cdh5* (Figure 3B).

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After 8 hours of tricaine exposure (40 hpf), embryos were moved to fresh water. Heartbeat and blood flow resumed in washed out embryos within one hour post-washout (41 hpf) and blood flow looked identical to flow in control embryos by two hours post-washout (42 hpf). I then washed remaining embryos out of tricaine to allow heartbeat to restart and collected embryos at 42 and 48 hpf for in situ hybridization for these same genes. Gene expression was scored as positive or negative (+/-) in each arterial vessel segment, and data are presented as percent of embryos with expression in each segment.

At 34 hpf, *alk1* expression was detectable in untreated embryos in all expected arterial vessel segments. In contrast, *alk1* expression was lost in some segments after 2 hrs tricaine exposure, with effects greater in distal versus proximal segments (% embryos with *alk1* expression, AA1, LDA > ICA > CaDI >BCA). At 40 hpf, *alk1* expression was detectable in most vessel segments in untreated embryos but was absent from nearly all vessel segments after 8 hrs of tricaine treatment (Figure 3B), with an average of 5.26% of embryos retaining some expression in AA1 only (Figure 3D). The pan-endothelial marker, *cdh5*, was unaffected by tricaine treatment.

At 42 hpf, *alk1* expression was detected in all arterial vessel segments in all untreated embryos and in most arterial vessel segments in most tricaine-treated embryos, with more complete recovery in proximal (AA1, ICA, LDA) versus distal (CaDI, BCA) segments (Figure 3C, E). *alk1* expression was fully recovered by 8 hrs post-tricaine exposure (48 hpf). *cdh5* expression remained unchanged in tricaine-treated versus untreated controls at all times.
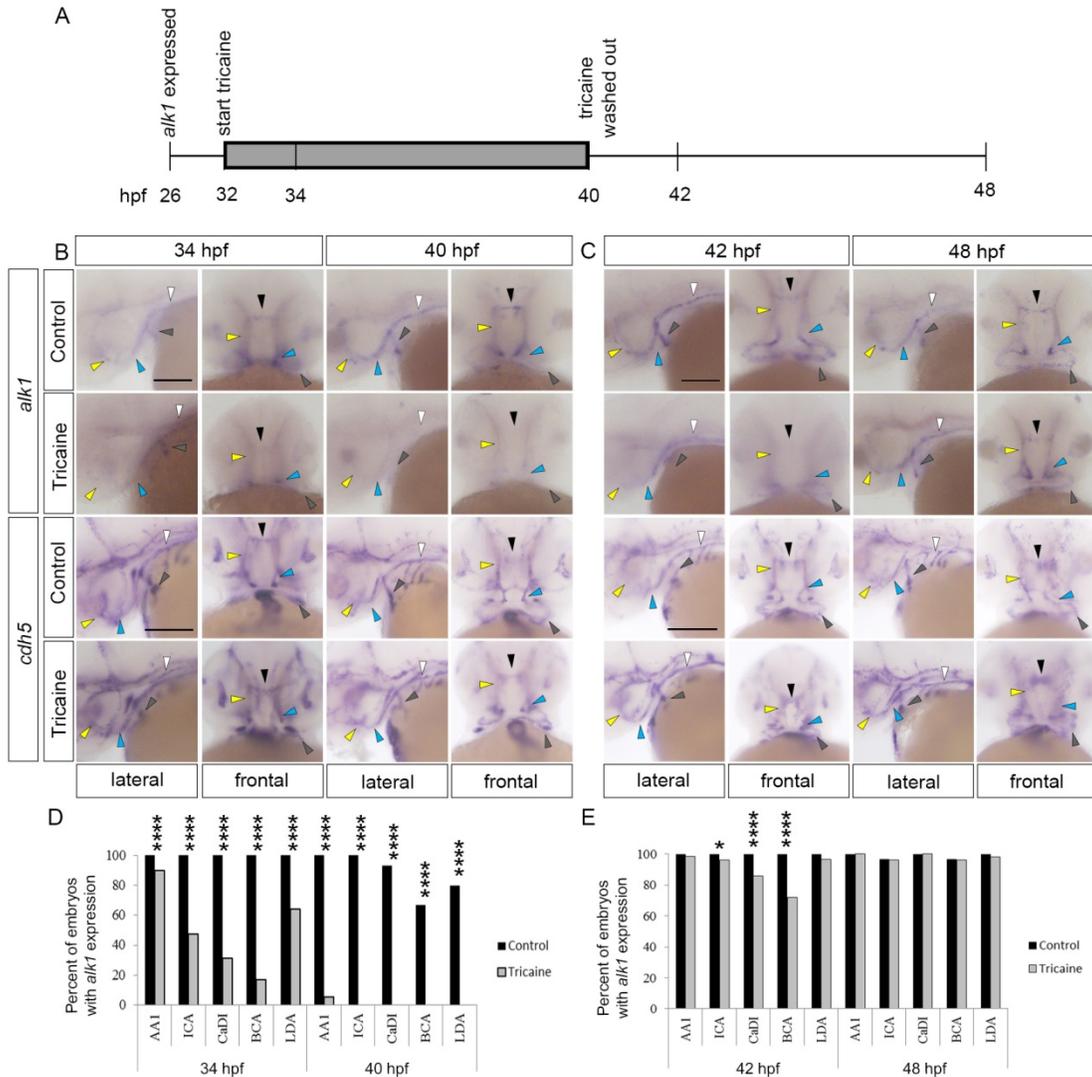
Figure 3. Cessation of blood flow results in loss of *alk1* expression, with distal arteries more sensitive to flow loss than proximal arteries.

(**A**) Schematic of experimental design showing times of treatment, washout, and embryo collection. (**B**) Embryos were treated with 800 µg/ml tricaine in 30% Danieau/0.003% PTU at 32 hpf to stop blood flow, fixed at 2 hours and 8 hours post treatment, and stained via whole mount in situ hybridization for *alk1* or *cdh5*. Lateral and frontal views, as indicated. (**C**) Embryos were removed from tricaine after 8 hours of exposure to restore blood flow, fixed at 2 hours and 8 hours post washout, and stained via whole mount in situ hybridization for *alk1* or *cdh5*. Arrows denote basal communicating artery, black; caudal division of the internal carotid artery, yellow; internal carotid artery, blue; first aortic arch, grey; lateral dorsal aorta, white. Scale bar = 100 µm. (**D, E**) Embryos were scored for the presence or absence of *alk1* in arterial segments at 2- and 8-hrs post-tricaine treatment (D) and at 2- and 8-hrs post-washout (E); data are presented as percent of embryos with positive expression. n ≥ 50 for each time point over three independent experiments. Embryos scored for *alk1* expression were analyzed by Fisher's exact test with significance set at 0.05. (**** signifies $p<0.0001$, * <0.05)

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Together, these data demonstrate that arterial endothelial *alk1* mRNA expression is exquisitely sensitive to blood flow. *alk1* expression is significantly diminished within 1-2 hrs of flow cessation, particularly in arterial segments distal to the heart, and nearly undetectable within 8 hrs of flow cessation. Conversely, reinitiating heartbeat and blood flow allows *alk1* re-expression within 2 hrs, with more rapid effects in proximal versus distal arterial segments.

3.2   FLOW REGULATION OF *alk1* EXPRESSION OCCURS AT THE LEVEL OF mRNA TRANSCRIPTION

To determine whether regulation of *alk1* mRNA expression by blood flow occurs transcriptionally or post-transcriptionally, I treated *Tg(alk1e5:egfp)*^pt517^ embryos, in which *egfp* expression is driven by a basal promoter and zebrafish *alk1* enhancer (intron 1), with tricaine between 32 and 40 hpf and collected embryos during tricaine exposure (34 hpf, 40 hpf) and after washout (42 hpf, 48 hpf). These embryos were siblings of embryos analyzed for *alk1* and *cdh5* expression. In situ hybridization revealed that *egfp* mRNA exhibited the same temporal response to cessation and reinitiation of blood flow as *alk1* mRNA (Figure 4A, B). However, *egfp* mRNA was more refractory to flow loss than *alk1* mRNA in AA1, and *egfp* recovery after flow initiation was slower and less robust than *alk1* (Figure 4C, D). To ensure that effects of flow manipulation on *egfp* mRNA expression were not due to *egfp* transcript instability in the absence of flow, I treated *Tg(fli1a:negfp)*^y7^ embryos with tricaine, 32-40 hpf. At 40 hpf, I found nearly complete loss of *alk1* expression (Figure 5B), as expected, but no effect of flow loss on *fli1a*-driven endothelial *egfp* expression (Figure 5A). Taken together, these results suggest that *alk1*
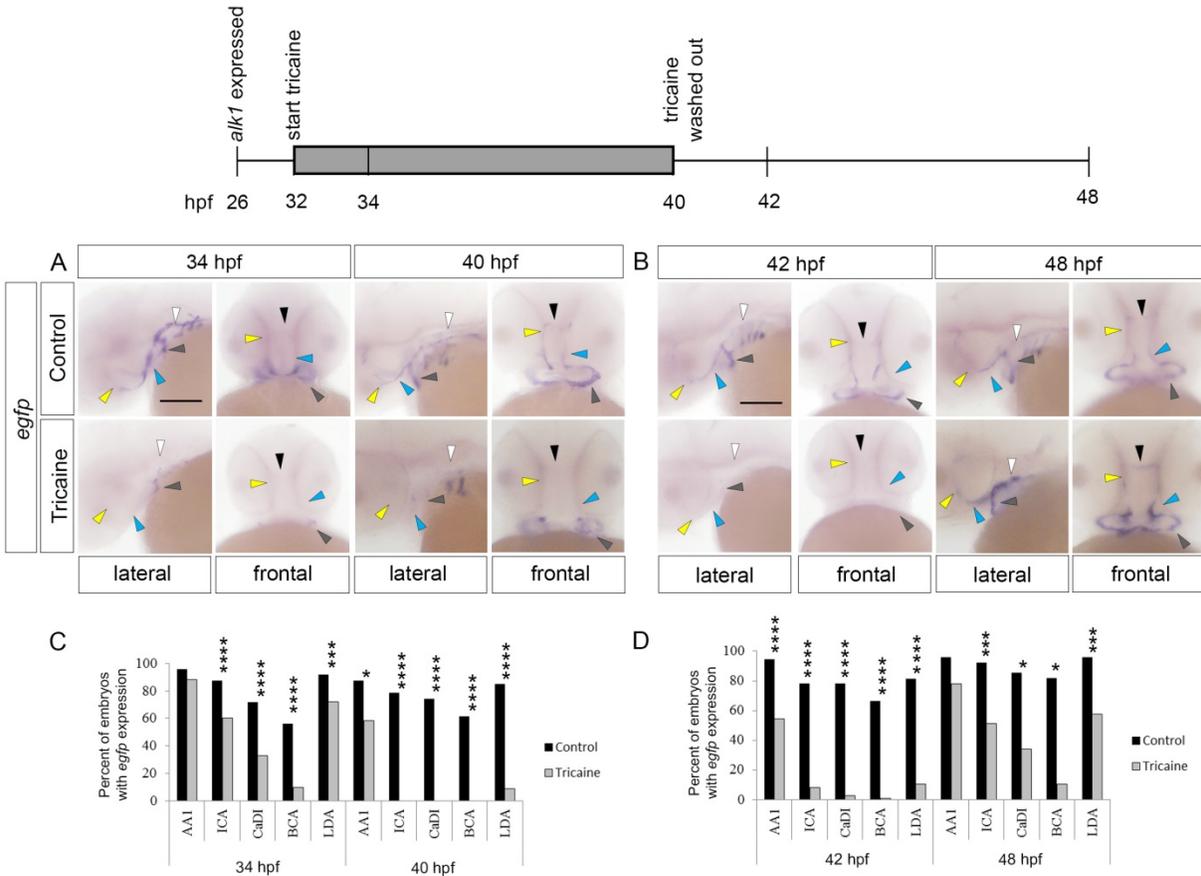
Figure 4. Blood flow regulates *alk1* expression at the level of transcription.
(**A**) *Tg(alk1e5:egfp)^{pt517}* embryos were treated with tricaine at 32 hpf to stop blood flow and fixed in 4% paraformaldehyde at 2 hours and 8 hours post treatment. (**B**) Embryos were removed from tricaine treatment after 8 hours of exposure and blood flow was restored. Embryos were fixed at 2 hours and 8 hours post washout. Whole mount in situ hybridization for *egfp* was performed and embryos were scored for the presence of expression in arteries in lateral and frontal views. n ≥ 55 for each timepoint over three independent experiments. Arrows denote basal communicating artery, black; caudal division of the internal carotid artery, yellow; internal carotid artery, blue; first aortic arch, gray; lateral dorsal aorta, white. Scale bar = 100 µm.   (**C-D**) Embryos scored for *egfp* expression were analyzed by Fisher's exact test with significance set at 0.05. (**** signifies p-value <0.0001, *** 0.0001 to 0.001, * 0.01 to 0.05).

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regulation by blood flow occurs at the level of transcription and is mediated by *cis* elements in *alk1* intron 1.
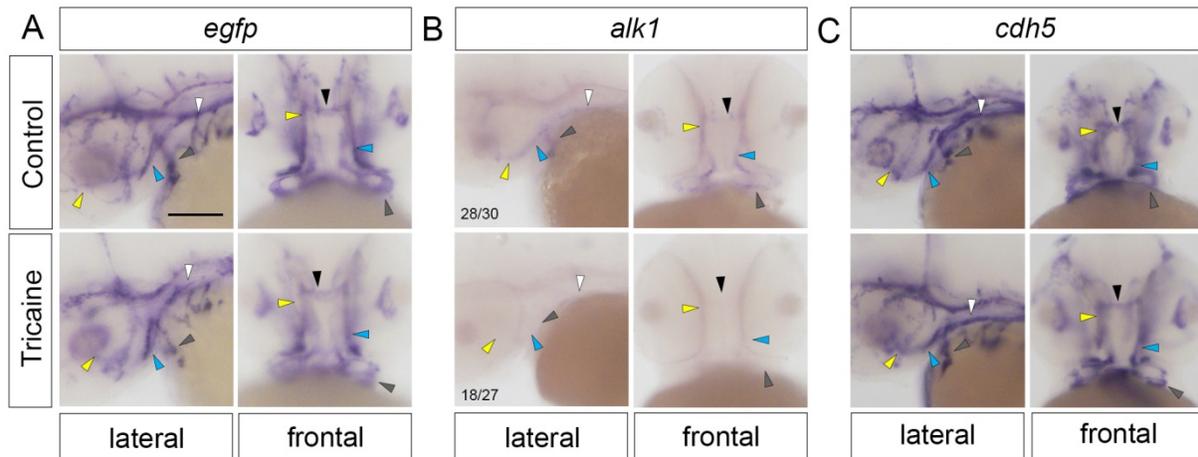
Figure 5. Loss of blood flow has no effect on *fli1a*-driven *egfp* expression in endothelial cells.
(A-C) *Tg(fli1a:egfp)^y7* embryos were treated with tricaine at 32 hpf and then to stop blood flow and fixed in 4% paraformaldehyde at 8 hours post treatment. Whole mount in situ hybridization for *egfp* (A), *alk1* (B), or *cdh5* (C) was performed and embryos were scored for the presence of expression in arteries in lateral, and frontal views. In control and treatment groups, n = 31 and 35 for *egfp*, 30 and 27 for *alk1*, and 27 and 26 for *cdh5*, respectively. Arrows denote basal communicating artery, black; caudal division of the internal carotid artery, yellow; internal carotid artery, blue; first aortic arch, grey; lateral dorsal aorta, white. Scale bar = 100 µm.

3.3 INTACT Bmp10/Alk1 ACTIVITY IS REQUIRED FOR *alk1* EXPRESSION

I reasoned that Alk1 activity may be required to maintain *alk1* mRNA expression by a positive feedback mechanism given previous findings that demonstrated that *alk1* mRNA expression is decreased in *alk1^{y6}* mutants [41]. This missense transcript generates a kinase-dead, full length Alk1 mutant protein [42] and therefore *alk1* loss is unlikely due to inherent transcript instability. To test this hypothesis, I crossed *alk1$^{y6/+}$;Tg(alk1e5:egfp)^{pt517}* zebrafish to *alk1$^{y6/+}$* to produce embryos that were either wild type, heterozygous, or homozygous mutant for the *alk1^{y6}* allele. In situ hybridization with subsequent genotyping for the *alk1^{y6}* allele showed that *alk1* expression was significantly decreased in all arterial segments at 36 hpf in *alk1^{y6}* homozygous mutants compared to wild type or heterozygous siblings (Figure 6A, D), whereas *cdh5* was unaffected (Figure 6C). There was no significant difference in *alk1* expression between wild type and heterozygote siblings (Figure 6D), suggesting that a partial level of *alk1* activity is not sufficient to disrupt the proposed positive feedback loop via a haploinsufficiency in the embryonic zebrafish model. This is expected given that *alk1^{y6}* homozygous mutants develop AVMs, but their heterozygous siblings do not.

   egfp expression behaved similarly to *alk1*, although expression was less sensitive to *alk1* genotype and did not decrease in number of embryos to a degree as significant as *alk1*. To ensure that the *egfp* mRNA transcript itself was not affected by Alk1 activity, I crossed *alk1$^{y6/+}$;Tg(fli1a:negfp)^{y7}* zebrafish to *alk1$^{y6/+}$* to produce embryos that were either wild type, heterozygous, or homozygous mutant for the *alk1^{y6}* allele. I found loss of *alk1* expression as described previously, as expected, but no effect of loss on *fli1a*-driven endothelial *egfp* expression (Figure 7).
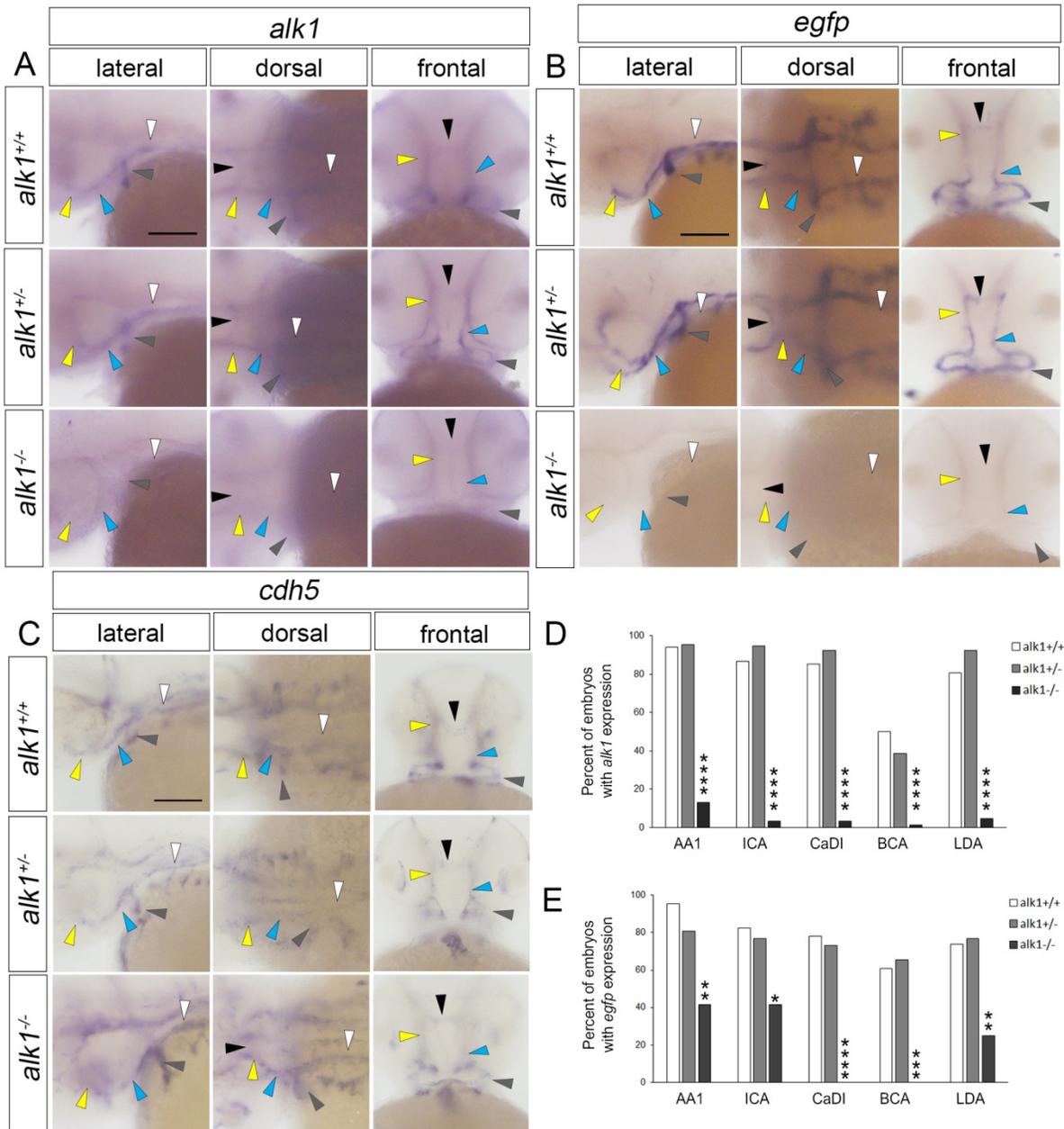
Figure 6. Evidence suggests that Alk1 participates in a self-regulatory pathway to maintain expression.
alk1$^{y6/+}$;Tg(alk1e5:egfp^{pt17}) fish were outcrossed to *alk1$^{y6/+}$* non-trangenics and then sorted for the presence of the *egfp* transgene at 30 hpf. Whole mount in situ hybridization was performed on resulting embryos for *alk1* (**A**), *egfp* (**B**), and *cdh5* (**C**) fixed at 36 hpf. Embryos were scored for the presence or absence of expression in vessels in lateral, dorsal, and frontal views and genotyping was subsequently performed on all embryos for *y6* allele. For each y6 genotype, n ≥ 82 over three experiments for *alk1*, n ≥ 14 over two experiments for *egfp*, and n ≥ 12 over two experiments for *cdh5*. Arrows denote basal communicating artery, black; caudal division of the internal carotid artery, yellow; internal carotid artery, blue; first aortic arch, grey; lateral dorsal aorta, white. Scale bar = 100 µm. (**D-E**) Embryos scored for *alk1* or *egfp* expression were analyzed by Fisher's exact test with significance set at 0.05. (**** signifies p-value <0.0001, *** 0.0001 to 0.001, ** 0.001 to 0.01, * 0.01 to 0.05).

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To investigate the concept of a circulating ligand, specifically Bmp10, as the component of blood flow required for *alk1* expression, I crossed *bmp10^{pt527}* heterozygous fish to *bmp10^{pt527:}alk1e5:egfp^{pt517}* to yield offspring that were wild type, heterozygous, or homozygous mutant for the *pt527* allele. Embryos were then either injected with *bmp10-like* morpholino or control morpholino at 1-8 cell stage. In situ hybridization of 36 hpf embryos was performed for *alk1, cdh5, and egfp* with subsequent genotyping for the *pt527* allele. I observed that based on *bmp10* genotype, *alk1*expression was decreased only in homozygous mutants, but not significantly except in the ICA (with a p-value of 0.047) which was just slightly significant (Figure 8A, D). It should be noted that the smaller number of embryos that were injected with control morpholino and were then determined to be homozygous mutant for the *pt527* allele after scoring for expression (n = 7 for mutants with control morpholino versus n = 19 wild types and n = 24 heterozygotes) may have affected the potentially significant trend. With the additional knockdown of *bmp10-like,* however*,* there was a significant decrease in homozygous mutants in the number of embryos with *alk1* expression in all vessels (Figure 8A, D). The combination of loss of *bmp10-like* and *bmp10* heterozygotes was not significant in any vessels except for the BCA (with a p-value of 0.034). *cdh5* expression was unaffected by *pt527* genotype and/or *bmp10-like* morpholino (Figure 8C).

Looking at *bmp10* genotype alone, there was some noticeable decrease in *egfp* expression in *bmp10* homozygous mutants compared to wild type and heterozygous siblings, but it was not significant or consistent across all of the vessels. The combination of *bmp10* and *bmp10-like* loss yielded some significant decrease in *egfp* expression in the AA1, ICA, and LDA, but it was not as strong or consistent as with *alk1* (Figure 8B, E).
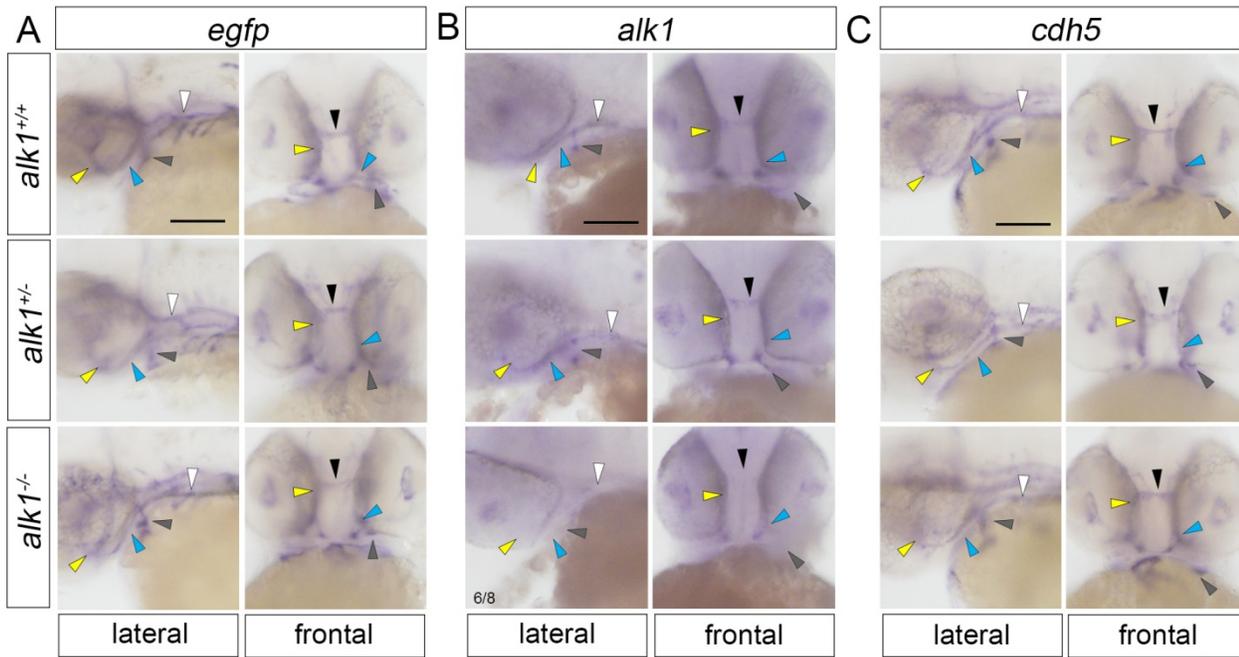
Figure 7. Loss of *alk1* activity has no effect on *fli1a*-driven *egfp* expression in endothelial cells.
(**A-C**) *alk1^{y6/+}*;*Tg(fli1a:negfp)^{y7}* zebrafish were crossed to *alk1^{y6/+}* to produce embryos that were either wild type, heterozygous, or homozygous mutant for the *alk1^{y6}* allele. Whole mount in situ hybridization was performed on resulting embryos for *egfp* (A), and *alk1* (B), and *cdh5* (C) fixed at 36 hpf. Embryos were scored for the presence or absence of expression in vessels in lateral and frontal views and genotyping was subsequently performed on all embryos for y6 allele. For *alk1* n = 7, 28, 8 for wild type, heterozygotes, and mutants, respectively. For *egfp* n = 29, 74, 23 for wild type, heterozygotes, and mutants, respectively. For *cdh5*, n = 8, 8, 4 for wild type, heterozygotes, and mutants, respectively. Arrows denote basal communicating artery, black; caudal division of the internal carotid artery, yellow; internal carotid artery, blue; first aortic arch, grey; lateral dorsal aorta, white. Scale bar = 100 µm.

Overall, these data support *bmp10/bmp10-like* as being required for activation of Alk1

activity and subsequently maintenance of *alk1* expression.
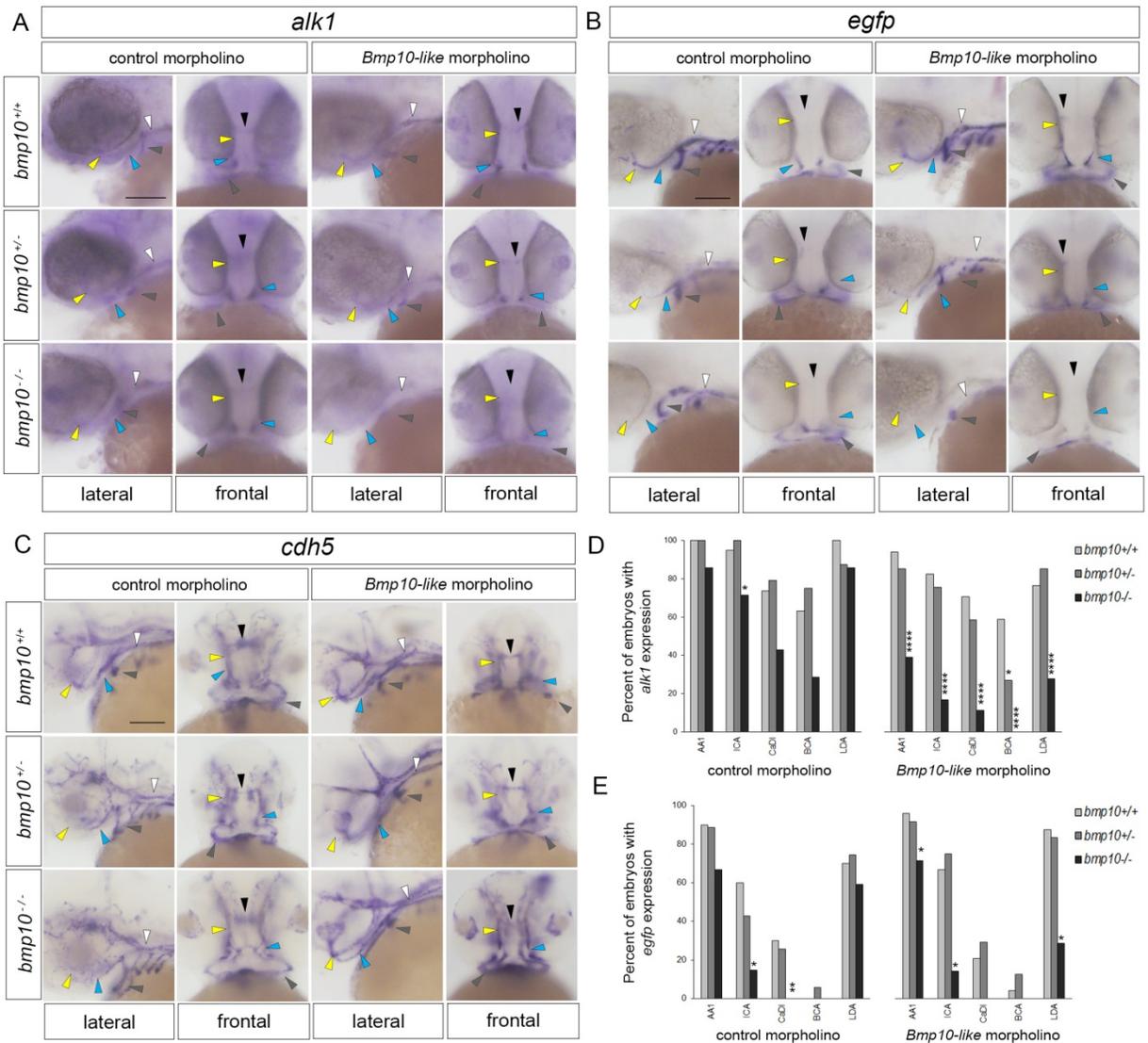
Figure 8. *alk1* expression requires Bmp10.

(**A-C**) *Tg(alk1e5:egfp); bmp10*^{+/-} were crossed to *bmp10* [+/-] non-transgenics and then injected with control morpholino (MO) or *bmp10-like* morpholino (MO) and then fixed at 36 hpf. Whole mount in situ hybridization was performed for *alk1* (A), *egfp* (B), and *cdh5* (C) and embryos were scored for the presence of expression in arteries in lateral and frontal views. Embryos were then genotyped for the *bmp10*[pt527] allele and for *egfp*. For wild type, heterozygous, and mutants, respectively, in control MO injected embryos: n = 19, 24, 7 (*alk1*); 10, 35, 27 (*egfp*); 15, 18, 3 (*cdh5*) over three independent experiments. For wild type, heterozygous, and mutants, respectively, in *bmp10-like* MO injected embryos, n = 17, 41, 18 (*alk1*); 24, 24, 7 (*egfp*); 16, 24, 8 (*cdh5*) over three independent experiments. (**D-E**) Embryos scored for *alk1* (D) or *egfp* (E) expression were analyzed by Fisher's exact test with significance set at 0.05. (**** signifies p-value <0.0001, ** 0.001 to 0.01, * 0.01 to 0.05). Scale bar = 100 µm.

4.0   DISCUSSION

Decreased transforming growth factor β (TGF-β) signaling in vascular endothelial cells in humans, specifically a haploinsufficiency of *ALK1* expression, results in hereditary hemorrhagic telangiectasia type 2 (HHT2). This disease is modeled by zebrafish embryos with a homozygous loss of *alk1* in which arteriovenous shunts form in the cranial arteries at a predictable time in development. Studies have shown that *alk1* is predominantly expressed in the endothelial cells of arteries undergoing angiogenesis, but its exact role has yet to be delineated. Because HHT2 is considered to be a result of a haploinsufficiency, studying the mechanisms that regulate *ALK1* expression is an area of research that could yield promising candidates for clinical treatment that is targeted towards HHT2 patients.

4.1   SENSITIVITY OF *alk1* TO FLOW

In zebrafish, Alk1 receptors are only expressed in endothelial cells in vessels that are nearest to the heart. The dependence of a*lk1* on blood flow has been documented [41] and I have further characterized this relationship through time course experiments. *alk1* expression is quickly lost subsequent to absence of blood flow. Loss of expression is first noticed in the vessels most distal from the heart (BCA, ICA, CaDI) followed by vessels more proximal to the heart (AA1 and LDA). Furthermore, when anesthetic is removed and blood flow returns to the embryo, *alk1*

expression returns to the most proximal vessels first before the more distal vessels. This gradient-like pattern from the heart outwards could support a number of hypotheses regarding which component of blood flow – whether a circulating element or mechanical forces including shear stress and/or circumferential strain – is specifically responsible for *alk1* expression.

4.2   ENDOCRINE LIGAND AS KEY FACTOR IN *alk1* EXPRESSION

Given that Alk1activity is required for *alk1* expression [40], this suggests a feedback mechanism may be involved. If so, then loss of Bmp10 (*bmp10$^{-/-}$*) should produce the same effect. My study has examined the possibility of this endocrine factor as the underlying activator of *alk1* expression. As mentioned previously, Bmp10 is a strong candidate for this role. We have demonstrated that loss of Bmp10 results in vascular defects at the embryonic stage, similar to *alk1*mutants [40]. Furthermore, Bmp10 is derived from the heart and is detected in blood [40]. One could speculate that Bmp10 is the critical factor for *alk1* expression due to the observed pattern of loss of *alk1* expression in my time course experiments. I observed that with loss of blood flow *alk1* expression initially persisted in vessels most proximal to the heart, which is also the source of Bmp10 ligand. My data support the concept of a gradient of Bmp10 ligand in arteries with highest concentrations of Bmp10 ligand closest to the heart and then diminishing as it follows along to more distal vessels. Loss of blood flow would therefore restrict the location of Bmp10 to the heart and only the most proximal arterial segments. The concept of a ligand gradient explains why I observed retained *alk1* expression in vessels most proximal to the heart after blood flow cessation. When blood flow resumes, Bmp10 is carried from the heart and

circulates through arteries, which explains why I observed *alk1* expression return first in the most proximal vessels and then later in the most distal vessels.

I then hypothesized that if Bmp10 was the critical factor for promoting *alk1* mRNA expression, then loss of Bmp10 signaling activity would result in loss of *alk1* expression. I observed a decrease in *alk1* in *bmp10* homozygous mutants but the change was not statistically significant. One reason for this result may be that there were too few *bmp10* mutants in our control group to prove significance. Another reason may be that *bmp10* is not the only regulator of *alk1*expression. *bmp10-like*, a redundant *bmp10* paralog ligand in the zebrafish genome that is also derived from the heart, may also serve to compensate for Bmp10. In this study I found that loss of *bmp10-like* alone does not significantly decrease *alk1* expression (Figure 8A, statistics not shown) but the combination of *bmp10* and *bmp10-like* loss does significantly decrease *alk1* expression. It may be that Bmp10 is the primary critical ligand with Bmp10-like is a secondary ligand in promoting Alk1 activity. We know that *bmp10* is expressed starting at 28 hpf, and *bmp10-like* is expressed later starting at 36 hpf [40] I did my analysis on 36 hpf embryos, so it may be that at this stage in embryonic development, *bmp10-like* may not yet play a critical role in *alk1* regulation in arterial endothelial cells.

As mentioned earlier, I observed that loss of *bmp10-like* alone (in wild type embryos) does not have an effect on *alk1* expression. I also observed that *bmp10-like* loss in *bmp10* heterozygotes did not show any significant difference in *alk1* except in the BCA where there was a significant difference. There are a several explanations for this finding. First, given that the BCA is the *alk1*-positive artery most distal from the heart, one could speculate that the concentration of Bmp10 ligand as it is carried away from the heart by flow is lower in the BCA than the more proximal arterial vessels. Perhaps the concentration of Bmp10 in the BCA, in

combination with Bmp10-like, is just enough to activate *alk1* expression in the wild type state. If there is a loss of Bmp10-like, then half of the Bmp10 ligand in *pt527* heterozygotes is no longer sufficient to maintain *alk1* expression in this vessel. Secondly, the loss of *alk1*expression in the BCA may be due to technical execution of the in situ hybridization. The BCA is deep within the head of the embryo and therefore getting the probe into this region to detect *alk1* expression is more difficult. This could explain why mRNA expression for this vessel is more variable than other vessels closer to the embryo's outer surface, but it does not explain the differences I observed between control and treated embryos.

Overall, my data support the idea of a circulatory component distributed in a gradient pattern across proximal and distal blood vessels as playing a necessary role in *alk1* activation.

4.3 ROLE OF MECHANICAL FORCES

Although my data support the notion that *alk1* expression is self-regulated and/or regulated by a circulating factor, my study does not rule out the possibility that mechanical force may still play a role. The proposal that *Alk1* is regulated by mechanical force has been suggested in the past. In mouse models, *Alk1* expression is upregulated in arteries presumably experiencing high shear stress [49]. Also, shear stress has been found to induce *alk1* expression in endothelial progenitor cells [55]. Additionally, *alk1* expression is more likely related to shear stress rather than circumferential strain due to the observation that *alk1* loss affects the arterial lumen but not the thickness of the arterial wall [41]. On the other hand, *gata1a* mutants, which lack erythrocytes but still have plasma flow, have normal *alk1* expression despite theoretically having lower blood viscosity and consequently lower shear stress on vessels. However, there is no observed change

in expression of known shear-responsive genes (*edn1, cxcr4a,* and *kil2a*) in *gata1a* mutants [41] which indicates that more research is needed to define the relationship between shear stress in the early embryo and its effects on downstream gene expression.

Mechanical forces could potentially work in conjunction along with a circulating ligand in a complex to activate Alk1 activity and stabilize *alk1* mRNA transcripts. Another theory may be that mechanical forces may be important in activating the circulating ligand i.e., releasing the growth factor domain of Bmp10 from its propeptide.

4.4   REGULATION OF *alk1* OCCURS AT THE LEVEL OF TRANSCRIPTION

I also aimed to determine whether regulation of *alk1* occurs at the level of transcriptional activity. Stopping the blood flow of *Tg(alk1e5:egfp)^{pt517}* embryos, a reporter line in which EGFP expression is driven by an enhancer element from *alk1* intron 1, showed that changes in *egfp* expression mimic changes in *alk1* expression that result from a loss of blood flow. These data suggest that blood flow regulates *alk1* at the level of transcription. Examination through whole mount in situ hybridization for *egfp* of *Tg(alk1e5:egfp^{pt517})* embryos in conjunction with the *y6* mutations suggested that loss of *alk1* expression is not due to decay of an unstable transcript but rather is due to a loss of *alk1*activity. Additionally, I showed that *egfp* transcriptional stability itself is not regulated by blood flow.

My experiments utilizing *Tg(alk1e5:egfp^{pt517})* embryos were valuable in helping to delineate the level at which *alk1* is regulated, but I have observed that *egfp* does not always mimic *alk1* expression perfectly, most often following the same trend but being less significantly different than *alk1*. I note that there is more inherent variability in the expression of our

transgene when compared to endogenous *alk1*. Certain lines of fish, or even individual adult fish, may yield embryos that greatly differ in the robustness of their transgenic expression. I did not pre-sort embryos for the presence of EGFP and instead genotyped them after in situ hybridization processing, and phenotypic scoring. Because I was not able to pre-sort transgenics, there is the potential that embryos within the same clutch expressed very different levels of EGFP. This variability may explain some of the differences I see between *alk1* and *egfp* expression, but one also cannot rule out the possibility that additional regulatory elements for *alk1* are missing in our transgenic line or *egfp* transcript has a longer half-life. Overall, my data suggest that Alk1 activation by Bmp10 is required for *alk1* expression in a self-regulatory process.

4.5   LIMITATIONS OF THE STUDY AND FUTURE DIRECTIONS

In this study I used whole mount in situ hybridization for analysis of gene expression. While this method is powerful in its ability to illustrate patterns of gene expression specific to tissue location it is not a quantitative method. Real-time quantitative PCR (qRT-PCR) would be an invaluable method to confirm my observations and quantitate changes in gene expression.

Additionally, it would be worth trying to identify particular *cis* elements within *alk1* intron 1 – the genomic fragment enhancer that drives *egfp* in our transgenic line *Tg(alk1e5:egfp)^{pt517}*– that regulates *alk1* by blood flow and Alk1 activity.

Also, further examination into the role of Bmp10 and Bmp10-like as the key ligand for *alk1* regulation is needed. Our lab has developed a *bmp10-like* mutant line of zebrafish to allow for the production of double *bmp10* and *bmp10-like* mutants, which would be a more robust

method of studying the roles of these genes than using morpholinos. We plan to replicate my experiments with double mutants to further support Bmp10 as a regulator of *alk1* expression. We also need to show that *egfp* stability is not changed by Bmp10/Bmp10-like loss.

4.6   APPLICATIONS FOR CLINICAL MANAGEMENT OF HHT

Delineating the mechanisms that regulate *alk1* will allow for the exploration of possible avenues to upregulate *alk1* as a means of preventing or treating AVMs. Because Bmp10/Alk1 signaling is required for *alk1* expression, increasing the level of Bmp10 ligand could help to increase *alk1* expression in arterial endothelial cells. Bmp10 binds specifically to Alk1, which makes it an ideal therapeutic for targeted treatment. Earlier in the TGF-β pathway (See Figure 1), an increase in Bmp9 is thought to upregulate its receptor endoglin [56], so the proposal that Bmp10 may be a candidate for jumpstarting *alk1* is a promising one.

4.7   APPLICATIONS FOR GENETIC COUNSELING

It is thought that HHT affects as many as 1 in 5,000 people. It would not be uncommon for a genetic counselor to interact with a patient with a personal or family history of the disease, but the condition often goes undiagnosed given its variable expressivity. Genetic counselors routinely ask about health information for common indications such as early-onset cancer, multiple miscarriages, or a pattern of intellectual disability when eliciting family history. A greater awareness of HHT would prove useful so that counselors may refer patients and families

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that have a history of AVMs, telangiectasias, and/or epistaxis to the proper specialists. Use of the Curaçao criteria [20] is a quick way to determine which individuals should be referred for genetic testing of the genes implicated in HHT. One challenge that health providers may face is judging whether the patient/family is experiencing epistaxis and separating these cases from those with common nosebleeds. Online tools exist (Nosebleed Severity Score: https://www2.drexelmed.edu/HHT-ESS/) as an easily accessible reference for health care providers to address these discrepancies. Another challenge for genetic counselors and physicians when encountering a person/family with possible HHT is that clinics or physicians that are specifically knowledgeable about HHT (such as a Center for Excellence designated by CureHHT/The HHT Foundation, http://curehht.org/) may not be available in many cities, so there needs to be increased advocacy and funding to establish more clinics and increase physician awareness of the disease.

4.8   PUBLIC HEALTH SIGNIFICANCE

As discussed previously, HHT is a disease that affects a population that is large relative to other genetic conditions. The implementation of newborn screening (NBS) programs is heralded as the most successful public health intervention to come out of advances in genetic screening. Most of the diseases that are included in NBS have a prevalence that is the same or smaller than HHT, including phenylketonuria (PKU), the first screening test introduced on the population scale. Because it is as common as other genetic diseases for which we screen the public, one could argue that HHT deserves the attention of those who study public health. A good screening test that is both easy to administer and accurate in its detection, however, has not yet been developed.

There has been some investigation into identifications of plasma markers for HHT screening including various cytokines and pro-angiogenic factors (i.e., VEGF) which are elevated in the serum of HHT patients [57, 58]. Furthermore, the lack of available treatments and inability to predict disease course limits the utility of a screening measure, particularly in unaffected or mildly affected people. At this time, the best approach for discovering unidentified individuals with HHT likely lies in the hands of physicians and genetic counselors with knowledge of the disease and subsequent cascade screening of at-risk relatives.

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