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# The Role and Regulation of Etv2 in Zebrafish Vascular Development: A Dissertation

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# THE ROLE AND REGULATION OF ETV2 IN ZEBRAFISH VASCULAR DEVELOPMENT

A Dissertation Presented

By

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**THE ROLE AND REGULATION OF ETV2 IN ZEBRAFISH VASCULAR DEVELOPMENT**

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This work is dedicated to my father.

**John E. Moore Jr.**

March 11th, 1952 – September 28th, 2005

He taught me the meaning and value of hard work.

Thanks Dad.

I miss you.

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**Abstract**

*Etv2* is an endothelial-specific ETS transcription factor that is essential for endothelial differentiation and vascular morphogenesis in vertebrates. However, *etv2* expression dynamics during development and the mechanisms regulating it are poorly understood. I found that *etv2* transcript and protein expression are highly transient during zebrafish vascular development, with both expressed early during development and then subsequently downregulated. Inducible knockdown of Etv2 in zebrafish embryos prior to mid-somitogenesis, but not later, causes severe vascular defects, suggesting a role for Etv2 in specifying angioblasts from the lateral mesoderm. I further demonstrate that the 3'UTR of *etv2* is post-transcriptionally regulated in part by the *let-7* family of microRNAs. Ectopic expression of *let-7a* represses endogenous Etv2 transcript and protein expression with a concomitant reduction in endothelial cell gene expression. Additionally, overexpressed Etv2 in HEK293T cells is ubiquitinated and degraded by the proteasome. Accordingly, endogenous zebrafish Etv2 protein is rapidly degraded in the presence of the translation inhibitor cycloheximide in vivo. Taken together, our results suggest that *etv2* acts during early development to specify endothelial lineages and is subsequently downregulated through post-transcriptional and post-translational mechanisms, to allow normal vascular development to proceed.

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## List Of Abbreviations

| Abbreviation | Term |
|---|---|
| -DBD | Minus the DNA Binding Domain |
| 3'UTR | 3' Untranslated Region |
| bHLH | basic Helix-Loop-Helix |
| BMP | Bone Morphogenetic Protein |
| cMO | caged Morpholino |
| ChIP | Chromatin Immunoprecipitation |
| D2EGFP | Destabilized Enhanced Green Fluorescent Protein |
| DA | Dorsal Aorta |
| DLAV | Dorsal Longitudinal Anastomotic Vessel |
| DC | Ducts of Cuvier |
| DMEM | Dulbecco's Modified Eagles Medium |
| ETS1 | E26 Transformation Specific 1 |
| EMSA | Electromobility Shift Assay |
| EGFP | Enhanced Green Fluorescent Protein |
| FBS | Fetal Bovine Serum |
| FGF | Fibroblast Growth Factor |
| FACS | Fluorescent Activated Cell Sorting |
| Fox | Forkhead |
| Fli1 | Friend leukemia integration site 1 |
| GSK | Glycogen Synthase Kinase |
| GFP | Green Fluorescent Protein |
| HAT | Histone Acetyltransferase |
| hpf | Hours Post Fertiliztion |
| HUVECs | Human Umbilical Cord Endothelial Cells |
| ISV | Intersegmental Vessel |
| Kb | Kilo base |
| KLF | Krüppel-like factor |

| | |
|---|---|
| LDA | Lateral Dorsal Aorta |
| MZ | Maternal Zygotic |
| MO | Morpholino |
| NIL | Notch Indicator Line |
| PBS | Phosphate Buffered Saline |
| PBSTw | Phosphate Buffered Saline Tween |
| PCV | Posterior Cardinal Vein |
| PEST | Proline (P), Glutamic Acid (E), Serine (S), Threonine (T) |
| SDS-PAGE | Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis |
| ss | Somite Stage |
| Sox | Sry-related HMG box |
| SEM | Standard Error of the Mean |
| TALEN | Transcription Activator-Like effector Nucleases |
| VEGF | Vacular Endothelial Growth Factor |
| VE-Cadherin | Vascular Endothelial-Cadherin |
| WT | Wild Type |
| ZFN | Zinc Finger Nucleases |

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**Zebrafish Camera Lucida pictures** - originally published in: Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. Dev Dyn *203*, 253-310. Digital images obtained from the zebrafish information network (www.zfin.org).

## Contributions

**Etv2 protein fragment cloning and isolation for antibody production.**
Dr. Nathan Lawson, University of Massachusetts Medical School

***Etv2* caged morpholino.**
Dr. Ilya Shestopalov; Chen Lab, Stanford University

**Overexpressed *etv2* mosaic transplant analysis.**
Sarah Sheppard; Lawson Lab, University of Massachusetts Medical School

**Microarray normalization and statistical analysis**
Dr. Lihue J Zhu; University of Massachusetts Medical School

***Etv2* 3'UTR identification, cloning, 3'RACE, RT-PCR, *Let-7* binding site analysis and *let-7* binding site mutagenesis.**
Sarah Sheppard, Lawson Lab

**Endothelial cell autonomous 3'UTR analysis, cloning, injection, imaging, quantification and statistical analysis**.
Sarah Sheppard, Lawson Lab, University of Massachusetts Medical School

**Overexpressed *Let-7* duplex mosaic transplant analysis.**
Sarah Sheppard, Lawson Lab, University of Massachusetts Medical School

**Maternal zygotic *dicer1* generation.**
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Gift from Dr. Ingolf Bach, University of Massachusetts Medical School

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Tom Smith, Lawson Lab, University of Massachusetts Medical School

**The rest of the work presented in this thesis was performed by:**
**John C. Moore**

# CHAPTER I: INTRODUCTION

## Primer

Due to their large size and complexity vertebrate animals cannot receive oxygen to support metabolic activity by simple diffusion alone. As a result they have evolved a system of interconnected tubular structures called the circulatory system. The circulatory system is an essential conduit for the systemic distribution of oxygenated blood, nutrients, hormones, immunological factors and the removal of metabolic waste. Cells therefore are located no more than100-200 μm from the vessels of the circulatory system, the diffusion limit of oxygen. The fundamental cellular unit that defines the circulatory system is the endothelial cell. The human circulatory system is a highly ramified network of blood vessels containing approximately ~1 X $10^{13}$ endothelial cells [1].

The vascular system is one of the first organ systems to form during embryogenesis. Development of this system starts with the specification of angioblast, endothelial cell precursors from the lateral plate mesoderm. Angioblasts migrate and coalesce forming a vascular cord *de novo* through a process called vasculogenesis [2]. This initial vascular plexus is remodeled and extended upon through a process termed angiogenesis [3]. Finally, vascular cords formed by either the vasculogenic or angiogenic process are hollowed out though a process called tubulogenesis.

The development of the cardiovascular system involves the genetic regulation and coordination of multiple endothelial cellular behaviors including

migration, proliferation, and differentiation. Perturbation of the genetic regulatory mechanisms driving vascular development often result in vascular dysfunction and embryonic lethality. Post-embryonic vascular dysfunction or deregulation can lead to arteriovenous malformations, arteriosclerosis, stroke, inflammation, cancer growth and metastasis [4, 5]. In fact, heart disease, cancer, and stroke are three of the top four leading causes of death in the United States (National Vital Statistics Report, 2010).

To date, many of the genetic regulatory and signaling mechanisms that drive vasculogenesis and angiogenesis have been identified. However, little is known about the transcriptional regulation of angioblast specification and endothelial cell differentiation. In this introduction I will: **(1) describe the ontology of angioblast specification, (2) Explain vascular morphogenesis in the zebrafish and the processes driving vascular development, (3) Discuss the transcriptional regulation of endothelial cell differentiation with a particular emphasize on the ETS family of transcription factors, specifically *etv2*.** *Etv2* is essential for vascular development and will be the main focus of the following data chapters [6-8].

## Benefits of Zebrafish Vascular Development Model

Understanding how the vascular system develops is essential because perturbations in the morphogenetic mechanisms responsible lead to embryonic lethality or post-natal vascular dysfunction and death. However, morphogenetic studies of vascular development in many vertebrate species are difficult due to animal opacity or in utero development. Recently, the small *Teleost* (ray-finned fish), zebrafish (*Danio rerio*) has greatly improved our understanding of the mechanisms of vertebrate development and disease [9]. Given its size, the zebrafish allows the maintenance of thousands of individual fish both affordable and manageable on a large scale. Zebrafish produce large clutches of externally fertilized embryos that are optically clear allowing visualization of organogenesis using a simple light microscope. Importantly, the zebrafish undergoes rapid development forming most organs within 24 hours. All these inherent characteristics make the zebrafish an exquisite organism to study vertebrate vascular development, including vasculogenesis, angiogenesis, endothelial cell differentiation and circulation [10, 11]. In addition to the above characteristics, the zebrafish is genetically tractable. Transgenic zebrafish are readily available and easily producible that express exogenous transgenes in an endothelial-specific manner, allowing morphogenetic studies *in vivo* [12]. Endothelial cell specific transgenic zebrafish embryos have been employed in large-scale mutagenesis screens, which have yielded numerous genes that are required for proper vascular

development [13-16]. Vascular studies in the zebrafish also benefit from the ability to perform targeted gene knockdown and over-expression experiments through the injection of morpholinos or mRNAs into single cell embryos, respectively [17]. This allows for the straightforward dissection of genetic pathways through epistasis experiments [18]. Until recently the zebrafish was limited by the inability to perform target gene mutations or knock-ins. This limitation has been overcome by the implementation of zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs), which have been used to generate mutations and locus specific knock-ins [19, 20]. Lastly, the genetic manipulations and information obtained are relevant in the zebrafish because the cellular and molecular processes underlying the patterning of the vascular architecture is conserved throughout vertebrate species [21]. Therefore the use of zebrafish is highly advantageous in the study of cardiovascular development and endothelial cell differentiation.

# Vascular Morphogenesis in the Developing Zebrafish

## Vasculogenesis

The basic pattern of the vascular system is conserved among vertebrates, which makes it possible to identify homologous vessels and make direct comparisons in different species [22]. The metameric arrangement of intersegmental vessels (ISVs) and stereotypical formation of the dorsal aorta (DA) and posterior cardinal vein (PCV) makes the zebrafish an ideal model to describe the processes of vascular morphogenesis. In this section I will introduce the relevant aspects of zebrafish vasculature development with an emphasis on the trunk vasculature, which will help define terms and processes that will be encountered throughout this manuscript.

Vascular development is a multistep process that starts with the specification of endothelial precursors early in development. Angioblasts are specified from the lateral mesoderm at early somitogenesis stages. (Figure I-1A; [23, 24]). Etv2, one of the earliest known markers of angioblasts, is detected by *in situ* hybridization at the 2 somite stage (ss) in zebrafish [6]. After initial emergence of angioblasts from the lateral plate mesoderm, they are positioned as two anterior posterior aligned stripes as early as 5 ss and are often described as resembling the stitched seams of a baseball (Figure I-1B). At 14-18hpf the lateral stripes of angioblasts start to migrate towards the

midline following guidance cues that emanate from the midline endoderm positioning them ventral to the hypochord (Figure I-1C; [25]. Upon reaching the midline, angioblasts start to coalesce into a vascular chord and shortly thereafter begins to lumenize (Figure I-1D,E) into the axial vasculature composed of the DA and PCV. Specification of arterial angioblasts fated to form the DA is dependent on vascular endothelial growth factor (VEGF) and Notch. The ligand VEGF-A activates its receptor Flk-1 (VEGFR2/Kdr; hereon referred to has Flk-1 (Fetal liver kinase -1)) inducing *notch* expression in angioblasts causing them to adopt an arterial identity [13]. Venous cell fate is independent of VEGF-A signaling and involves the activation of the nuclear hormone receptor COUP-TFII, which suppresses *notch* and arterial gene expression [26, 27]. The DA is lumenized by chord hollowing, a process that involves the cells within the primitive vascular chord obtaining apical-basal polarity, and forming the lumen through membrane separation and fluid influx [28]. The PCV vein is formed through a process called ventral sprouting, where starting at 20 hpf PCV precursors migrate ventrally from the primitive vascular cord coalescing around previously positioned red blood cells. (Figure I-1D; [29]. Upon the completion of a primitive vascular loop circulation begins (Figure I-1E).

Circulation in the zebrafish starts around 26 hpf concomitant with full lumenization of the DA and PCV. Initially, circulation is contained in a simple circulatory loop starting with blood being pumped from the two chambered

heart through to the paired anterior lateral dorsal aorta (LDA). The LDA then merge to form the DA of the trunk. Blood then continues caudally, whereupon reaching the tip of the tail it makes a 180 degree turn into the cardinal vein (CV). The CV flows directly into the PCV with flow terminating at a pair of large venous sinuses called the ducts of Cuvier (DC). Blood then flows ventrally down the yolk where it is returned to the heart (Figure I-1E). Vasculogenesis positions the major blood vessels and provides the core structures for angiogenesis to then elaborate on.

**The hemangioblast**

The intimate temporal and spatial association of hematopoietic and endothelial cell development has led to the idea that a single precursor, the "hemangioblast" gives rise to both cellular lineages. This hypothesis was put forward in some of the earliest studies in amniotes from the observation that mesodermal cells in the yolk sac proliferate and from mesodermal cell masses called "blood islands" [30]. The inner cells of the blood islands will start to express hemoglobin, and the outer cells flatten and form endothelial cells [31]. The hemangioblast hypothesis was further supported genetically by the fact the Flk-1 a receptor tyrosine kinase (RTK) is expressed in both hematopoietic and endothelial cell lineages, although its expression is maintained in endothelial cells only [32, 33]. Additionally, gene ablation of Flk-1 causes failure of both the hematopoietic and vascular systems to form

in mice [34]. The hemangioblast hypothesis has not been disproven but *in vivo* clonal analysis reveals that hemangioblast progenitor contribution to both the endothelial and hematopoietic lineages is an exceedingly rare event [35]. The literature probing the mechanisms of endothelial and hematopoietic differentiation often still refers to the hemangioblast as a tangible cellular progenitor. Therefore this discussion of vascular development and the molecular mechanisms controlling it will largely ignore hematopoiesis unless relevant.

**Figure I - 1: Stages of zebrafish vascular development**

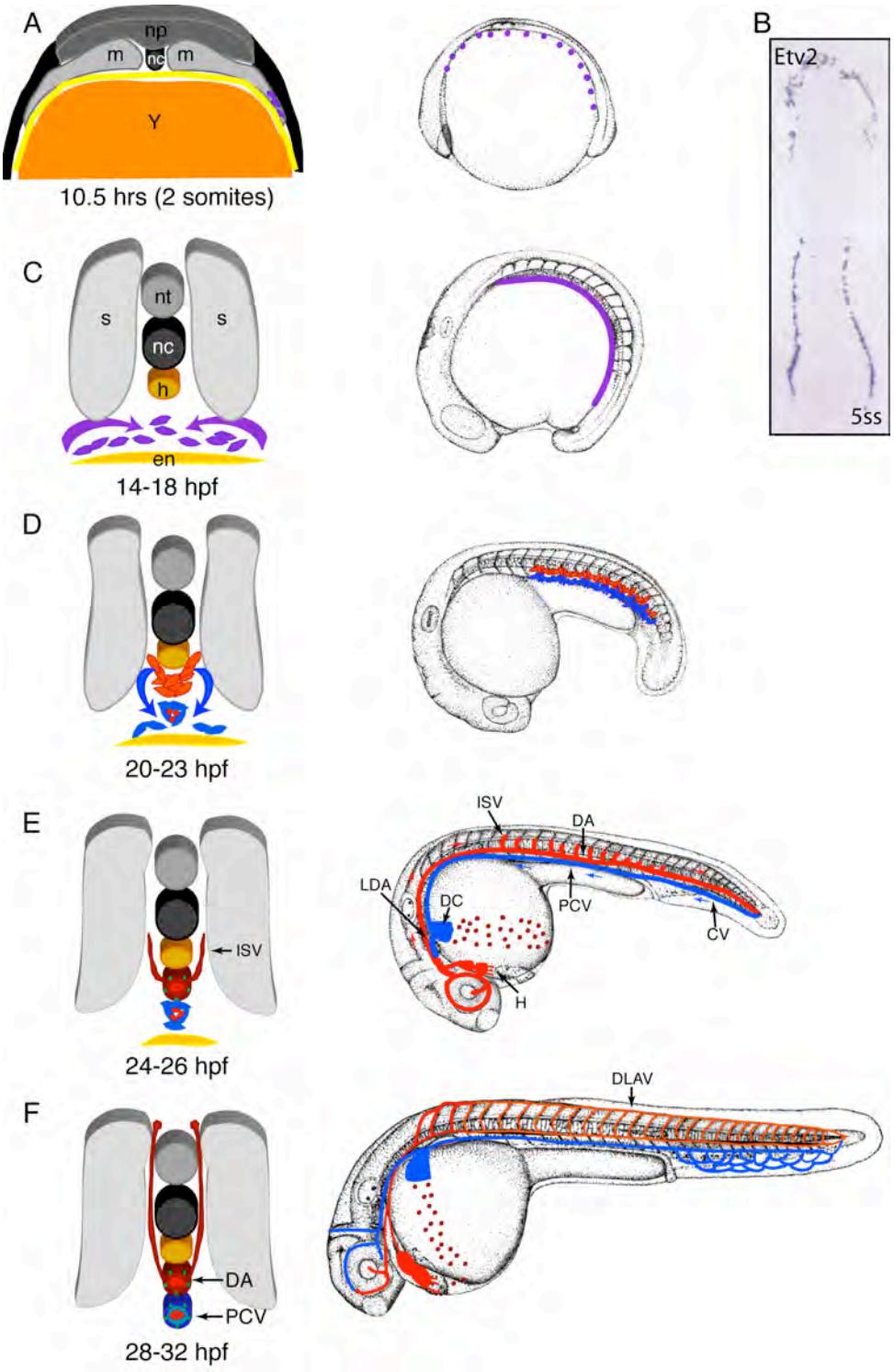

**Figure I-1: Stages of zebrafish vascular development.** (A, C-F; Left side) Schematic cross sections of the trunk at indicated developmental time points. The following tissues are labeled: neural plate (np), neural tube (nt) mesoderm (m), notochord (n) yolk (y) endoderm (yellow), hypochord (h) and somites (s). (A, C-F; right side) Camera Lucida drawings overlaid with schematic representations of the zebrafish vasculature at indicated developmental time points, angioblasts (purple), arterial vessels/cell (red), venous vessels/cells (blue) and blood (maroon). (B) *In situ* hybridization for *etv2* transcript in *Wt* embryos at 5 ss. Staining marks the bi-lateral stripes of angioblasts. (A) Angioblasts (purple) are specified from the lateral mesoderm at early-somitogenesis stages. (C) Midline migration. From 14 hpf onward, angioblasts migrate over the endoderm towards the midline just below the hypocord (h) where they coalesce to form a vascular cord (D). (D) Arterio-venous segregation and ventral sprouting. At ~17 hpf, angioblasts start to express markers for differentiated artery (red) or vein (blue) endothelial cells. Arterial endothelial cells are located in the dorsal portion of the vascular cord and will give rise to the dorsal aorta (DA), where as venous endothelial cells are located more ventrally and will give rise to the posterior cardinal vein (PCV) and cardinal vein (CV). (E) The DA forms and lumenizes prior to the PCV and CV in the absence of blood cells (smaller bright red cells) by chord hollowing. The DA simultaneously starts sending sprouts dorsally to start the process of angiogenesis to make intersegmental vessels (ISVs). Venous angioblasts aggregate and coalesces around the blood cells to ultimately form a tube. After the DA and PCV are fully lumenized a basic circulatory loop is established starting at the heart (H) and ending at the ducts of Cuvier (DC) (F, E; right side, arrows). (F) Functional vasculature. At ~32hpf the ISVs have extended to their full height at the dorsal roof of the neural tube and have branch anteriorly and posteriorly to form the dorsal longitudinal anastomotic vessel (DLAV). This figure was based on and modified from [22].

## Angiogenesis

The primary axial-vessels are elaborated on by the sprouting of secondary vessels, a process called angiogenesis. The sprouting of the intersegmental vessels (ISV) from the DA has become a classic model for studying the cellular and molecular mechanism driving angiogenesis in zebrafish [36-38]. ISV formation starts around 22 hpf with endothelial cells sprouting dorsally from the DA (Figure I-1D, E; Figure I-2A). Endothelial cells move dorsally between the somite boundaries and are prevented from crossing the boundary by antagonistic semaphorin-plexin signaling. Zebrafish mutants in this pathway have non-sterotypical ISV morphogenesis with torturous and chaotic sprouts [39, 40]. The ISVs sprout until they reach the dorsal roof of the neural tube, where they branch anteriorly and posteriorly connecting to their neighbor forming the dorsal lateral anastomotic vessel (DLAV; Figure I-1F; Figure I-2C). Angiogenic sprouting of the ISVs is a highly dynamic process that involves extensive filopodial extensions, cellular migration and proliferation, which has been described in detail[41]. Sprouting requires the coordination of two endothelial cell types, called the tip and the stalk cells. The tip cell becomes highly proliferative and migratory. Conversely, the stalk cells must inhibit these behaviors to remain connected to the DA. ISV formation starts with one or two cells migrating dorsally out of the DA (Figure I-2A), dorsal growth is continued by proliferation and migration

of the tip cell, which is the main coordinator of this process (Figure I-2B). Cell divisions can occur at various time points meaning the stalk can consist of a variable number of cells, generating a large degree of morphological heterogeneity. Further cell divisions and cellular rearrangements will lead to a paired configuration of cells in the ISV prior to lumen formation and after the DLAVs connect (Figure I-2C; [22, 41]. Studies in mice and zebrafish demonstrate tip and stalk cell coordination during angiogenesis is governed in large part by the VEGF and Notch signaling pathways [15, 38, 42-44]. Additionally, microRNAs (miRNA) have been shown to play a role in this process [37]. The complexity of the vascular system is increased throughout development by the continuation of the angiogenic process.

**Figure I – 2: Angiogenesis**

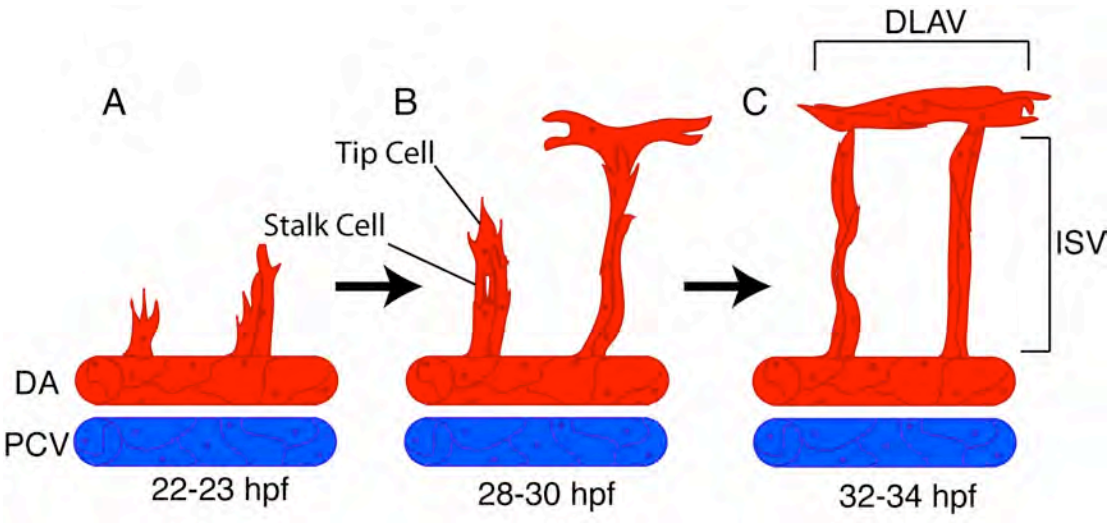


**Figure I-2: Angiogenesis.** (A-C) A model for the morphogenetic events leading to the formation of intersegmental vessels (ISVs) and the dorsal longitudinal anastomotic vessel (DLAVs) in the zebrafish trunk. (A) At 22 hpf endothelial cells of the dorsal aorta (DA) form sprouts that go along the somite boundaries up to the dorsal roof of the neural tube (B). (B) During these stages the sprout consists of 2-4 cells. (C) Upon reaching the height of the dorsal roof of the neural tube each sprout branches anteriorly and posteriorly to connect to its neighboring sprout to form the DLAV.

## Transcriptional Regulation of Endothelial Cell Differentiation

The development of the cardiovascular system is a complex multistep process involving the strict regulation of cellular behaviors that are genetically programmed. The signaling molecules that control the morphological processes of vasculogenesis and angiogenesis are known and well documented [45]. For example VEGF signaling is required for vasculogenesis, angiogenesis, tubulogenesis and endothelial survival. However angioblasts are still present in VEGF and VEGF receptor knockout mouse embryos, indicating that they are not strictly required for angioblast specification [34, 46-48]. Additionally, the signaling events that govern differentiation of the artery and vein have also been elucidated [13, 26]. However, little is known about the transcriptional regulation of angioblast specification including the factors required, their hierarchical arrangement or their potential combinatorial activation of endothelial gene programs. This section will discuss known transcription factors involved in endothelial cell differentiation, with a focus on the ETS family of transcription factors in particular *etv2*, which is essential.

**The ETS Family of transcription factors in endothelial cell differentiation**

Transcription factors determine gene expression by binding to specific DNA sequences within promoter or enhancer regions, activating transcription by recruitment of RNA polymerase or causing repression. Multiple transcription factor families have been implicated in the activation and maintenance of endothelial gene expression, including members of the Sox (Sry-related HMG box), Fox (Forkhead), GATA, KLF (Krüppel-like factor) and the bHLH (basic-Helix Loop Helix) families [49]. Yet, no transcription factor family seems to be as important, or regulate the endothelial cell transcriptional program as extensively as, the ETS family of transcription factors.

The first ETS factor, Ets1 (E26 transformation specific-1), was identified as the oncogenic progenitor of viral oncogene *v-ets* found in the genome of avian leukemia retrovirus E26 [50]. The majority of ETS factors are transcriptional activators, however some are repressive and still some can be both [51]. The ETS family of proteins is defined as having a conserved, approximately 85 amino acid (aa) DNA binding domain, called the ETS domain, consisting of a winged helix-turn-helix motif that binds a core DNA sequence of 5'-GGA(A/T)-3'[52]. The DNA binding domain consists of three alpha-helices and four anti-parallel beta-sheets, with the third helix primarily responsible for DNA-binding specificity [53]. All ETS factors contain a transactivation domain and a subset contains a domain called Pointed,

thought to be involved in protein-protein interactions. The ETS family is divided into four major classes based on DNA binding domain sequence conservation, overall conservation, domain presence and orientation (Figure I-3; [54]. The major classes not only share similar DNA binding domain sequences but also recognize and bind comparable DNA motifs [53]. ETS proteins play key cellular roles regulating growth, proliferation, apoptosis, migration and the differentiation of multiple cellular lineages [55]. Because of the critical role they play in basic cellular processes, ETS protein loss or enhancement is found in various types of cancers including breast, prostate and leukemias [56, 57]. Additionally the ETS factors play essential roles throughout development and in adult life, regulating a broad spectrum of processes particularly hematopoiesis and vascular development.

**Figure I – 3: Phylogeny of the zebrafish ETS Family**

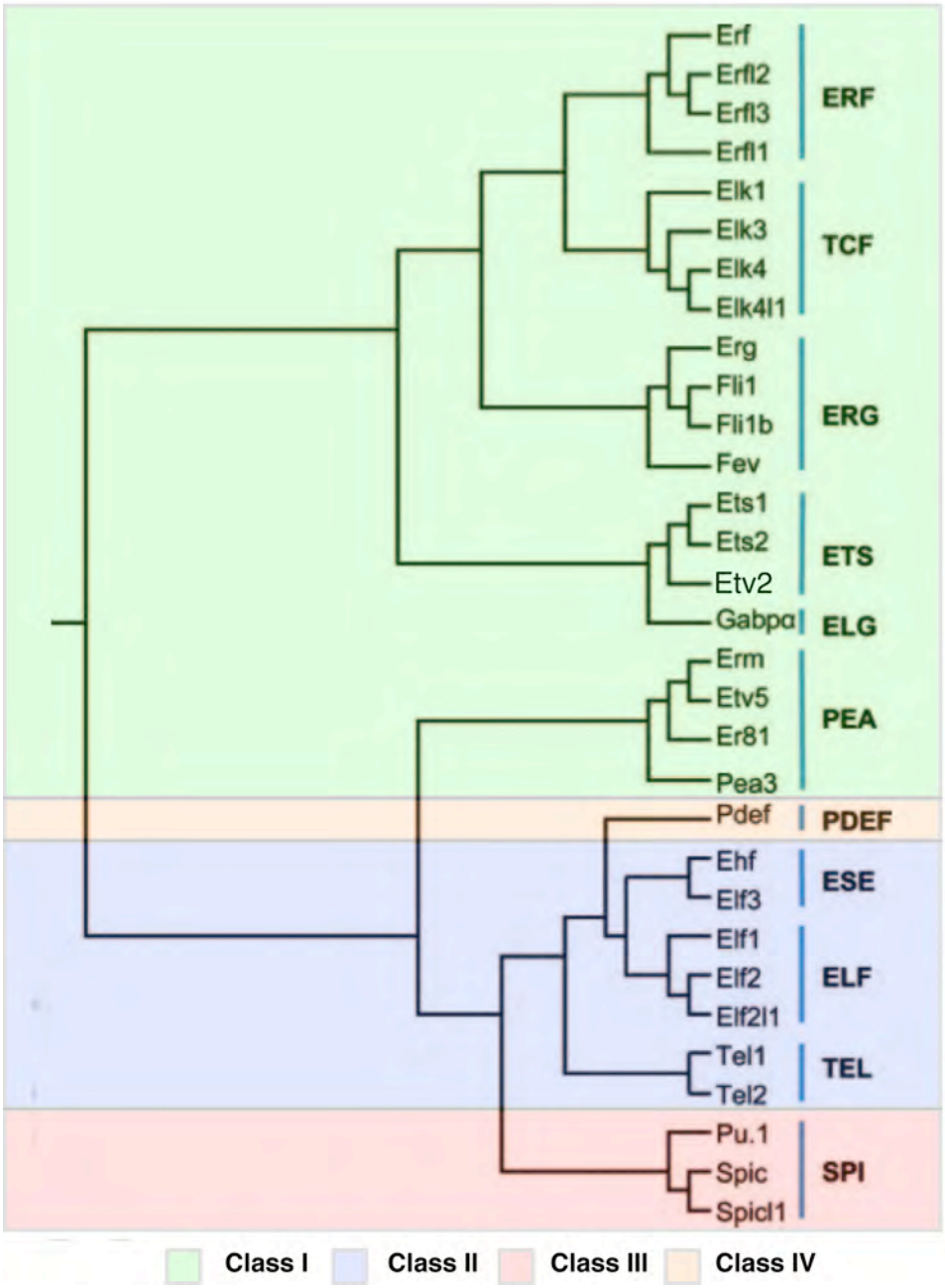


**Figure I – 3: Phylogenetic analysis of zebrafish ETS genes.** The tree was generated from the alignment of the amino acid sequence of the whole protein including ETS DNA binding domain and Pointed domain (PNT) using the CLUSTAL W method. Unmodified figure originally appeared in [58]

The ETS factor family is essential for the development of the vascular system, functioning during angioblasts specification, vasculogenesis, angiogenesis and endothelial cell differentiation. The human and zebrafish genome encodes 27 and 31 ETS family members, of which 19 and 12 are expressed in the endothelium of each species, respectively (Figure I – 3; [58-60]. Although no ETS factor is solely expressed in the endothelium, several are highly enriched including ETS1, ETS2, ETV2 (etsrp/ER71), ETV6 (TEL), FLI, ERG and ELK3 (NET/SAP2) [49, 51, 57]. Nearly every characterized endothelial gene promoter or enhancer contains essential ETS binding sites and ETS motifs are strongly associated with endothelial cell genes throughout the human genome [49, 61, 62]. As a result, it is thought that nearly every endothelial cell gene is regulated by ETS factors in some manner [57]. The following section will highlight a number of the endothelial cell enriched ETS transcription factors, which have significant roles in vascular development including Ets1, Ets2, Fli1 and Erg. The last segment will give an in depth review of the literature regarding Etv2 as it is the subject of the following three data chapters. Although, Etv2 is considered the most important of the endothelial ETS transcription factors, its role and regulation during vascular development is probably the least understood.

**Ets1 (E26 transformation specific-1)**

Ets1 has been shown to play a role in endothelial differentiation, migration, angiogenesis and survival and is the best studied. Ets1 is expressed early during development and can be detected in the DA, PCV and ISV of the zebrafish trunk vessels as well as in the quiescent endothelium of humans, albeit at low levels [58, 60, 63, 64]. Consistent with its role in various endothelial cell processes, disruption of Ets1 function results in vascular defects. Research studying angiogenesis in *in vitro* cell culture and the chicken choriallantoic membrane assay (CAM) were the first to discover a role for Ets1 in vascular biology. Inhibition of Ets1 by dominant negative Ets1 expression or by anti-sense oligonucleotide directed against Ets1 abrogated the ability of endothelial cells to migrate, adopt invasive behavior and form tubes in response to angiogenic growth factors in culture [65, 66]. Additionally, genetic down-regulation of Ets1 in chick results in the reduction of both the number and diameter of vessels. In mice, introduction of a dominant negative form of Ets1 through intravitreal injection suppresses retinal angiogenesis [67, 68]. However, homozygous Ets1 mutant mice are viable with no obvious vascular phenotype but have a significant reduction of natural killer cells in the spleen. The lack of a vascular phenotype in Ets1 null mice is likely due to functional redundancy with Ets2 (see below) [69]. Genetic dowregulation of *ets1* in the developing zebrafish causes a mild angiogenesis defect with minor semi penetrant disruption of the ISVs, and a complete loss

of circulation at high morpholino doses [7]. Taken together these loss of function studies demonstrate its important role for Ets1 in endothelial differentiation, angiogenesis and vascular function.

Ets1 loss of function phenotypes result from the inability of Ets1 to be up-regulated during vascular development and its subsequent lack of downstream gene activation. Expression of Ets1 can be induced by pro-angiogenic signals and can activate transcription of endothelial genes. For example, hypoxic tissues up-regulate hypoxia inducible factor-1 (HIF-1) and induce Ets1, resulting in new vessel invasion into the tissue. [70]. Pro-inflammatory and pro-angiogenic stimuli such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$), platelet derived growth factor and hepatocyte growth factor (HGF), can also up-regulate Ets1[71, 72]. Ets1 downstream targets are important regulators of vascular development, including receptors involved in VEGF signaling like Flk-1, Flt-1 (VEGFR1), and Nrp1[73, 74]. Furthermore, Ets1 induces a number of adhesion molecules such as, Pecam1 and VE-cadherin [75, 76]. Additionally, Ets1 regulates angiogenesis and tumor invasion by inducing genes capable of degrading the extra cellular matrix including matrix metalloproteases MMP-1, MMP-3 and MMP-9[77]. Interestingly, Ets1 is also capable of directly activating Fli1, another ETS protein that plays important roles in the differentiation of endothelial cells (see below) [78]. Experimental evidence demonstrates an important role for Ets1 in vascular development

and especially angiogenesis. However Ets1 is not required for angioblast specification and likely shares overlapping functions with other ETS family members.

**Ets2 (E26 transformation specific-2)**

Ets2 is expressed in endothelial cells and can activate endothelial gene expression similar to Ets1. Ets2 and Ets1 are closely related and highly conserved sharing ~67% sequence identity (data not shown, Clustal W protein alignment of human sequences)[53];b Ets1 and Ets2 are both activated by *Ras*-mediated phosphorylation [79] and demonstrate weak binding to each other [80], suggesting they may work to together to drive transcription. Additionally, Ets2 can activate endothelial cell gene promoters of Flt-1, Flk-1, Anpep (aminopeptidase) and Angpt 2 (angiopoietin-2) as measured by luciferase activity *in vitro* [74, 81-83]. Interestingly, Ets1 is able to activate a number of the same genes. Ets2 is expressed in the posterior lateral mesoderm, the sight of angioblast specification and in endothelial cells throughout zebrafish development [58]. In addition to its expression in presumptive angioblasts, Ets2 is also expressed in the vasculature of the developing mouse [84] and in the differentiated endothelium of humans [84-86]. Homozygous deletion of the conserved DNA binding domain of Ets2 is embryonic lethal in mice, caused by a defect in trophoblast function. However, tetraploid complementation experiments demonstrate that Ets2 is

dispensable for the development of the embryo proper [87]. This is likely due to the functional redundancy of Ets1 and Ets2, because double knockouts of both genes in mice are embryonic lethal. Ets1/Ets2 null mice mice develop vascular abnormalities at E10.5, including marked reduction in vascular complexity, defective branching, and dilated vessels. Additionally, RT-PCR on the endothelial cells of these double knock out embryos reveal a significant up-regulation of apoptotic genes indicating that Ets1 and Ets2 play a role in endothelial cell survival [84]. Furthermore, both Ets1 and Ets2 are able to activate mir-126, which plays a crucial role during angiogenesis and is the most abundantly enriched endothelial miRNA [88]. As a whole, these data suggest that Ets2 is functionally redundant to Ets1 during vascular development and likely compensates for the loss of Ets1 by turning on the similar transcriptional programs.

**Fli1 (Friend leukemia integration site-1)**

Fli1 like Ets1 was first found as an oncogene and a common site for viral integration in Friend Virus-induced erythroleukemias [89]. Fli1 shares 80% homology with the ETS protein Erg, and they share overlapping expression domains, suggesting they may have overlapping functions [90]. Fli1 is expressed at E7.5 in the murine mesoderm thought to give rise to both the hematopoitic and endothelial cells, then subsequently in the blood islands and the developing vasculature [91]. In the zebrafish Fli1 is encoded by two

genes, *fl1a* and *fl1b* due to a genome duplication event in Teleosts [92]. Zebrafish *fli1a and fl1b* have nearly identical overlapping expression patterns, starting in early angioblasts then throughout the endothelium and these proteins are functionally redundant [7, 58]. In humans Fli1 is constitutively expressed in the endothelium of arteries, veins, lymphatics as well as tumor vasculature [93]. Zebrafish transgenics harboring a Fli:egfp transgene have been used extensively in the vascular biology community to study the processes of vasculogenesis, angiogenesis and as a way to isolate endothelial cells for *in vivo* gene expression analysis [12, 94].

The expression pattern of Fli1 would seem to imply that it could be a major effecter of endothelial cell differentiation. However, loss of Fli1 causes only minor vascular defects. Although Fli1 shares 80% homology with Erg, and share overlapping expression domains, they do not seem to be functionally redundant because Fli1 knockout mice are not viable [90]. Fli1 homozygous mice die at E11.5 due to cranial hemorrhaging and/or a failure in hematopoiesis. However, specification of the endothelial lineage and early vascular patterning are normal in these mice [95]. Likewise, knockdown of the zebrafish *fli1a* and *fl1b* genes alone or together only causes minor vascular patterning defects with a semi-penetrant loss of circulation phenotype [7]. Simultaneous knockdown Fli1 and Erg in zebrafish have an additive angiogenesis phenotype compared to the phenotype of either one alone, therefore both are required and do not have overlapping functions [58].

Despite mild vascular defects in the absence of Fli1, overexpression of a constitutively active form of *fli1a* in the zebrafish can up-regulate early vascular markers such has *flk-1*, *scl* and *lmo2* [96]. Interestingly, in the zebrafish *cloche* mutant, *fli1a* is still expressed despite lack of all hematopoietic and endothelial cell linage [97]. This continued expression is paradoxical and has yet to be explained. Although studies interpret this to mean Fli1 is positioned atop of the hemangioblast transcriptional hierarchy, its limited mutant phenotypes suggest otherwise [96]. However, Fli1 is able to activate endothelial cell gene expression and remains expressed within the differentiated endothelium to reinforce vascular stability, demonstrating an essential function during vascular development.

**Erg (Ets-related gene)**

Erg is expressed in the vasculature throughout embryonic development and post-nataly. It is expressed as early as E9.5 in Flk-1 positive blood islands of the mouse extra-embryonic yolk sack and in the trabeculated endocardial surface of the heart and is continually expressed in close association with VE-cadherin-positive endothelial cells [98]. Consistent with its expression, Erg plays a role in endothelial survival, differentiation, migration and angiogenesis. shRNA knock down of Erg in embryoid bodies causes reduction of several endothelial genes including *Hey1* and *Hey2* [98], which have known roles in arterial differentiation [98-100]. Mice lacking Erg

die at E11.5, failing to initiate definitive hematopoiesis. However, blood islands and intra-embryonic vasculature are present in these mice, although severely dilated [101]. These results suggest that Erg is not required for the specification of early endothelial precursors and is consistent with loss of function studies in the zebrafish. In Erg morphant zebrafish embryos, the axial vasculature forms normally although subsequent angiogenesis and maintenance is impaired [58], suggesting Erg plays a role in later stages of endothelial differentiation maintaining vessel integrity.

Erg activates a number of endothelial cell genes in vitro including VE-cadherin, endgolin, and Von Willebrand factor (vWF) [102] and can ectopically induce Flk-1 expression *in vivo* [103]. Erg is the most expressed ETS protein in adult endothelial cells and has a role in the maintaince of endothelial cell quiescence and homeostasis by repressing NF-κB mediated activation of proinflammatory genes [86, 104]. This newly found repressive activity is of note because most ETS transcription factors are activators [51]. In addition to its role in endothelial cell maintenance, Erg expression inhibits vascular permeability by activating the tight junction protein Claudin 5 (CLDN5)[105]. In Human umbilical cord venous endothelial cells (HUVECs), shRNA knock down of ERG causes an increase in permeability with significant changes in cytoskeletal architecture [105]. Thus, *Erg* is not essential for vasculogenesis but instead plays a role during angiogenesis

perhaps by regulating cell-cell junctions and later to maintain fully differentiated endothelial cell homeostasis.

**ETS Factor redundancy**

Mutation or deletion of several ETS proteins gives a vascular phenotype and their overexpression leads to the induction of endothelial cell gene expression but these phenotypes are mild because of functional redundancy within the family. For example, homozygous null Fli1 mice die at E12.5, due to poor blood vessel integrity and hemorrhaging. Overexpression of a constitutive active from of Fli1 in zebrafish embryos causes the induction of the zebrafish homolouge of *vegf-receptor-2* (*kdrl*) in non-endothelial cells. Another ETS protein highly expressed the developing vasculature, which directly targets a number of endothelial cells genes is Ets1. In zebrafish Ets1 knockdown results in a loss of circulation phenotype with mild intersegment vessel defects, yet the majority of Ets1 homozygous mouse mutants are viable, and have no vessel defects [7, 69]. The minor phenotypic effects of Fli1 and Ets1 knockouts in the mouse is likely due to the functional redundancy between ETS factors. Several ETS transcription factors are highly expressed in the developing endothelium [49, 51, 57]. Additionally, studies have shown that different ETS factors can bind to and transactivate the same consensus sequences in promoters [59, 60]. Interestingly the major ETS proteins that effect vascular development are also evolutionarily

conserved meaning they also share similar DNA binding site affinities (Figure I-3). This ETS factor redundancy is typified by the double knockouts of the Ets1 and Ets2 genes in mice. Neither knockout alone gives a vascular phenotype but the combined mouse knockouts have are embryonic lethal between E11.5 and E15.5 and display a vascular phenotype consisting improper vessel remodeling and diminished angiogenic branching [84]. Similarly, zebrafish knockdown of four distinct ETS genes causes a much more severe vascular phenotype than knock down of any one individual ETS genes [7]. However, of all the ETS factors expressed in endothelial cells, only Etv2 is essential for the specification of angioblasts and is not redundant.

**Etv2 (Ets variant 2)**

Unlike the other ETS proteins involved in vascular biology, Etv2 has been the least studied. Currently if one searches Pubmed for Ets1, Fli1 or Erg and similar derivations thereof, the results return 1579, 1104 and 1743 articles for each gene, respectively. Similarly, if you search for Etv2, a total of 50 articles are referenced and only about half investigate its biological role, rather than simply use it as an early angioblast marker. This is in part because a role of Etv2 in vascular biology was only suggested in 2005. Additionally, Etv2 has not been found to be an oncogene or implicated in any pathological disease. Therefore, few researchers except for vascular biologists have focused on it.

Etv2 (er71 or etsrp) was first identified using degenerate oligonucleotides designed against two conserved regions within the DNA biding domain of ETS proteins, which were then hybridized against cDNA from an E8.5 mouse. This report from 1992 named the gene Er71 (ets-related 71) and described it as expressed only within the testes [106]. It took another ten years for a study to be published on Etv2. This report is the only published biochemical analysis of Etv2 and highlights several important features of the murine protein[107]. Etv2 is constitutively localized to the nucleus, mediated by a bipartite nuclear localization sequence within the C-terminal ETS DNA binding domain. The ETS DNA binding domain is able to bind to and activate transcription of the E74 and MMP-1 promoters. Both

promoters have been shown to bind other ETS proteins [108], highlighting the highly conserved nature of ETS protein DNA binding site recognition. They also found an N-terminal activation domain between residues 1-157; this domain can be fused to the GAL4 DNA binding domain and activate heterologous promoters. Three years later the same group identified and cloned the murine Etv2 promoter and after initial characterization found that it is a TATA-box-devoid promoter that is syntenic with human ETV2 and is bound by the ETS protein Sp1(PU.1) [109]. Interestingly, Sp1 is expressed mainly in hematopoietic cells but also in the germinal compartment of the testes after E12.5 [110], along with Etv2, indicating that Sp1 may activate Etv2 within these cells in a context dependent, manner but not in the vasculature.

Etv2's role in vascular development was first proposed after a microarray screen analyzing the zebrafish *cloche* mutant [111]. The *cloche* mutation affects a very early step in hematopoiesis and vasculogenesis; mutant embryos are devoid of blood cells and endothelial cells [23]. The *cloche* mutant is believed to be a loss of function mutation in the *lysocardiolipin acyltransferase* (*lycat*) gene but its mechanism of action is still unknown [112]. In a microarray study the *cloche* mutant was crossed with a transgenic zebrafish that expressed EGFP in hematopoietic cells. The expression of the transgene allowed the authors to identify homozygous mutants during early somitogenesis before they were morphologically

distinguishable, which allowed early genetic comparisons. Gene expression microarrays were performed comparing *cloche* homozygous mutants to their WT siblings. Etv2 (called etsrp at the time, for ETS related protein) was identified as a novel transcript that was significantly downregulated and expressed in the endothelium but not in *cloche* mutants [111].

Sumanas and Lin, who identified Etv2 in the microarray screen were the first to perform its characterization and functional analysis during vascular development [6]. The zebrafish Etv2 encodes a novel ETS transcription factor that shares 37% similarity to the human Ets1 protein, with 87% similarity within the DNA binding domain. Syntenic analysis revealed that Etv2 is located next to the *fli1b* gene in the zebrafish genome in opposite transcriptional orientation. The human and mouse Ets1 and Fli1 genes are in the same syntenic arrangement. Therefore, it is likely that Etv2 and Ets1 are evolutionarily related and arose through gene duplication but then functionally diverged [113]. Etv2 expression starts at the earliest somitogenesis stages in two distinct anterior and posterior populations that give rise to the hematopoietic and endothelial cell lineages. Etv2 is constitutively expressed in the endothelium until ~36hpf then becoming mostly absent except in the pronephric duct and the hematopoietic stem cell niche. We present data in Chapter II, that demonstrates Etv2 is not expressed past 24 hpf in any appreciable amount in the endothelium.

The early and endothelial cell specific expression of Etv2 suggests it may be required for vascular development. Accordingly, morpholino knockdown of Etv2 causes a defect in blood vessels morphology with a subsequent failure in circulation. This defect is caused by failure of the angioblasts to differentiate from the lateral mesoderm and migrate to the midline to form a vascular cord. Subsequently, embryos develop pericardial edema and eventually become necrotic and die. Prior to this, Etv2 morphants are morphologically normal except for the lack of a vascular system. The vascular phenotype of Etv2 morphants seems to be caused by a failure of endothelial gene expression. Expression of Flk-1, VE-Cadherin, and Flt4 (VEGFR3) is completely absent in Etv2 morphants. However, there was a minimal effect on hematopoietic cell gene expression, indicating Etv2 plays a more prominent role during endothelial differentiation in the zebrafish (Etv2's role in hematopoiesis will be discussed in more detail below). Importantly, global Etv2 overexpression is able to precociously and ectopically induce endothelial gene expression, even in non-mesodermal tissue; indicating Etv2 alone is sufficient to initiate vasculogenesis. By contrast, overexpression of other regulators of vasculogenesis such as VEGF and Scl can only induce expression of vascular markers within the lateral or somitic mesoderm [114, 115]. Since this study was published [6], a number of studies have been published corroborating and extending these initial findings. In the following

section I will discuss several important and relevant aspects of Etv2 biology found in the literature.

The expression of Etv2 is in the developing vascular and hematopoietic system is conserved in fish mammals and amphibians. In the developing mouse, Etv2 is expressed in the primitive streak, embryonic mesoderm, amnion, allantois, and yolk sac blood islands at E7.5 [8]. By E8.5, Etv2 is expressed in the endothelium of all major blood vessels including the dorsal aorta, segmental vessels, branchial arches and the endocardium [8, 116]. Etv2 expression is transient and undetectable in the mouse by E11.5. In murine embryonic stem cells, Etv2 is similarly expressed prior to expression of Flk-1 and is then downregulated upon differentiation into endothelial lineages [8]. Consistent with the mouse studies, ETV2 is not expressed in the differentiated endothelium of humans [60]. A transgenic mouse driving yellow fluorescent protein (YFP) using a 3.8 kb (kilo base) upstream proximal Etv2 promoter, faithfully recapitulates the Etv2 expression described above [117]. However, it is unclear whether transgene expression is also extinguished at later developmental stages. The authors do not present any experiments that use the line after E9.5 suggesting Etv2 may recapitulate endogenous regulation. In the developing zebrafish, Etv2 expression is detectable by in situ hybridization at the 2 ss, as two bilateral stripes in the lateral mesoderm, while at mid-somitogenesis Etv2 is expressed in hematopoietic and endothelial precursors. Etv2 expression is maintained in

angioblasts as they migrate to the midline, the nascent vascular cord during late somitogenesis and then in the endothelium of the trunk vessels. Similar to mice, zebrafish Etv2 expression is down-regulated in differentiated axial vessels, but is still visible in vessels that are newly developing (Chapter II; [6, 58]. Transgenic lines using regulatory sequences from the zebrafish Etv2 to drive expression of a florescent reporter gene mostly recapitulates endogenous Etv2 expression within the endothelium, however inappropriate expression is visible in the trunk and tail region of the neural tube and expression persists in the endothelium until adulthood [118]. Continued expression indicates the transgene promoter escapes inhibitory transcriptional mechanisms normally exerted on the endogenous gene. This is distinct from a second Etv2 transgenic [*tg(-2.3etsrp:gfp)*], where 2.3 kb of the upstream Etv2 promoter is fused to GFP [119]. In this reporter line, GFP is specifically expressed in the hemato-vascular tissue until 48hpf, suggesting transcriptional regulation of the transgene may reflect endogenous Etv2 expression. Lastly, expression of Etv2 has also been reported in *Xenopus* [120, 121], where its expressed shortly after gastrulation in the developing blood islands, angioblasts and throughout all the structures of the primitive vascular network. Consistent with reports in mouse and zebrafish, Etv2 expression in *Xenopus* is transient and mRNA levels become undetectable by tadpole stage. The transient nature of Etv2 expression during vascular development compared to other endothelial expressed ETS transcription

factors suggest that down-regulation of Etv2 is caused by an active inhibitory mechanism (see chapters III and IV). In fact, down regulation of Etv2 expression is required, because persistent expression in the mouse is deleterious to vascular development and endothelial maturation [122].

Loss of Etv2 function prevents the formation of the cardiovascular system. A large scale mutagenesis screen looking for vascular phenotypes using zebrafish *Tg(fli1:egfp)*$^{y1}$ transgenics recovered an Etv2 mutant, *tg(fli1:egfp)*$^{y11}$, where a premature stop codon eliminates 3/4 of the endogenous protein including the essential c-terminal ETS DNA binding domain and essentially recapitulates genetic downregulation of Etv2 [6, 7]. Zebrafish Etv2 morphants and mutants do not express endothelial genetic markers including Flk-1 and as result fail to form a vascular system. To date, mice with targeted deletion of Etv2 display the most severe vascular phenotypes reported for any vascular gene, including Flk-1 and other ETS factors, demonstrating its essential position in the genetic hierarchy controlling the endothelial cell lineage [8]. Etv2 homozygous mutant mice fail to express Flk-1, which the authors demonstrate can be directly activated by Etv2. These mice also fail to express genes associated with endothelial cell identity and consequently die at ~E9.5 due to the complete loss of embryonic and extraembryonic blood and vascular structures, including the endocardium of the heart [116]. Consistent with the mouse and zebrafish studies, *Xenopus* Etv2 morphants also fail to form vascular structures and to initiate endothelial

marker gene expression. The role of Etv2 during vascular differentiation is conserved between species but its role in hematopoiesis is not.

Vertebrate hematopoiesis proceeds in two waves. Primitive hematopoiesis serves as a transient early source of limited blood cell types, while definitive hematopoiesis contributes to all adult blood cell lineages [123]. In the mouse Etv2 is required for primitive hematopoiesis and specification of all blood cell lineages. As a result, blood cells are absent in both embryonic and extraembryonic structures of Etv2 mutant null mice after benzidine staining at E9.0. Additionally, no erythroid colonies formed when E8.5 yolk sacks were analyzed by hematopoietic replating, demonstrating the absence of hematopoietic progenitors [8]. Furthermore, Etv2-devoid embryonic stem cells fail to differentiate any hematopoietic cell types. The lack of hematopoietic and endothelial gene expression in Flk1-negative, Etv2-devoid, hematopoietic progenitors can be partially rescued by the expression of exogenous Etv2 [124]. This ability of Etv2 to *de novo* initiate hematopoiesis is caused in part by the direct activation of Flk-1 and Scl, which are critically required for blood cell formation [34, 61, 124-126]. Unlike in the mouse, zebrafish Etv2 is only required for the myeloid but not the erythroid cell lineages during hematopoiesis [127]. Etv2 works upstream of Scl during hematopoiesis and Etv2 morphants fail to initiate expression of genes required of definitive hematopoiesis [127, 128]. Interestingly, loss of Etv2 in *Xenopus,* has no effect on hematopoiesis with all blood cell lineages

developing normally although over expression of Etv2 is able to induce myeloid but not erythroid cell lineages [120]. In conclusion, these data suggest that unlike vascular development, Etv2 function is only partially conserved during vertebrate hematopoiesis.

Consistent with its role in cardiovascular system development and its ability to induce hemato-vascular gene expression, ubiquitous Etv2 mRNA expression causes ectopic induction of endothelial marker genes [6]. Specifically, zebrafish or mouse Etv2 mRNA is able to induce the expression of Flk-1 and Scl in WT and *cloche* mutant zebrafish embryos [6, 127]. Similarly, forced expression of Etv2 in Xenopus embryos or in mouse embryonic stem cells injected with Etv2 mRNA result in ectopic induction of endothelial cell gene expression[8, 120, 124]. However, continued expression of the usually transient expressed Etv2 in mice using the Tek promoter disrupts vascular development by preventing vascular maturation, where endothelial cells retain an immature gene expression profile. In this study mice are severely anemic, because constitutive Etv2 expression induces an endothelial program on hematopoietic cells [122]. Microarray and deep sequencing analysis of Etv2 overexpressing zebrafish embryos results in transcriptional up-regulation of hundreds of endothelial cell genes [129-131]. Some of the genes in these studies were previously unidentified endothelial genes, suggesting global induction of the endothelial gene program. We performed a similar microarray study and our results are

consistent with these published reports (Chapter II). Overall, these studies across species indicate that Etv2 has a conserved function as a potent activator of endothelial gene transcriptional programs.

The literature referenced thus far clearly demonstrates a role for Etv2 in endothelial cell transcriptional program activation. But what turns on Etv2? There is no definitive answer and only a limited amount of research has been dedicated to answering this question. In embryonic stem cells, addition of chemical inhibitors for the bone morphogenetic protein (BMP), Notch and Wnt, but not the Hedgehog families result in the reduction of Flk-1 positive hemato-endothelial cells [8]. The combined addition of these inhibitory chemicals had a more dramatic affect on cell number than any one chemical alone. The chemical inhibition of Flk-1 positive mesodermal cells was concomitant with the downregulation of normalized expression of Etv2, suggesting these signaling pathways directly or indirectly regulate Etv2 gene expression. Considering the regulatory breadth of these three signaling pathways, it is not surprising that a transcriptional activator acting on the Etv2 gene was not identified in this study. Recently, the homeobox transcription factor Nkx2-5 was found to directly bind and activate Etv2 transcription and Nkx2-5 mutant mice do not express Etv2 in the heart [116]. Nkx2-5 is the earliest expressed genes in the cardiac lineage and its deletion in mice perturbs heart morphogenesis and prevents the formation of the endothelial derived endocardial cushion, resulting in embryonic lethality at E9.5 [132,

133]. Although Nkx2-5 is likely a bona fide Etv2 transcriptional activator, it is specifically expressed within the developing heart field and therefore not likely to be responsible for Etv2 activation in the entire endothelium during embryogenesis. Another possible candidate for Etv2 expression in the endothelium are the members of Forkhead (Fox) transcription factor family, specifically murine Foxc1 and Foxc2 and their zebrafish homologs *foxc1a* and *foxc1b* (foxC1a/b). The murine and zebrafish Foxc genes are expressed in the developing vasculature although not exclusively [134, 135]. *Foxc1a/b* genes bind to a conserved endothelial enhancer in the Etv2 locus by EMSA and ChIP in the zebrafish and deletion of this enhancer region significantly reduces transgene expression [119]. Additionally, mice composite ETS:FOX DNA binding motifs strongly predict endothelial target genes from the genome and Foxc1/Etv2 synergistically and directly activate endothelial gene expression, suggesting Etv2 and Foxc cooperatively activate transcription during vascular development [61]. It is possible that Foxc1 maintains or enhances Etv2 transcriptional activation instead of being its sole initiator, because double Foxc1/Foxc2 homozygous mice have a much less severe vascular phenotype than Etv2 mutants [134]. Hematopoiesis and vascular development proceeds normally in Foxc1/Foxc2 homozygous mutant mice, unlike Etv2 mutants, which fail to form cell types associated with cardiovascular development [8, 134]. Not surprisingly, considering their seemingly ubiquitous role in endothelial gene expression, ETS proteins can

directly activate Etv2 transcription. For instance, Etv2 is directly activated by the ETS factor Spi1 (Pu.1), a gene essential for the development of the myeloid lineage [109, 136, 137]. However, Spi1 is only expressed in hematopoietic cells and therefore its activation would not explain the initial expression of Etv2 within the endothelium. Interestingly, Etv2 has been shown to activate its own expression when overexpressed in Zebrafish [129]. This opens up the possibility that alternative ETS members could activate Etv2, considering the substantial gene activation overlap of the family. Etv2 activation is downstream of at least three major signaling pathways and is directly activated by three separate families of transcription factors. The complexity of Etv2 activation mirrors the complexity of its down-regulation (discussed in chapters III and IV). It will be interesting to see how many more players are found and how they may cooperatively interact to activate Etv2 transcription and thus, vascular development.

## Project Goals

The non-autonomous molecular signaling mechanisms that drive the process of vasculogenesis and angiogenesis have largely been elucidated. However, the transcription factors that drive angioblast specification are not well understood. Research surrounding the ETS transcription factor *etv2,* has led to the idea that it is the master regulator of endothelial cell lineage commitment. That is because loss of *etv2* causes severe cardiovascular defects, with a failure to specify angioblasts consistently; overexpression precociously and ectopically induces endothelial gene expression. However, the expression dynamics of *etv2* and its regulation have not been fully investigated. In order to increase our understanding of *etv2* function during vascular development, I conducted detailed expression studies in embryonic zebrafish. Additionally, I used a conditional loss of function approach to determine the developmental time window for Etv2 function to more clearly defining the requirement for etv2 during vascular development. I also investigated how *etv2* expression is regulated by examining both post-transcriptional and post-translational mechanisms.

**Questions addressed in the thesis:**

- When is *etv2* expressed during development of the zebrafish?
- When is *etv2* functionaly required during development?
- What is the role of Etv2 during vascular differentiation and what are its transcriptional targets?
- Is *etv2* post-transcriptionally or post-translationaly regulated? If so,what are the mechanisms?

# CHAPTER II: ETV2 IS THE MASTER REGULATOR OF ENDOTHELIAL CELL FATE.

A portion of the work contained within this chapter was submitted for publication:
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## Introduction

The morphological events defining vasculogenesis and angiogenesis are well defined but the transcriptional regulation of angioblast specification and their eventual differentiation into endothelial cells is poorly understood. Multiple transcription factor families have been implicated in the activation and maintenance of endothelial gene expression, including members of the Sox, Fox, GATA, and KLF families [49]. To date, no single transcription factor family seems to regulate the endothelial cell transcriptional program as extensively as the ETS family of transcription factors. Several ETS transcription factors are highly expressed in the developing endothelium including Ets1, Ets2, Etv2(etsrp/er71), Etv6(Tel), Fli1, Erg and Elk3(Net/Sap2) [49, 51, 57]. Among these, only Etv2 has no redundant function and is essential for the specification of angioblasts (detailed in Chapter I).

Etv2 is required for vascular morphogenesis and its expression is detectable at the onset of vasculogenesis. In mice Etv2 is expressed in the blood islands, extra-embryonic vessels and in primitive vessels of the embryo early during development [8, 116]. Similarly, Etv2 expression in the zebrafish is detectable at the 2 somite stage (ss) at the initiation of angioblast specification in the lateral plate mesoderm [6]. Etv2 knockout mice fail to specify hemangioblast cells from the mesoderm manifesting in a complete lack of hematopoietic and endothelial cell lineages and die at E9.5 [8, 116]. Zebrafish Etv2 mutants and morphants have complementary phenotypes that

mirror that of the mouse, in that they fail to properly form the vascular system [6, 7, 111]. However, in the zebrafish Etv2 seems to play a more important role in vasculogenesis than hematopoiesis, compared to the mouse [127]. Loss of Etv2 expression coincides with a complete absences of endothelial cell gene expression including, *kdrl*, *flt4*, *cdh5*, and *plxnd1* [6-8, 116]. Global overexpression of mouse and zebrafish Etv2 in zebrafish embryos leads to the expansion of both hematopoietic and endothelial cell lineages [127]. Additionally, Etv2 overexpression leads to the induction of hundreds of vascular and myeloid genes, some of which are activated by direct promoter or enhancer binding [61, 116, 124, 129-131, 138]. The role of Etv2 as transcriptional initiator and as endothelial cell lineage determinant is exemplified by research demonstrating that Etv2 in combination with Fli1 and Erg can transdifferentiate amniotic fluid cells into reprogrammed vascular endothelium cells [139].

Etv2 is expressed only early during mouse development up to E9.5 and expression in the zebrafish axial vasculature is downregulated by 36hpf [6, 8, 116]. This suggests that Etv2 has only an early role in development and is then dispensable. Interestingly, mouse embryos are viable following conditional endothelial ablation of Etv2 using a Flk1:Cre driver[124]. Flk-1 is required for angioblast specification but is induced by Etv2, suggesting that Etv2 function is restricted to very early stages of vascular development [124, 140]. Although previous studies have suggested dynamic control of *etv2*

expression during embryogenesis [8, 116, 124, 125, 140], little is known about its expression dynamics throughout development. We sought to perform carefully quantified, staged studies to define Etv2 expression during zebrafish vascular development while further investigating its role in endothelial differentiation by defining transcriptional targets.

Here we describe the expression of Etv2 transcript and protein during zebrafish vascular development. Both mRNA analysis and immunohistochemistry reveal that Etv2 is expressed in the primitive vasculature of developing zebrafish at 18 hpf and is strongly downregulated in differentiated endothelial cells by 24hpf. Conditional knockdown of Etv2 using a caged morpholino defined a functional window of Etv2 during vascular development that is shorter than it's expression window and suggests Etv2 functions to specify angioblasts from the lateral plate mesoderm. Accordingly, mosaic transplant analysis reveal that Etv2 overexpressing cells are much more likely to contribute to the vasculature than cells injected with a control mRNA. We then present microarray analysis as evidence that Etv2 sits atop a transcriptional hierarchy controlling endothelial cell lineage determination.

## Materials and Methods

### Zebrafish Handling and Maintenance

Zebrafish and their embryos were handled according to standard protocols [141] and in accordance with the University of Massachusetts Medical School IACUC guidelines. *Tg(fli1a:egfp)*$^{y1}$, *Tg(fli1a:negfp)*$^{y7}$, *Tg(fli1a.ep:DsRedEx)*$^{um13}$ and *Tg(kdr:grcfp)*$^{ZN1}$ lines have been described previously [12, 16, 142, 143].

### Plasmid Construction.

The *etv2* open reading frame was amplified from 24 hpf whole embryo cDNA and used in a BP recombination reaction with plasmid pDONR221 (Invitrogen) to make pME-*etv2* (primer sequences are in Appendix II). pME-*etv2*, or pME-*mcherry* [144] were used in LR reactions with pCSDest or pCSMTDest [145] to generate pCS-*etv2*, pCSMT-*etv2*, and pCS-*mCherry*. pCS-*EGFP* has been described [146].

### Antibody production

The Fli1b antibody has been previously described [15]. A fragment encoding the N-terminal 218 amino acids of zebrafish Etv2 was amplified from 24 hpf zebrafish cDNA (primer sequences are in Appendix II), cloned into pCR2.1 by TOPO cloning (Invitrogen), and sequence verified. The *etv2* fragment was

subcloned into pGEX-6P-1 using BamHI and XhoI sites. pGEX-*etv2* was transformed into BL21(DE3) *e. coli* and glutathione S-transferase (GST) fusion protein expression was induced with IPTG. Expressing bacteria were lysed using Bug Buster (Novagen), and proteins were purified using Glutathione Sepharose 4B (GE Healthcare), followed by release of the Etv2 fragment and removal of the GST using PreScission Protease (GE Healthcare). Purified Etv2 protein was used for rabbit polyclonal antibody production (Caprologics, Gilbertville, MA). Etv2 antiserum was validated using Western analysis of lysates from HEK293T over-expressing myc-tagged zebrafish Etv2 and EGFP. The myc epitope was detected using a 1:10,000 dilution of anti-myc monoclonal antibody (Sigma, 9E10) and Etv2 protein was detected using a 1:5,000 dilution of anti-Etv2 polyclonal antibody serum. The EGFP protein was detected using a 1:10,000 dilution of GFP polyclonal antibody (Invitrogen, A11122)

**Whole mount immunohistochemistry**

Staged zebrafish embryos were fixed overnight at 4°C in 2% paraformaldehyde (w/v) dissolved in phosphate buffered saline containing 0.1% Tween-20 (PBSTw). Embryos were washed 4 times for 5 minutes at room temperature in PBSTw and in PBS containing 0.5% TritonX-100 (PBSTw) for 30 minutes. Embryos were blocked for a minimum of 2 hrs in blocking solution (PBSTw, 0.1% TritonX-100, 10% normal goat serum, 1%

BSA, 0.01% sodium azide) at room temperature. Fli1b and Etv2 rabbit polyclonal serum was diluted 1:1000 and 1:500, respectively, in blocking solution and embryos incubated over night at 4°C. Embryos were washed 6 times in PBSTw for at least 4 hrs at room temperature and then incubated overnight with Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen) anti-rabbit secondary antibody diluted 1:1000 in blocking solution. Immunostained embryos were imaged on a LSM7 MP microscope (Zeiss; Objective: 20x/1.0 DIC(UV) VIS-IR 421452-9800) equipped with a Chameleon Ti:Sapphire pulsed laser (Coherent, Inc.). Alexa Fluor 488 and Alexa Flour 568 were alternatively excited at 904 nm and 1057 nm, respectively, on each section during image acquisition.

**Morpholino injections**

The Etv2 caged morpholino (cMO) used in this study has been previously reported [147]. 230 fmol (~2 ng) of Etv2 cMO was injected into *Tg(fli1a.ep:DsRedEx)*$^{um13}$ embryos at the 1-cell stage. Embryos were subjected to UV illumination for 10 seconds at indicated stages using a Zeiss Axioskop2 Plus compound microscope with a DAPI filter and an Achroplan (Zeiss) 20x water immersion objective. Following photoactivation, embryos were grown in egg water at 28.5°C. Control embryos were left in the dark. 5 ng of scrambled control or 5ng Etv2 MO [6] were injected as negative and positive controls, respectively. Vascular morphology was assessed at 30 hpf.

The standard Etv2 MO and scrambled control MO were used to test the specificity of the Etv2 polyclonal antibody in *Tg(fli1a:negfp)*$^{Y7}$ embryos at 18hpf after Etv2 whole mount immunohistochemistry at the same concentractions. Embryos were imaged using an MZFLIII fluorescent dissection microscope or using a using a Leica DMIRE2 confocal microscope (Objective: HC PL APO 20x/0.70CS). Circulatory defects were observed using a MZ12 stereomicroscope (Leica) and captured with a DMK21F04 camera (Imagesource) using Quicktime Pro or iMovie.

**Quantification of endothelial gene expression**

mRNA was quantified using the NanoString nCounter gene expression system (Nanostring Technologies, Seattle, WA) [148]. Total RNA was isolated from embryos at indiciated time points using a Qiagen RNAeasy kit. For each experiment, 100ng of total RNA was hybridized for 12 to 20 hrs with the Nanostring probeset (Appendix III) at $65^0$C in a thermocycler. Samples were then loaded into the nCounter prep station and fluorescence signal was quantified using the nCounter Digital Analyzer. Gene normalization and fold change calculations were done using Nsolver Analysis Software (Nanostring Technologies). In all cases, biological triplicates were performed and gene counts were normalized to *eukaryotic translation elongation factor 1 alpha 1 like 1* (*eef1a1l1*) and *actin, beta 2* (*actb2*). The average normalized gene

count was plotted and error bars represent the Standard Error of the Mean (SEM).

**mRNA synthesis and injections**

Capped mRNA was synthesized from pCS plasmids that had been linearized with NotI using the SP6 mMessage mMachine kit (Ambion). mRNAs were injected into 1-cell stage embryos according to standard protocols [141]. For the Etv2 overexpression microarray 50pg of *Etv2* or *mCherry* mRNA was injected into *Tg(fli1a:egfp)*$^{y1}$ embryos and RNA isolated at shield stage.

**In situ hybridization**

An antisense DIG-labeled *etv2* riboprobe was synthesized by linearizing pCS2-*etv2* with EcoRI followed by in vitro transcription using T7 polymerase. Whole mount *in situ* hybridization was performed according to standard protocols [149].

**Mosaic analysis**

*Tg(fli1a:egfp)*$^{y1}$ embryos were used as donors in all cases and 0.35% miniRuby (dextran, tetramethylrhodamine and biotin 10,000MW) (Invitrogen D-3312) was co-injected as a lineage tracer. To assess the effect of Etv2 overexpression, we injected 100pg of *myc-etv2* or *mCherry* mRNA into 1-cell stage donor embryo. At sphere stage, approximately 20 cells were transplanted from the ventral blastoderm margin of donors into wild type

hosts, which were subsequently screened at 30 hpf for the appearance of red and green fluorescence. Embryos were imaged using an MZFLIII fluorescent dissection microscope or using a using a Leica DMIRE2 confocal microscope (Objective: HC PL APO 20x/0.70CS). The proportion of successfully transplanted embryos (i.e. exhibiting miniRuby-positive cells in any trunk tissue) with contribution to blood vessels was determined in three separate experiments and significance was calculated by Fisher's Exact test. $p < 0.05$ was deemed significant.

**Embryo dissociation and Fluorescence Activated Cell Sorting (FACS)**

Wild type *Tg(kdr:grcfp)*$^{ZN1}$ embryos were grown to 24 hours post fertilization(hpf) in Egg water and dechorinated by Pronase . Embryos were washed several times in calcium free Ringers (116mM NaCl, 2.9mM KCl, 5mM HEPES, pH 7.2) and passed through a 200uL pipette tip to remove the yolk sack and rewashed in calcium free ringers. Embryos were transferred into a 35mm culture dish containing 2mL of Protease solution(Phosphate Buffered saline[PBS], 1mM EDTA, 0.25% Trypsin, pH 8) and incubated for 30-60 minutes at 28.5$^0$C with occasional trituration. Upon visualization of a single cell suspension, dissociated embryos were washed three times in 15mls of suspension medium (Colorless L-15 media[Gibco], 0.8mM $CaCl_2$, 50 U/mL penicillin, 0.0 5 mg/mL, 1% fetal calf serum). Cells were centrifuged for 3 min. at 3000rpm, in between washes. After the final wash, cells were

suspended in resuspension media at approximately $10^7$ cells/mL. FACS of single cell suspensions was performed at room temperature under sterile conditions using a FACSVantage SE /DIVA (BectonDickinson) with a Coherent Innova 70 laser at 488nm and 200mW power. GFP+ and GFP− cells were separately collected in collection media(L15, 0.8mM $CaCl_{2,}$50 U/mL penicillin, 0.0 5 mg/mL, 10% fetal calf serum) Equal numbers of GFP+ and GFP- cells were centrifuged and resuspended in 250µL of Trizol Reagent (Invitrogen) and stored at -80$^0$C until all samples were collected.

**RNA Isolation and microarray hybridization**

RNA was isolated using Trizol Reagent according to manufacturer's instructions. RNA pellets were DNAse treated, phenol chloform extracted and resuspeneded in 20µL of RNase free water (Ambion). For GFP+ and GFP- cell sorted RNA samples 825ng/sample of RNA was amplified using Ambions MessageAmp II aRNA Kit (Cat#: AM1751). Etv2 overexpression and control RNA samples were handled in the same manner except RNA was not amplified. All GFP+ and GFP- aRNA (amplified RNA) generated and 5µg Etv2 overexpressing whole and control RNA was given to the UMass Medical School Genomics Core for sample hybridization to Affymetrix (Santa Clara, CA) Zebrafish GeneChips as per manufacturer's instructions. GFP+ cell population's gene expression levels were compared to GFP- populations and

Etv2 overexpressing gene expression was compared to mCherry control. All comparisons where performed in triplicate. Gene expression as analyzed using the RMA method [150] in the Affy package from Bioconductor was used in R to summarize the probe level data and normalize the dataset to remove across array variation. Log transformed data was used in the subsequent analysis. Limma package from Bioconductor [151] with randomized block design (n=3) was used to determine whether a gene¹s expression level differs between treatments. Genes with adjusted p-value using B-H method (Benjamini & Hochberg 1995) < 0.05 and fold change >= 2 were considered significant.

## Results

*Etv2 transcript is transiently expressed*

Based on previous studies that suggested dynamic control of *etv2* expression during embryogenesis, we carefully investigated its expression during zebrafish vascular development [8, 116, 124, 125, 140]. To examine, the dynamics of *etv2* expression, we first applied the NanoString nCounter gene expression system assay to quantitatively measure *etv2* transcript levels during embryogenesis. Using this approach, we observed that *etv2* expression increases between tail bud and 10 somite stage (ss). *Etv2* RNA levels peak at 18ss and it is expressed nearly 2 fold greater than endothelial transcripts encoding *fli1a, fli1b*, and the zebrafish Vegf receptor-2 ortholog, *kdrl*. *Etv2* expression drastically decreases by 24 hpf and is expressed five fold below *kdrl* transcripts at 48hpf (Fig. II-1A). By contrast, *fli1a*, *fli1b*, and *kdrl* transcripts continued to modestly increase from 10 hpf until 48 hpf (Fig. II-1A). Thus, the *etv2* transcript displays an initial burst of expression during the time in which endothelial specification and vasculogenesis are taking place [12] , but is subsequently downregulated towards the onset of angiogenesis.

We next wanted to determine if Etv2 protein had a similarly dynamic expression pattern as its transcript. We therefore raised a rabbit polyclonal antibody that specifically recognized the divergent N-terminal domain of Etv2, because the C-terminal located DNA binding domain of many ETS

transcription factors share strong sequence homology [106]. The Etv2 polyclonal serum recognizes a single band in HEK293T lysates overexpressing a myc-tagged version of zebrafish Etv2. The same sized band is also detected following immunodetection for the myc-epitope using an anti-c-myc monoclonal antibody (Fig. II-1B). Additionally, Etv2 antibody staining is clearly visible in angioblasts marked by the *fli1a:egfp* transgene in embryos injected with control MO, but absent in embryos injected with 5ng of an Etv2 translation blocking morpholino (Fig. II-1C). Therefore the Etv2 polyclonal serum specifically recognizes zebrafish Etv2.

**Figure II - 1**

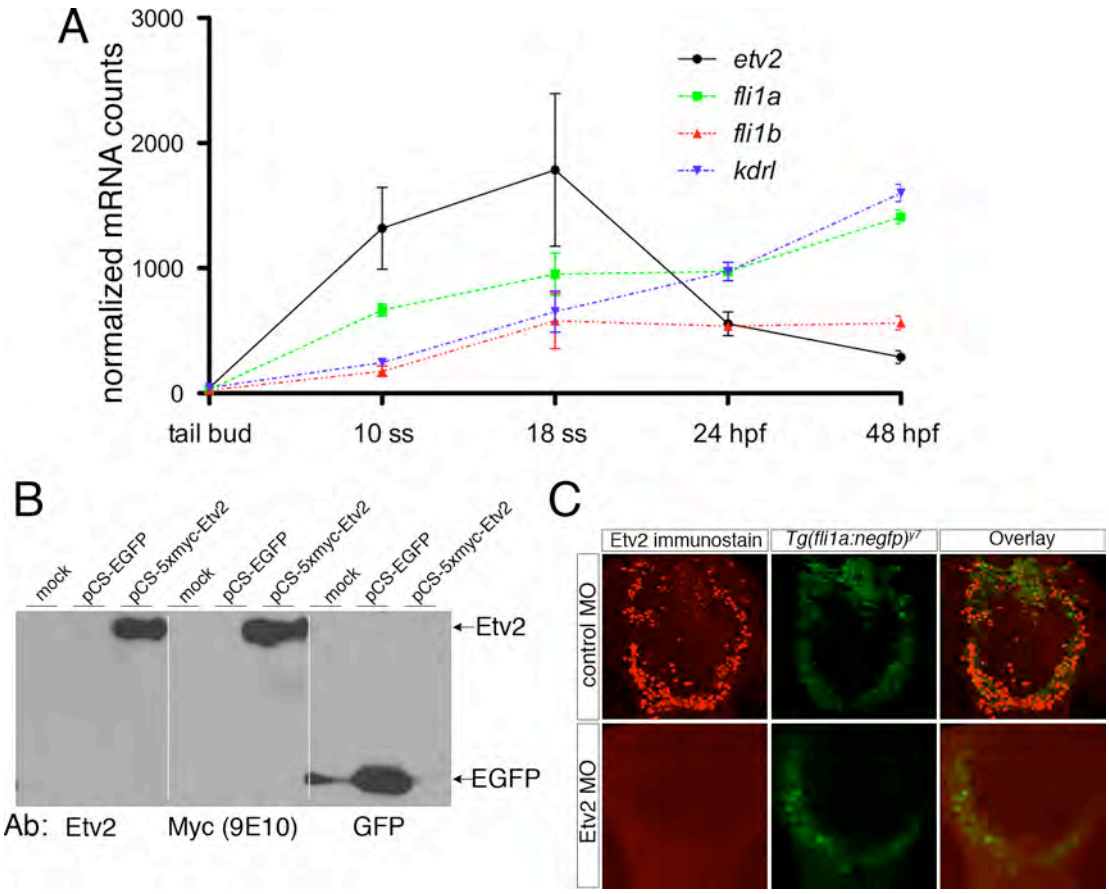


**Figure II – 1: Etv2 transcript is downregulated during vascular development and a polyclonal antibody to zebrafish Etv2 is specific**. (A) nCounter quantification for *etv2*, *fli1a*, *fli1b*, and *kdrl* at the indicated developmental stages. Values are normalized to *actb2* (*beta-actin*) and *eef1a1l1*(*ef1alpha*). (B) SDS-PAGE gel of HEK293T lysates transfected with mammalian expression vectors for EGFP (pCS-EGFP), myc-tagged zebrafish Etv2 (pCS-5xmycEtv2), or left untransfected (mock). Lysates from each sample were run on triplicate immunoblots, which were individually probed with Etv2 polyclonal antiserum, a monoclonal against the myc-epitope (9E10), or a polyclonal against GFP. (C) *Tg(fli1a:negfp)*$^{y7}$ embryos at 18 hpf injected with 5 ng of control or Etv2 MO followed by immunostaining using Etv2 polyclonal serum and Alexa-568 secondary. View, dorsal is up, facing posterior end of tail.

*Etv2 protein is transiently expressed*

We used the anti-Etv2 serum to perform whole mount immunostaining on zebrafish embryos during early embryonic stages. Similar to *etv2* transcript, we observed Etv2 protein in the anterior and posterior lateral mesoderm within nuclei of presumptive endothelial progenitors at the 5ss (Figs. II-2 A, B) and during initial formation of the vascular cord in the trunk at 18ss (Fig. II-2C, D). However, Etv2 protein levels were drastically decreased by 24 hpf and were undetectable in endothelial cells lining blood vessels by 48 hpf (Fig. II-2E, F; red channel), while an endothelial-expressed nuclear localized EGFP (*Tg(fli1a:negfp)*$^{y7}$) was easily detectable at both stages in the same embryos (Fig. II-2E, F; green channel). Furthermore, we observed robust expression of Fli1b protein in endothelial nuclei of *Tg(fli1a:negfp)*$^{y7}$ embryos at the same time points (Figs. II-2G, H). Although vascular expression of Etv2 is mostly diminished by 24hpf (Fig. II-2E), Etv2 protein staining is detectable in a subset of cells posterior to the caudal vein plexus composing the hematopoietic niche (Fig. II-2I), consistent with Etv2s role in maintaining hematopoietic stem cells [152]. Taken together, these observations demonstrate that *etv2* transcript and protein are expressed during early endothelial specification and vasculogenesis, but are subsequently downregulated as development proceeds.

**Figure II - 2**

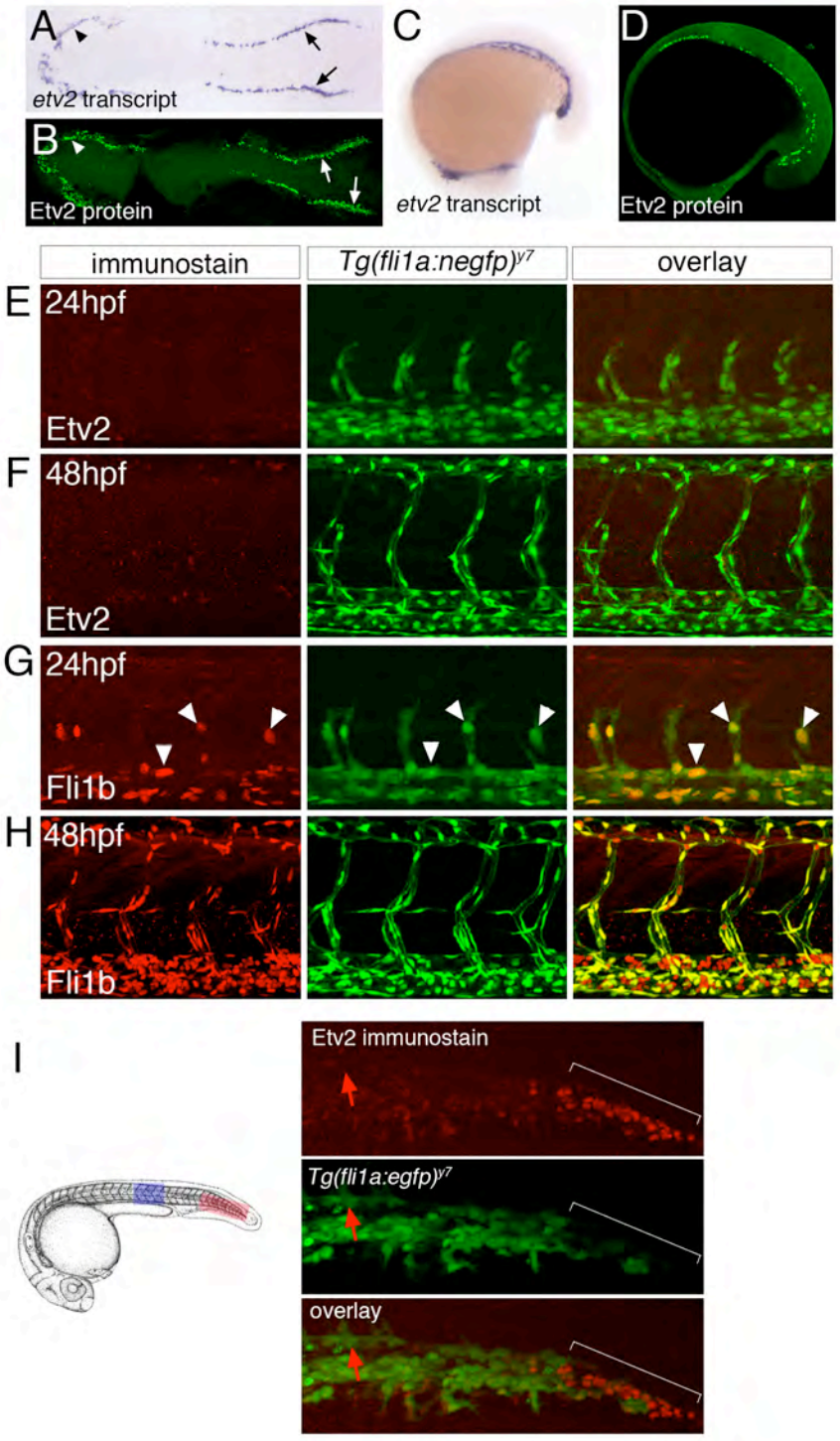
A
etv2 transcript
B
Etv2 protein
C
etv2 transcript
D
Etv2 protein
immunostain
Tg(fli1a:negfp)y7
overlay
E
24hpf
Etv2
F
48hpf
Etv2
G
24hpf
Fli1b
H
48hpf
Fli1b
I
Etv2 immunostain
Tg(fli1a:egfp)y7
overlay

**Figure II – 2: Etv2 protein is downregulated during vascular development.** (A, C) Whole mount *in situ* hybridization using an antisense *etv2* riboprobe at 5ss and 18ss. (B, D) Embryos at 5ss and 18ss immunostained with Etv2 antibody and anti-rabbit Alexa-488. (A, B) Dorsal views of flat mounted embryos, anterior to the left. (C, D) Lateral views, anterior to the left. (E-H) Two-photon micrographs of trunk vessels in fixed *Tg(fli1a:negfp)*$^{y7}$ embryos immunostained with antibodies against Etv2 (E, F) or Fli1b (G, H). Left panels, immunostained protein detected with Alexa-568 secondary antibody. Middle panels, transgenic expression of nuclear localized EGFP. Right panels, overlay of Alexa-568 and EGFP signals. Embryos at 25 hpf (E, G) or 48 hpf (F, H), arrows indicate double positive cells. (I) Left, Camera Lucida drawings of embryo at approximately 24 hpf, blue boxed area demarcates area imaged in (E-H), red box area in (I, right). (I) Bottom, immunostaining of an *Tg(fli1a:egfp)*$^{y1}$ embryo with Etv2 polyclonal serum and alexa-568 secondary at 24hpf. Faint Etv2 expression can be observed in many EGFP-positive cells within the caudal vein plexus, while strong Etv2 expression is apparent in a separate EGFP-negative population of cells (indicated by a white bracket). Etv2 expression is not detectable in the dorsal aorta at this time point (red arrows).

*Etv2 is required early for endothelial lineage commitment but is dispensable for later vascular development*

The dynamic expression of *etv2* suggested that its function might only be required during early stages of vascular development. To investigate this possibility, we utilized a caged Morpholino (cMO) that is activated by exposure to UV light to conditionally block Etv2 translation at different developmental stages [147, 153]. We injected *etv2* cMO into 1-cell stage *Tg(flia:DsRedex)*$^{um13}$ zebrafish embryos, exposed them to UV light at distinct developmental stages, and subsequently assessed vascular morphology and function. In control *Tg(flia:DsRedex)*$^{um13}$ embryos injected with scrambled control morpholino (MO) we observed normal vascular morphology at 30 hpf and normal circulation at 48 hpf (Data not shown and Fig. II-3E – 1$^{st}$ Bar, Supp. Movie 1). By contrast, embryos injected with a standard Morpholino targeting *etv2* exhibited loss of intersegmental vessels and a poorly formed dorsal aorta at 30 hpf and no circulation at 48hpf (Fig. II-3A, E – 2$^{nd}$ Bar, Supp. Movie 2). *Tg(flia:DsRedex)*$^{um13}$ embryos injected with Etv2 cMO that were not exposed to UV light, or those that were uninjected and exposed to UV at the indicated stages, were phenotypically normal (Fig. II-3B, E – 3$^{rd}$ bar, data not shown, Supp Movie 3). However, embryos injected with Etv2 cMO and exposed to UV light at 11 hpf or earlier exhibited defects in vascular morphology and loss of circulation (Fig. II-3C-E-4$^{th}$-6$^{th}$ bar, Supp. Movie 4), similar to embryos injected with an uncaged Etv2 MO (Fig. II-3B) or *etv2*$^{y11}$

mutant embryos [7]. In all cases, we did not observe any overt effects on general morphology (data not shown). Many fewer Etv2 cMO-injected embryos exposed to UV light at 12 hpf displayed defects in circulation and UV activation at later time points did not cause any defects (Fig. 3E-7$^{th}$—10$^{th}$ bar, Supp. Movie 5). Thus, the requirement of Etv2 for embryonic blood vessel formation is restricted to a defined early window during vascular development and suggests that Etv2 is required for initial angioblast specification. Furthermore, inappropriate expression of Etv2 can ectopically induce endothelial gene programs [6]. Consistent with this possibility, mosaic transplant analysis of Etv2 overexpressing cells significantly enhanced the ability of donor *Tg(fli:egfp)*$^{Y1}$ cells to contribute to endothelial and hematpoietic lineages when compared to donor cells from embryos injected with *mcherry* mRNA (Fig. II-3F). Cells from either mCherry or Etv2 mRNA injected hosts are able to contribute equally to any cell type (Fig. II-3G).

**Figure II - 3**

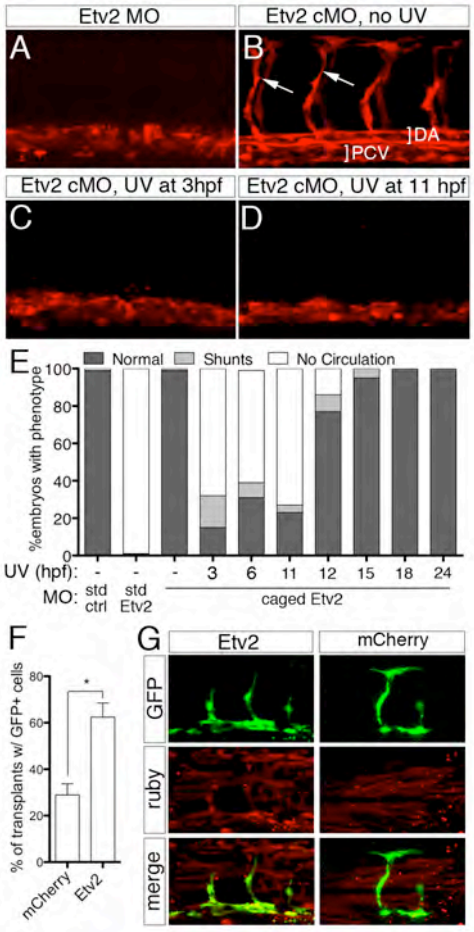


**Figure II – 3: Etv2 is required only during early stages of vascular development.** (A-D) Confocal images of trunk blood vessels in *Tg(fli1a.ep:DsRedex)*$^{um13}$ embryos at 30hpf. Lateral views, dorsal is up, anterior to the left. Embryos injected with (A) 5 ng standard Etv2 Morpholino (MO) or (B) 2 ng caged Etv2 MO, but not illuminated with UV light. ISVs (arrows), dorsal aorta (DA; bracket) and posterior cardinal vein (PCV; bracket) are indicated. (C, D) Embryos injected with Etv2 cMO exposed to UV light at (C) 3hpf or (D) 11hpf. (E) Penetrance of indicated circulatory defects in embryos at 48hpf following injection with MO and UV exposure as indicated. (F) Proportion of mosaic miniRuby-positive host embryos showing successful transplantation of *Tg(fli1:EGFP)*$^{y1}$ donor cells. Donor embryos were injected with 100 pg of *mcherry* or *etv2* mRNA. *$p < 0.05$. (G) Representative confocal images of wild type hosts with contribution to both vascular (green) and non-vascular (red) tissue.

*Etv2 induces endothelial transcription factors*

Etv2 is required early for angioblast specification and is a strong transcriptional activator, so to better understand the transcriptional targets of Etv2 we performed two comparative gene expression analyses. The first study was to define genes enriched in the endothelial cells and the second sought to identify genes induced by Etv2 overexpression. The *Tg(kdr:grcfp)*$^{ZN1}$ transgenic lines expresses GFP in the endothelial cells lining the blood vessels (Fig. II-4A; [143]). This line can be dissociated into a single cell suspension using trypsin and cells fluorescent activated cell sorted (FACS) to yield a 90-95% pure population of GFP-positive cells (Fig. II-4B, C; data not shown). RNA was isolated from GFP-positive cell populations and GFP-negative populations and then hybridized to Affymetrix zebrafish GeneChips. The gene expression profiles from the two cell populations where compared and 448 genes where enriched (Log2 ≥ 1) in the GFP-positive endothelium compared to the rest of the embryo (Fig. II-4E). To find genes induced by Etv2, we injected *etv2* mRNA or *mCherry* mRNA as a control into embryos, allowed them to develop until shield stage and then isolated RNA from each sample to compare gene expression profiles by microarray (Fig II-4D). We chose to isolate RNA from shield stage embryos in an attempt to find direct targets of Etv2 activation. Our analysis revealed that 74 genes are induced upon Etv2 overexpression and 27 of them are also enriched (Log2 ≥ 1) in the endothelium (Fig. II-4E). Interestingly, if you take

the top ten genes that are induced by Etv2 that are also enriched in the endothelium, five of them are transcription factors (Table II-1). Additionally, Etv2 induced transcription factors *scl, lmo2* and *hey2* have all been shown to play a role in endothelial cell differentiation [154-156]. Our microarray results are consistent with similar studies that demonstrate Etv2 induces a number of transcription factors known to be important for endothelial or hematopoietic cell differentiation [130, 131, 157]. Accumulatively, the data suggest that Etv2 initiates a transcriptional program that specifies angioblasts and after the initial requirement of Etv2 its function is dispensable.

**Figure II - 4**

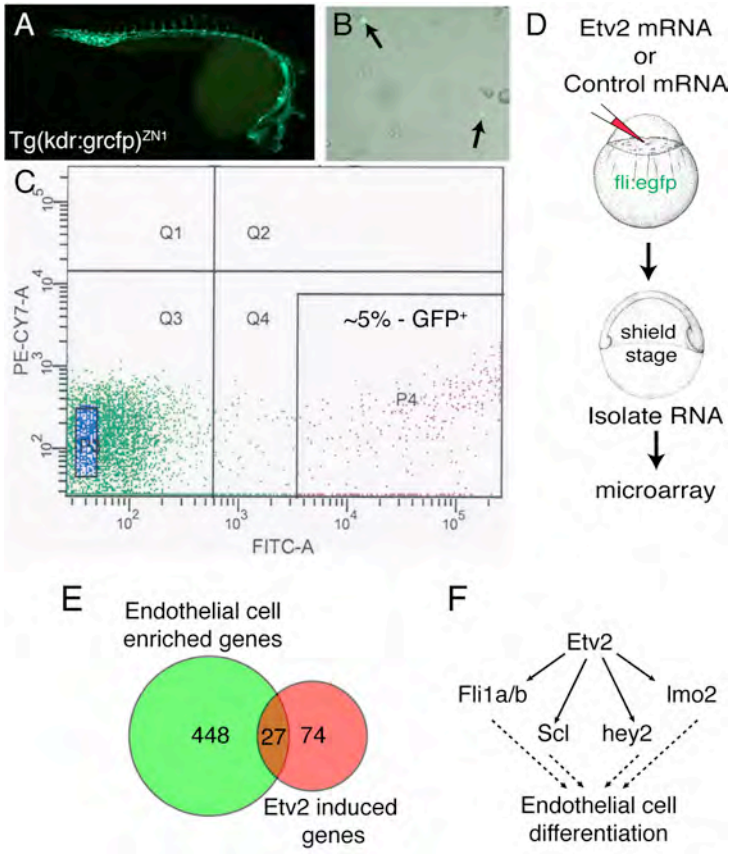


**Figure II – 4: Endothelial enriched and Etv2 induced microarray strategies and results.** (A) Epi-fluorescent image of 24hpf *Tg(kdr;grcfep)*$^{ZN1}$ embryo. (B) Bright field and epi-fluorescent overlayed images of *Tg(kdr;grcfep)*$^{ZN1}$ cells after dissociation with trypsin. Single GFP positive endothelial cells can be seen (arrows). (C) Diagnostic fluorescence activated cell sorting of dissociated *Tg(kdr;grcfep)*$^{ZN1}$ embryos. P3 and P4 demarcate cells sorted as GFP− and GFP+, respectively. (D) Experimental workflow for Etv2 overexpression microarrays. Embryos injected with Etv2 mRNA or control mRNA developed to shield stage and then RNA was isolated. The RNA from the two separate conditions where hybridized to Affymetrix microarrays and compared. (E) Venn diagram indicating that 448 genes are enriched after gene expression profiling of GFP+ vs. GFP- cell populations and 74 genes are induced when Etv2 is overexpressed, only 27 gene over lap from the two data sets. (F) Hypothetical transcriptional pathway controlling endothelial cell differentiation.

**Table II - 1**

Table 1: Top ten endothelial enriched, Etv2 induced genes

| Gene | LogFC +Etv2 | p-value +Etv2 | LogFC GFP+ | p-value GFP+ |
|---|---|---|---|---|
| *aqp8a* | 4.400 | 0.000 | 5.977 | 0.004 |
| *fli1a* | 4.282 | 0.000 | 4.516 | 0.003 |
| *yrk* | 3.360 | 0.000 | 5.442 | 0.001 |
| *lmo2* | 3.124 | 0.000 | 2.764 | 0.003 |
| *srgn* | 2.392 | 0.000 | 3.326 | 0.010 |
| *scl* | 2.419 | 0.000 | 2.099 | 0.004 |
| *fli1b* | 1.958 | 0.000 | 4.344 | 0.006 |
| *clic1* | 1.892 | 0.001 | 1.608 | 0.007 |
| *vamp5* | 1.461 | 0.002 | 1.174 | 0.076 |
| *hey2* | 1.224 | 0.001 | 1.801 | 0.007 |

**Table II – 1: Top ten endothelial enriched, Etv2 induced genes.** The table comprises the top ten genes induced by Etv2 after *etv2* mRNA overexpression in zebrafish with RNA isolated at shield stage, that are also enriched in the GFP+ cell population from FACS sorted *Tg(flk1:grcfp)*$^{ZN1}$ 24hpf embryos. Enrichment and induction was determined by gene expression analysis on Affymetrix zebrafish microarrays. Genes with adjusted p-value < 0.05 and fold change >= 2 are considered significant. Genes in blue boxes are transcription factors.

## Discussion

The ETS transcription factor Etv2 is essential for vascular development, but little is known about its expression dynamics or transcriptional targets. Using the zebrafish as a model system, we find that both *etv2* transcript and protein are expressed during angioblast specification and vasculogenesis but are subsequently downregulated at later stages. This expression pattern is mirrored by its functional requirement, which we find is restricted to early stages corresponding to angioblast emergence from the lateral mesoderm. We further provide evidence that Etv2 in a cell autonomous fashion enhances endothelial lineage commitment. We go on to demonstrate Etv2 induces transcription of several endothelial transcription factors and we hypothesize that etv2 sits atop a transcriptional hierarchy.

The phenotypes of *etv2*-deficient zebrafish and mouse embryos suggest that it should be considered as a master regulator of endothelial cell fate. In both species *etv2* is essential, with genetic ablation leading to severe vascular morphogenesis defects, an absence of circulation and global loss of endothelial gene expression [6-8]. Conversely, exogenous *etv2* expression can precociously and ectopically induce endothelial gene programs [129-131]. Appropriately, our mosaic transplant analysis shows enhanced endothelial cell lineage commitment of cells overexpressing Etv2. Similar studies overexpressing Etv2 in mouse embryoid bodies also show an increase in

commitment to hematopoietic and endothelial cell lineages [138]. Although these results suggest that Etv2 is required for early specification of angioblasts from the lateral plate mesoderm, Etv2 function seems to only be required during a short developmental time window. Conditional knockdown of Etv2 during or prior to angioblast specification, but not later, causes severe vascular defects. This demonstrates that Etv2 is functionally required only for the specification of angioblasts from the lateral mesoderm and is then dispensable for continued differentiation of the endothelial cell lineage. This functional requirement reflects the highly dynamic expression pattern of Etv2. Etv2 transcript and protein expression is initiated at early-somitogenesis and is gone by late-somitogenesis, the developmental window in which angioblasts are specified. Etv2 expression is absent in differentiated endothelial cells of the zebrafish, consistent with the loss of Etv2 expression in mice at E11.5 suggesting Etv2 downregulation is evolutionarily conserved [116]. Furthermore, our results are consistent with recent studies in mouse embryonic stem cells where *etv2* expression can be detected in *Brachyury-positive* mesodermal cells that have not yet initiated expression of endothelial cell marker genes, such as *vegf receptor-2* (*vegfr2*) [124]. Finally, conditional loss of Etv2 in endothelial cells using a Flk1:Cre driver does not appear to effect embryonic vascular development or viability, demonstrating that *etv2* is not required for later steps of vascular development in mammals as well [124]. Taken together with our studies, these results suggest that *etv2* plays

an essential role in specifying early lateral mesoderm progenitors to an endothelial cell lineage, yet is not required for later steps in vascular development.

Few studies have tried to understand how *etv2* specifies angioblasts from the lateral plate mesoderm. It is well understood that *etv2* is a transcriptional activator [129-131] but what sort of gene programs does it initiate? We sought to define endothelial enriched transcriptional targets of *etv2*. Our endothelial enriched gene list is smaller than similar studies due to our analysis of the *Tg(kdr:grcfp)*$^{ZN1}$ zebrafish transgenic lines opposed to the *Tg(fli1:egfp)*$^{Y1}$ line [94]. The *Tg(fli1:egfp)*$^{Y1}$ has expression in the pharyngeal arches and blood cells whereas the Tg(kdr:grcfp)$^{ZN1}$ is much more vascular restricted [12, 143]. Therefore our endothelial-enriched data set is more representative of the actual endothelial transcript profile at 24 hpf than previously published reports. Comparison of the endothelial enriched data set with the expression profile of early stage embryos overexpressing *Etv2* lead us to an interesting conclusion. The most highly induced endothelial genes by *Etv2* are transcription factors *scl*, *lmo2*, *hey2*, *fli1a*, and *fli1b* . Not only are they transcription factors but they have all been implicated in playing a role in endothelial cell differentiation [7, 95, 155, 158, 159]. *Scl* and *lmo2* are both expressed in early endothelial progenitors, they interact to form an active transcriptional unit, and both are required for the proper formation of the dorsal aorta [154, 155, 160, 161]. Additionally, *scl* and *lmo2* can induce

the formation of bi-potential hemangioblast cells in non-axial mesoderm of zebrafish that differentiate into endothelial cells. *Hey2* (*grl*) mutant embryos have no trunk circulation because of an improperly formed dorsal aorta [159]. Additionally, *Hey1* and *Hey2* are redundant during mouse development and double knockout mice are embryonic lethal with a global lack of vascular remodeling and massive hemorrhaging, although initial vasculogenesis appears unaffected [99]. Furthermore, Fli1 homozygous mutant mice exhibit a loss of blood vessel integrity and die after embryonic day 11 [162]. However, zebrafish *fli1a/b* morphants have a weakly penetrant circulation defect and normal ISV sprouts [7]. Although our data demonstrating Etv2 strongly induces the preceding transcription factors is consistent with other published Etv2 overexpression gene profiles [129-131], little is known about how these transcription factors drive vascular differentiation.

Understanding the molecular targets of Etv2 is likely to provide new insights into the transcriptional control of endothelial development. We hypothesize that Etv2 sits atop a transcriptional hierarchy by inducing *scl*, *lmo2*, *hey2*, *fli1a* and *fli1b* to continue endothelial cell differentiation (Fig II-4F). Although Etv2 likely directly activates the transcription of the aforementioned genes in part because how early we analyzed the transcriptional profile after *Etv2* overexpression, it cannot truly be determined until chromatin immunoprecipitation for those genes can be carried out using an *Etv2* antibody or similar method. Except for *lmo2,* which has been shown

to be a direct target of *etv2* by ChIP, EMSA and promoter transactivation assays in mice [138]. Additional studies are needed to better understand if these genes work in a linear fashion to induce each other or if they work separately after *Etv2* induction to initiate their own transcriptional targets or in some combination. How these Etv2 induced transcription factors work to drive endothelial cell differentiation is likely going to be complicated. For example, *Fli1* and *Scl* form a recursive transcriptional regulatory loop during mouse hematopoiesis and *Scl*'s obligate transcriptional partner *Lmo2,* is directly activated by *Fli1* in endothelial cells [163, 164]. Etv2 is a master regulator of endothelial cell lineage commitment that functions within a short developmental time frame to initiate a transcriptional program that specifies angioblasts from the lateral mesoderm. Thus, a better understanding of Etv2's transcriptional targets interactions and their role and regulation during angioblast specification will help better elucidate the mechanisms driving vascular development.

# CHAPTER III: POST-TRANSCRIPTIONAL REGULATION OF ETV2 BY THE LET-7 FAMILY OF MICRORNAS

A portion of the work contained within this chapter was submitted for publication:
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## Introduction

*Etv2* is an endothelial cell specific ETS transcription factor that can be called a master regulator of endothelial cell lineage determination and is essential for the proper formation of the vascular system. *Etv2* autonomously enhances endothelial cell lineage commitment by inducing an endothelial transcriptional program (Chapter II). Conversely loss of *etv2* causes severe defects in vascular morphogenesis and a concomitant loss of endothelial gene expression [7, 8, 116, 127]. Interestingly, unlike the ETS transcription factor *Spi1* (*Pu.1*), which has reiterative roles during myelopoiesis [136, 137], *etv2* is only required for the initial formation of the vascular lineage and is not required for its maintenance. We have found that in zebrafish, *etv2* transcript and protein are expressed only during early-somitogenesis when angioblasts emerge from the lateral mesoderm until late-somitogenesis (Chapter II). *Etv2* is not expressed in differentiated endothelial cells of the zebrafish or mouse [116]. Furthermore, conditional knock down using caged morpholinos defines a short developmental time window *etv2* functions within, that mirrors its temporal expression (Chapter II). Altogether, *etv2* is a transient activator of endothelial cell differentiation.

No negative regulatory mechanism has been described for *etv2* but several recent studies emphasize the deleterious effects of continued *etv2* expression. Conditional *Tek* driven *Etv2* expression in mouse leads to an abnormal yolk sac vasculature morphology and altered endothelial cell gene

expression. Endothelial cells retain an immature identity and as consequence fail to express genes responsible for sheer stress response, extracellular-matrix attachment and metalloproteinase activity [122]. *ETV2* together with the ETS transcription factors *ERG* and FLI1 can transdifferentiate human amniotic fluid cells into functional endothelial cells. However, in order to culture fully differentiated endothelial cells past an endothelial cell progenitor state, *ETV2* is required to be turned off after initial reprogramming [139]. The lack of *etv2* expression in differentiated endothelial cells and the negative consequences of its persistent expression suggest an active negative regulatory mechanism represses *etv2* expression later during development. A recently identified zebrafish Etv2 enhancer recapitulates endogenous *etv2* expression [119]. Transgene expression is extinguished by 48 hpf in the vasculature suggesting transcriptional repression or inactivation as one possible negative regulatory mechanism. However, multiple developmental mechanism may exist to downregulate *etv2* considering the negative consequences of misexpression on vascular development.

In this work we investigate the mechanisms down-regulating *etv2* expression in the endothelium during vascular development. We present evidence that the 3'UTR of *etv2* is alternatively polyadenylated and expresses multiple isoforms during development. We use an endothelial cell autonomous 3'UTR sensor construct to show that the 3'UTR of *etv2* is post-transcriptionally regulated. Persistent Etv2 protein expression in maternal

zygotic *dicer1* mutant zebrafish suggests *etv2* is regulated by miRNAs. Accordingly, the 3'UTR of *etv2* contains binding sites for the *let-7* family of miRNAs and mutation of *let-7* binding sites inhibit post-transcriptional regulation of the sensor. Furthermore, the *let-7* family of miRNAs can repress the 3'UTR of *etv2*. Moreover, endogenous *etv2* transcript and protein are repressed upon *let-7a* overexpression, with subsequent reduction of endothelial cells and endothelial marker gene expression, due to a reduction of *etv2* expression and a failure to specify angioblast from the lateral mesoderm. Additionally, *Let-7a* overexpressing endothelial cells are less likely to commit to the endothelial cell lineage after mosaic transplantation. Overexpression of the *let-7* inhibitor *lin28a* causes a significant down-regulation in *let-7* expression but does not cause a concomitant increase in *etv2* transcript or protein levels. Together our results indicate that *etv2* is post-transcriptionally regulated in part by the *let-7* family of miRNAs, to allow for proper vascular development to occur.

## Materials and Methods

### Zebrafish handling and maintenance

Zebrafish and their embryos were handled according to standard protocols [141] and in accordance with the University of Massachusetts Medical School IACUC guidelines. The *Tg(fli1a:egfp)*$^{y1}$ line has been described[12]. Maternal zygotic (MZ) *dicer1* embryos were made using the germline replacement technique as previously described [165, 166] using *dicer1*$^{hu715}$ donors [167].

### Plasmid construction

The pCS-*etv2* and pCS-*mCherry* vectors were described (Chapter II).*The etv2* open reading frame minus the DNA binding domain (-DBD) was amplified from plasmid pME-*etv2* (described in chapter II) and used in a BP recombination reaction with plasmid pDONR221 (Invitrogen) to make pME-*etv2(-DBD)*. The zebrafish *lin28a* open reading frame was amplified from a full-length Zebrafish Gene Collection (ZGC) clone (Clone ID: 2643384; Thermo Scientific; see appendix II for primers), then subjected to BP recombination with plasmid pDONR221 to generate pME-*lin28a*. pME-*lin28a* and pME-*etv2(-DBD)* were used in an LR reaction with pCSDest [145] to generate pCS-*lin28a* and pCS-*etv2(-DBD)*. Alternative *etv2* 3' UTRs were cloned through PCR amplification using attB2 and attB3 primers (appendix II) followed by BP recombination into pDONRP2r-P3 (Invitrogen) to give p3E-EST *etv2* 3'UTR, p3E-short *etv2* 3'UTR and p3E-long *etv2* 3'UTR. *let-7*

binding sites were identified by miRANDA, RNAhybrid, and a perl script. Bases 1, 3, 4, 5, 6 were mutated to adenines within 5 identified *let-7* binding site seed sequences identified by all three methods [168]. The mutant *let-7* etv2 3' UTR fragment was synthesized by Genewiz (pUC57-kan-etv2_3putr_mut_let7) followed by subcloning into p3E-mcs1 with AscI and XhoI to give p3E-mut*let-7 etv2* 3' UTR. To generate mRNA sensors constructs, p3E-EST*etv2* 3'UTR or p3E-short*etv2* 3'UTR were recombined with pCSDEST2 and pENTR-*EGFP2* [145] to yield pCS2-egfp-EST*etv2* 3'UTR and pCS2-egfp-short*etv2* 3'UTR. Endothelial 3' UTR sensor constructs were generated by performing an LR Gateway recombination reaction between pTolBasPegfpfliEPmcherryR2-R3 and one of the following 3' entry clones: p3E-mcs1, p3E-shortEtv2-3'UTR, p3E-ESTEtv2-3'UTR, p3E-longEtv2-3'UTR, p3E-mut-let7-Etv2-3'UTR.

**mRNA synthesis and injections**

Capped mRNA was synthesized from pCS plasmids that had been linearized with NotI using the SP6 mMessage mMachine kit (Ambion). mRNAs were injected into 1-cell stage embryos according to standard protocols [141].

**3'UTR Sensor assays**

For whole embryo sensor assay, 50 pg of *mCherry* mRNA and 50 pg of indicated *gfp etv2 3' UTR* mRNA was co-injected along with 50μM of indicated miRNA duplexes into 1-cell stage zebrafish embryos. Embryos were visualized at 24 hpf using an MZFLIII dissection microscope equipped with epifluorescence and digital images were captured using an AxioCam mRC (Zeiss). Alternatively, equal numbers of dechorinated embryos were lysed by boiling in 2x Laemmli buffer. Lysates were run on an SDS-PAGE gel and transferred to Western blots, which were probed with antibodies against EGFP (Invitrogen, A11122) and mCherry (Clontech, 632496). Blots were stripped in between each antibody detection. Expression levels were quantified by measuring the optical density of bands using ImageJ following incubation with a horseradish peroxidase conjugated secondary antibody and chemiluminscence detection. For endothelial autonomous sensor assays, 25 pg of indicated pTol sensor construct was co-injected with 25 pg *transposase* mRNA into one-cell stage wild type embryos. Individual 3'UTR constructs were always injected with control sensor in parallel. At 24 hpf, embryos were transferred to egg water containing 0.2mM 1-phenyl-2-thiourea (PTU) to inhibit pigment formation. At 48-50hpf, approximately five embryos from each group per experiment displaying robust transgenesis were imaged by confocal microscopy. Gain settings were set using embryos injected with the control sensor and remained constant throughout the experiment.

Quantification of fluorescence levels was performed using Imaris by creating a surface based on GFP fluorescence and examining the average values intensity sum of green and red channels. The red/green ratio of an experimental embryo was normalized against the red/green ratio of a control embryo imaged on the same day. All sensor experiments were done and quantified in quadruplicate, except the EST-3'UTR which was done in triplicate. Significance was calculated by a Welsh test and significance determined by a p value < 0.03.

**Antibody production and whole mount immunohistochemistry**

The methods are the same as described in Chapter II.

**miRNA Duplexes**

RNA oligonucleotides (Integrated DNA technologies) corresponding to the mature and star sequences of zebrafish *let-7a*, *let-7c*, *let-7f*, and, *let-7g* (see appendix II) were diluted to 250 mM in nuclease-free water. Equal volumes of mature and start oligonucleotides were combined, heated to 95°C and annealed at 37°C for 30 minutes. miRNA duplexes were aliquotted and stored at -80°C. 2 nl of miRNA duplexes were injected into embryos at a concentration of 50 μM . A mis-match duplex in which 4 out of 8 bases in the

seed sequenced were changed (see appendix II) was used as a negative control (referred to as “control duplex”).

**Quantification of endothelial gene expression**

mRNA was quantified using the NanoString nCounter gene expression system (Nanostring Technologies, Seattle, WA) [148]. Total RNA was isolated from embryos using a Qiagen RNAeasy kit. For embryos injected with 50μM *let-7a* or *mm-let7a* duplex, RNA was isolated at 15 ss. To assess over-expression of *etv2* and *let-7a*, embryos were co-injected with *let-7a* duplex as above along with 50 pg of mRNA encoding *etv2* or *etv2* minus its DNA binding domain [*etv2*(-DBD)] and RNA was isolated at shield stage. For each experiment, 100 ng of total RNA was hybridized for 12 to 20 hrs with the Nanostring probeset (appendix III) at $65^0$C in a thermocycler. Samples were loaded into the nCounter prep station and fluorescence signal was quantified using the nCounter Digital Analyzer. Gene normalization and fold change calculations were done using Nsolver Analysis Software (Nanostring Technologies). In all cases, biological triplicates were performed and gene counts were normalized to *eukaryotic translation elongation factor 1 alpha 1 like 1* (*eef1a1l1*) and *actin, beta 2* (*actb2*). Either the average normalized gene count or the average fold change of triplicate biological replicates was plotted and error bars represent the Standard Error of the Mean (SEM).

**In situ hybridization**

An antisense DIG-labeled *lin28a* riboprobe was synthesized by linearizing pCS2-*lin28* with SacI followed by in vitro transcription using T7 polymerase. A *gata1a* riboprobe was synthesized as described elsewhere [169]. Whole mount in situ hybridization was performed according to standard protocols [149]

**Mosaic analysis**

*Tg(fli1a:egfp)*$^{y1}$ embryos were used as donors in all cases and 0.35% miniRuby (dextran, tetramethylrhodamine and biotin 10,000MW) (Invitrogen D-3312) was co-injected as a lineage tracer. To assess *let-7a* overexpression we injected 2 nl of either 50µm control or *let-7a* duplex. At sphere stage, approximately 20 cells were transplanted from the ventral blastoderm margin of donors into wild type hosts, which were subsequently screened at 30 hpf for the appearance of red and green fluorescence. Embryos were imaged using an MZFLIII fluorescent dissection microscope or using a using a Leica DMIRE2 confocal microscope (Objective: HC PL APO 20x/0.70CS). The proportion of successfully transplanted embryos (i.e. exhibiting miniRuby-positive cells in any trunk tissue) with contribution to blood vessels (i.e. exhibiting EGFP-positive cells) was determined in three

separate experiments and significance was calculated by Fisher's Exact test. ($p < 0.05$).

**Northern**

Northern blot analysis for microRNA expression was performed as previously described [170]. Zebrafish RNA was isolated using a miRNeasy Micro kit (Qiagen) and 5 µg of total RNA was loaded per lane. Blots were hybridized with a DIG labeled *let-7a* locked-nucleic acid probe (Exiqon), stripped using boiling water, and hybridized with a DIG-labeled 5s rRNA DNA probe (see appendix II). Chemilumenscence detection was performed following incubation with a horseradish peroxidase-conjugated antibody against DIG. Northern blots were performed using RNAs from three separate experiments and quantified by measuring the optical density of bands using ImageJ to compare levels in uninjected versus *let-7a* injected embryos. Average fold difference from three independent experiments was plotted and error bars represent SEM. Significance was measured using a student t-test.

**3' RACE and Etv2 3' UTR cloning**

3' RACE was performed using the SMART RACE kit (Clontech). *etv2*-specific primers for primary and nested PCR are listed in appexdix II. Amplified fragments were gel purified, cloned into pGEM-t (Promega) and sequence verified.

**Quantitative PCR of miRNAs**

RNA was purified from uninjected zebrafish embryos injected at 24 hpf or those injected with 1 ng *lin28a* mRNA using a miRNeasy micro kit (Qiagen). qRT-PCR to detect mature miRNAs was performed using the miScript System (Qiagen). Two µg of whole RNA was used to synthesize cDNA. qPCR was performed from 100 ng of cDNA template with a commercially available primers for indicated miRNA (Qiagen) and the miScript universal primer using the miScript SYBR green PCR Kit (Qiagen). *snord61.2* expression was assessed in parallel and used to normalize microRNA expression levels. PCR quantification was performed on a StepOnePlus real time PCR system (Applied Biosystems). Each reaction was run in triplicate and performed on at least two experimental replicates and 2-log fold change calculated by comparing uninjected to Lin28a injected.

**RT-PCR**

RNA was isolated from *Wt* or MZ *dicer1* embryos at the indicated developmental stages using Qiagens RNaeasy Kit. cDNA was made using 1µg of whole RNA, reverse transcriptase III (Invitrogen) and oligo (dT). Primers were designed to amplify the three distinct etv2 3'UTR isoforms from the cDNA and run on agarose gels (see Supplementary Table 1 for primers).

## Results

*The etv2 transcript has multiple length 3'UTRs and post-transcriptionally regulated*

*Etv2* is not functionally required for later steps in vascular development, and persistent expression in endothelial cells leads to abnormal vascular morphology in the mouse, suggesting mechanisms are in place to actively down-regulate *etv2* expression (Chapter II; [122]). To determine if *etv2* is regulated by post-transcriptional mechanisms, we first tested the ability of the *etv2* 3' UTR to negatively regulate expression. For this purpose, we employed a previously described bi-cistronic endothelial cell autonomous reporter assay in which a 3' UTR of interest is placed downstream of a red fluorescent protein (mCherry) reporter [37]. EGFP and the mCherry-3'UTR fusion gene are driven in opposing directions by a zebrafish *fli1a* gene enhancer. In the process of cloning the appropriate regulatory sequences for this assay, we observed evidence suggesting the existence of alternative *etv2* 3'UTRs (Fig. III-1A). In ENSEMBL (version 69, Zv9), the annotated *etv2* 3'UTR spans only 298 nucleotides, while two separate expressed sequence tags (ESTs) extend past this sequence by an additional 315 nucleotides (Fig. III-1A). The presence of an A-rich sequence immediately downstream of this sequence suggested that this longer form may arise from spurious binding by oligo(dT) primers during cDNA reverse transcription [171]. Therefore, we

performed 3’ rapid amplification of cDNA ends (RACE) from 24hpf zebrafish embryos to further characterize expressed *etv2* 3’ UTR sequences. We confirmed the in vivo transcription of the annotated 3’UTR (Short *etv2* 3’ UTR) and the EST indicated 3’UTR (EST *etv2* 3’ UTR; Fig III-1B). Additionally a third isoform (Long *etv2* 3’ UTR) encoding a 3’ UTR of approximately 1030 nucleotides was discovered and can be detected up until 48 hpf ( Fig. III-1C). To determine the possible regulatory potential of these UTRs, we cloned each downstream of mCherry and quantified their effect on reporter expression in endothelial cells *in vivo* compared to an internal EGFP cassette. (Fig. III-1D). The endothelial cell autonomous sensor assays revealed that compared to control, the short *etv2* 3’ UTR did not significantly contribute to repression, while the EST and long *etv2* 3’ UTRs were capable of reducing transgene expression significantly to similar levels (Fig. 3E-G, data not shown). These results suggest that post-transcriptional regulation of alternative *etv2* 3’UTRs may contribute to its regulation during vascular development.

MicroRNAs (miRNA) are short non coding RNAs with a well described role in the post-transcriptional regulation of target mRNA during development [172]. miRNAs repress target transcripts by binding 3’UTR sequences. Thus we analyzed the 3’UTR of *etv2* for candidate microRNA binding sites. We found 5 putative binding sites for members of the *let-7* family of microRNAs in the longest defined *etv2* 3’UTR [173, 174]; see methods). Consistent with isoform length, the short, EST and long *etv2* 3’UTRs have two, three and five

binding sites, respectively (Fig. III-1A). Additionally, *let-7* binding site analysis on the mouse and human *etv2* transcripts, reveal the presence of *let-7* binding sites in the 3'UTR is a conserved feature (data not shown). Endothelial cells isolated from *Tg(kdrl:egfp)*$^{s893}$ embryos at 24 hpf express several members of the *let-7* family of microRNAs [37]. Additionally, high levels of *let-7* microRNAs are also expressed in primary human endothelial cells [175-177]. The *let-7* family of microRNAs are known to promote differentiation in a variety of cell types, in part, through the repression of transcripts encoding regulators of pluripotency and proliferation [178]. Regulation of *etv2* by *Let-7* would be consistent with *let-7*'s known role in promoting differentiation as continued Etv2 expression leads to the maintenance of an endothelial progenitor identity [122, 139].

**Figure III - 1**

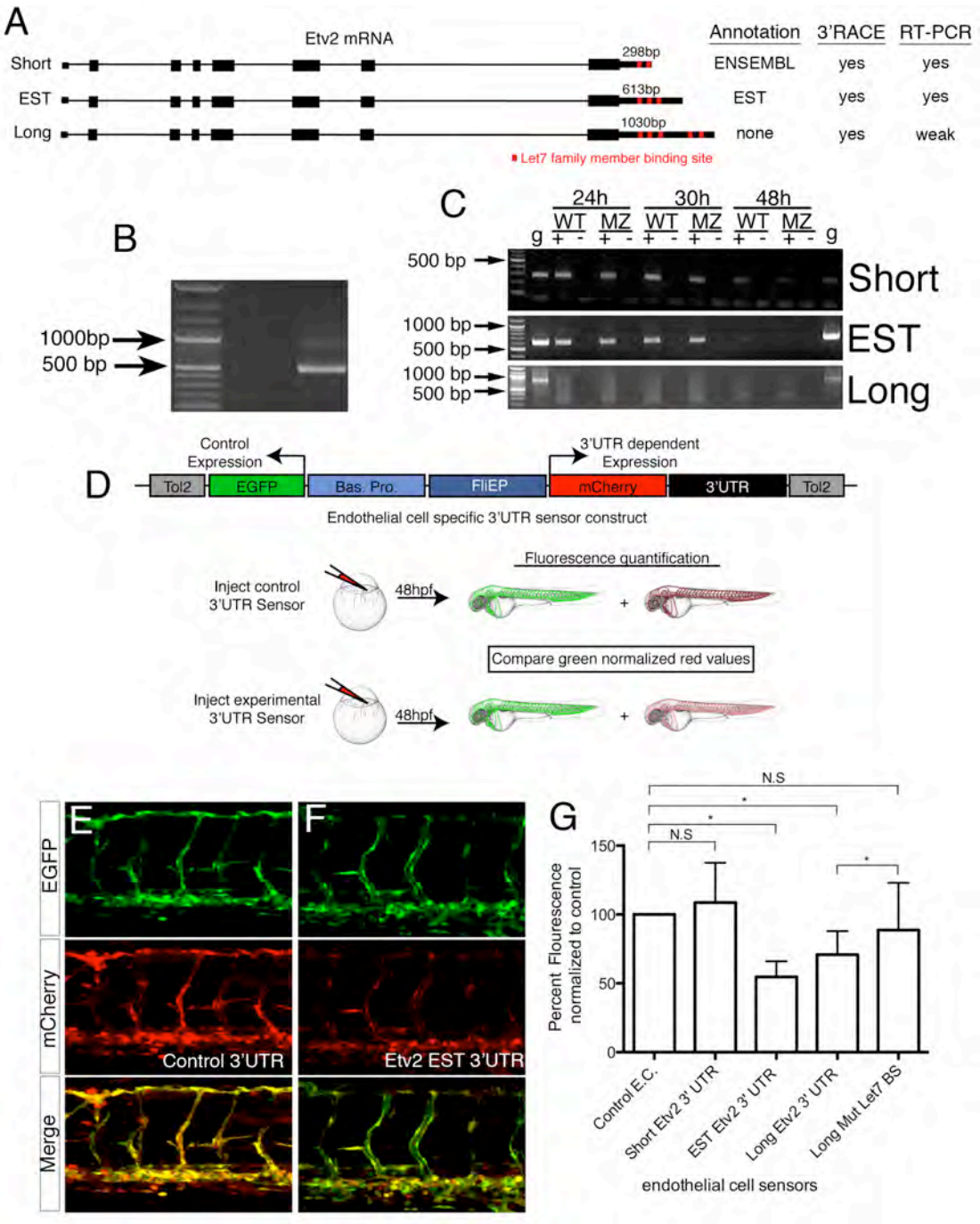

A
Etv2 mRNA
Short
EST
Long
298bp
613bp
1030bp
Let7 family member binding site
Annotation
3'RACE
RT-PCR
ENSEMBL
yes
yes
EST
yes
yes
none
yes
weak
B
1000bp
500 bp
C
24h
30h
48h
WT
MZ
g
500 bp
1000 bp
500 bp
Short
EST
Long
D
Control Expression
3'UTR dependent Expression
Tol2
EGFP
Bas. Pro.
FliEP
mCherry
3'UTR
Tol2
Endothelial cell specific 3'UTR sensor construct
Inject control 3'UTR Sensor
48hpf
Fluorescence quantification
Compare green normalized red values
Inject experimental 3'UTR Sensor
E
F
EGFP
mCherry
Merge
Control 3'UTR
Etv2 EST 3'UTR
G
N.S
*
N.S
*
Percent Flourescence normalized to control
150
100
50
0
Control E.C.
Short Etv2 3' UTR
EST Etv2 3' UTR
Long Etv2 3' UTR
Long Mut Let7 BS
endothelial cell sensors

**Figure III – 1: Evidence for alternative 3'UTRs encoded by the zebrafish *etv2* locus and *etv2* 3'UTR can negatively regulate a heterologous reporter.** (A) Schematic depicting etv2 intron/exon structure and alternative 3'UTR lengths. Evidence for the existence of each isoform derived from annotation, 3'RACE, and RT-PCR is indicated. (B) 3'RACE products amplified from 24 hpf embryos. (C) RT-PCR from 24, 30, or 48 hpf wild type or *MZDicer* embryos was performed using primers specific to the short, EST, or long *etv2* 3'UTR. Genomic (g) DNA was used as a positive control. "+" denotes reverse transcribed cDNA template; "-" indicates template without reverse transcription to rule out genomic DNA contamination. (D) Diagram of endothelial cell autonomous 3' UTR sensor construct and experimental procedure for measuring post-transcriptional regulation of 3' UTRs. (E, F) Representative confocal micrographs of 48 hpf wild type embryos co-injected with 25 pg of a Tol2 bi-cistronic endothelial cell autonomous sensor construct encoding mCherry fused to a (E) control 3'UTR or the (F) EST *etv2* 3'UTR sensor and 25 pg of transposase mRNA. *Top*, endothelial expression of the control EGFP transgene. *Middle*, endothelial expression of the mCherry sensor transgene. *Bottom*, merge of green and red channels. Lateral views, dorsal is up, anterior to the left. (G) Quantification of relative mCherry fluorescence levels compared to EGFP following indicated 3' UTR sensor injection. *$p < 0.05$, N. S. = Not significant.

*Let-7 can negatively regulate exogenous etv2 3'UTRs*

To test the possibility that *let-7* regulates the 3'UTR of *etv2,* we fused the short and EST *etv2* 3'UTRs to *egfp* and injected mRNA into embryos in combination with *let-7a* duplex or a mis-match control duplex (mm-duplex) RNA and analyzed *egfp* repression. *mCherry* mRNA was simultaneously injected to act as an internal control. The coinjection of *egfp* fused to the EST-*etv2* 3'UTR with *let-7* duplex lead to a dramatic decrease in Egfp expression compared to control duplex (Fig. III-2 compare green channel A to B). Consistent with the number of binding sites in each *etv2* 3' UTR isoform, fusion of the Short *etv2* 3' UTR to *egfp* led to repression when coinjected with *let-7a* (Fig. III-2 compare green channel C to D) but to a lesser extent than the EST form (Fig. III-2 compare green channel A to B). No change of expression was seen in the mCherry control in any of the injections (Fig. III-2 compare red channel in A to B, and C to D). To better quantify the degree of repression mediated by the *etv2* 3' UTR, we performed Western blot analysis for Egfp and mCherry expression in lysates from embryos injected with the mRNA sensors (Fig. III-2E). Band intensity quantification of embryo lysate Westerns demonstrates that the EST *etv2* 3'UTR is negatively regulated five fold compared to an uninjected control and the Short *etv2* 3' UTR is only two fold repressed, but still significantly (Fig. III-2F). *Let-7* binding site number dependent regulation is consistent with our endothelial cell autonomous 3'UTR sensor assay findings (Fig. III-1G). Furthermore, we found that

several highly related members of the *let-7* family, all of which are expressed in endothelial cells [37], were all capable of repressing the EST *etv2* 3' UTR (Fig. III-3). In accordance with the ability of *let-7* to regulate the 3'UTR of *etv2*, deletion of all five of the putative let-7 family member binding sites in the *etv2* Long 3' UTR (Long Mut let7 BS) causes a significant increase in mCherry reporter expression in endothelial cells compared to the wild type Long 3'UTR (Fig. III-1G, data not shown). Overall, these data suggest that that *let-7* family of miRNAs can contribute to the repression of *etv2* expression in endothelial cells.

**Figure III - 2**

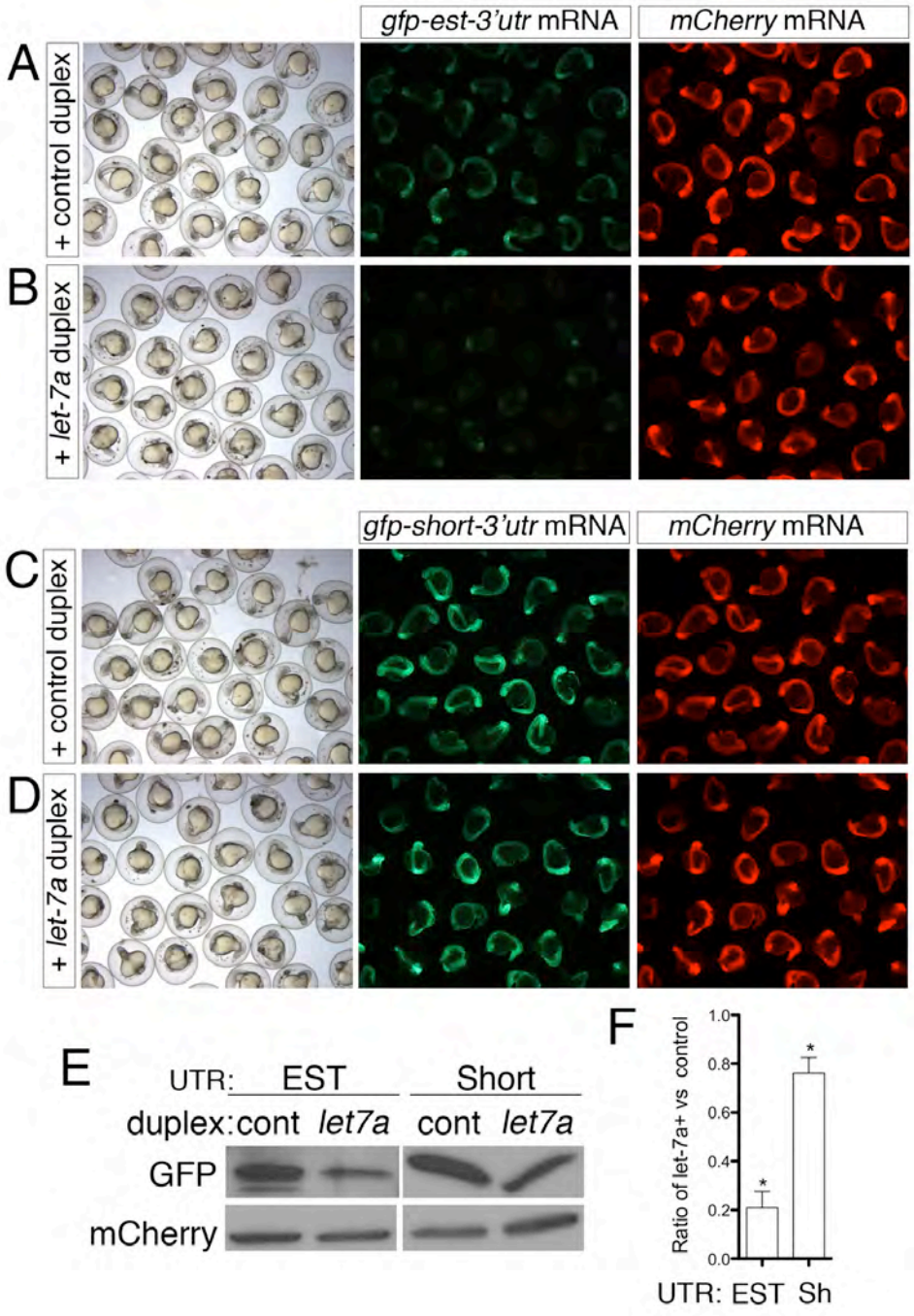

gfp-est-3'utr mRNA
mCherry mRNA
A
+ control duplex
B
+ let-7a duplex
gfp-short-3'utr mRNA
mCherry mRNA
C
+ control duplex
D
+ let-7a duplex
E
UTR: EST Short
duplex: cont let7a cont let7a
GFP
mCherry
F
Ratio of let-7a+ vs control
1.0
0.8
0.6
0.4
0.2
0.0
*
*
UTR: EST Sh

**Figure III - 2. *let-7a* negatively regulates the *etv2* 3'UTR.** (A-D) Transmitted light (left column), green fluorescence (middle column) and red fluorescence (right column) images of embryos injected with sensor mRNAs. (A, B) Embryos co-injected with 25 pg *gfp-est-etv2-3' UTR* and 25 pg *mcherry* mRNAs and 50 µM (A) mismatch or (B) *let-7a* duplex. (C, D) Embryos co-injected with 25 pg *gfp-short-etv2-3' UTR* and 25 pg *mcherry* mRNAs and 50 µM (C) mis-match control or (D) *let-7a* duplex. (E) Western analysis for GFP and mCherry protein on embryo lysates at 24 hpf following injection with *EST*- or *short-etv2 3' UTR* sensor mRNA, *mcherry* mRNA, and indicated duplex. (F) Quantification of Western analysis from three independent experiments. Bars represent the average ratio of GFP band intensities from embryos injected with control duplex compared to *let-7a* duplex from either the EST or Short GFP-*etv2 3' UTR sensor*. Significance was calculated using the student t-test. *$p < 0.05$

**Figure III - 3**

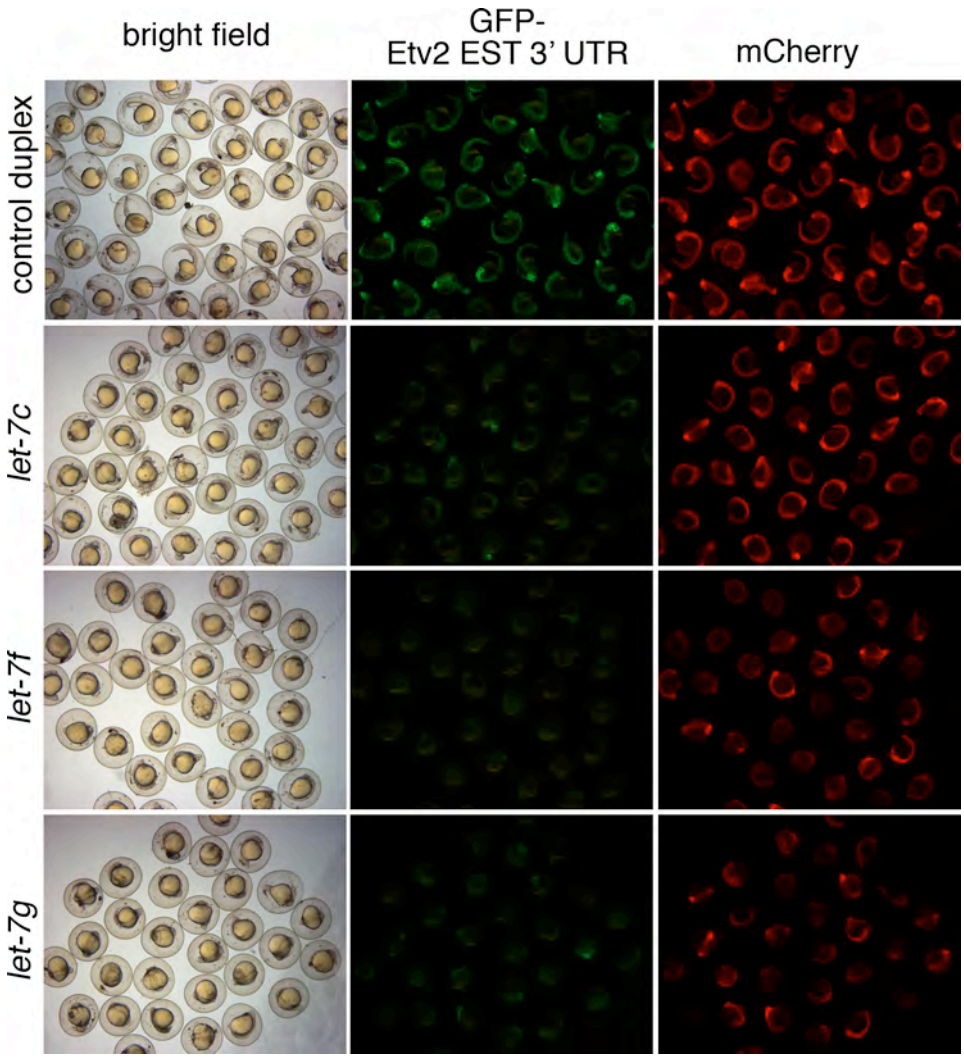



**Figure III – 3: Multiple *let-7* family members can repress the *etv2* 3' UTR.** Embryos were co-injected with gfp-est-*etv2* 3' UTR sensor (25 pg) and mcherry mRNAs (25 pg), along with indicated RNA duplexes (2 nl of 50µM). Bright field (left column), green fluorescent (middle column) and red fluorescent (right column) images of injected embryos were captured at 24 hpf.

*Let-7 negatively regulates endogenous etv2*

To determine if *let-7* could repress endogenous *etv2*, we injected *let-7a* duplex into zebrafish embryos and assessed both *etv2* transcript and protein levels. Exogenous *let-7a* overexpression reduced endogenous Etv2 protein levels in *Tg(fli1a:negfp)*$^{y7}$ embryos at 15ss (Fig III-4A left) while those injected with control mis-match duplex exhibited robust Etv2 expression (Fig III-4A right). We also noted reduced nuclear EGFP in let-7a duplex injected embryos (Fig III-4A compare middle panels), which is likely due to endothelial differentiation defects associated with reduced Etv2 expression. Endogenous *etv2* transcript was significantly down-regulated at 15 ss following injection of the *let-7a* duplex compared to embryos injected with control mismatch duplex (Fig. III-4B). Furthermore, we noted concomitant reduction in *fli1a, fli1b, hey2, lmo2, tal1, kdrl,* and *flt4* in *let-7a* duplex-injected embryos (Fig. III-4B), consistent with the observation that Etv2 can induce expression of these genes [129-131, 179]. Accordingly, co-injection of *etv2* mRNA containing a heterologous 3'UTR along with let-7a duplex rescues the expression of these *etv2* responsive genes, ruling out the possibility that they may also be targeted by let-7a (Fig. III-4C). While we observed repression of several endothelial genes, there was no change in the early hematopoietic markers such as *gata1a and gata2a*, following injection of *let-7a* duplex ( Fig. III-4B). Whole mount *in situ* hybridization of *etv2* expression in *let-7a* injected embryos revealed both a down-regulation of expression, along with

decreased number of cells expressing *etv2*, while *gata1a* expression was normal (Fig. III-4D), consistent with the gene expression data (Fig. III-4B). To investigate the cell autonomy of these effects, we transplanted cells from *Tg(fli1a:egfp)*$^{y1}$ embryos injected with *let-7a* into wild type embryos and assessed the frequency of successfully transplanted host embryos with EGFP-positive donor cells. Consistent with our observation that *let-7a* can repress endogenous *etv2,* significantly fewer host embryos transplanted with *let-7a* overexpressing donor cells displayed contribution to vascular tissue compared to mis-match control injected embryos (Fig. III-4F). This is likely caused by *let-7a* negatively regulating *etv2* and preventing the specification of endothelial cell lineages [6]. Despite the negative effect of *let-7a* over-expression on endothelial cell contribution, both *Let-7a* or mis-match *Let-7a* injected donor cells were otherwise able to contribute to other cell types (Fig. III-4F). Taken together these data suggest that *let-7* family members can act to limit the ability of *etv2* to induce endothelial specification during development.

**Figure III – 4**

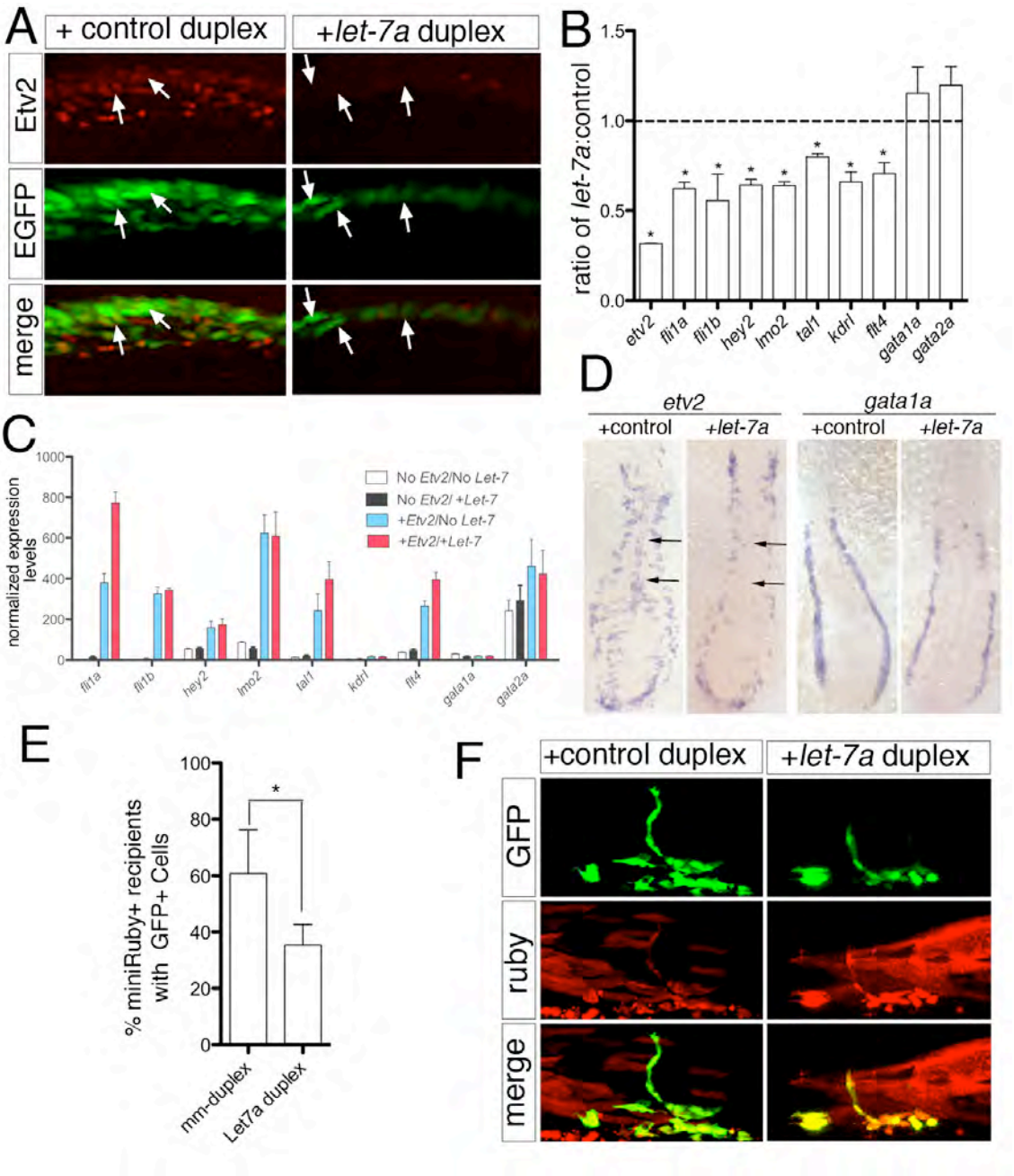

A
+ control duplex
+let-7a duplex
Etv2
EGFP
merge
B
ratio of let-7a:control
1.5
1.0
0.5
0.0
etv2
fli1a
fli1b
hey2
lmo2
tal1
kdrl
flt4
gata1a
gata2a
C
normalized expression levels
1000
800
600
400
200
0
No Etv2/No Let-7
No Etv2/ +Let-7
+Etv2/No Let-7
+Etv2/+Let-7
fli1a
fli1b
hey2
lmo2
tal1
kdrl
flt4
gata1a
gata2a
D
etv2
gata1a
+control
+let-7a
+control
+let-7a
E
% miniRuby+ recipients with GFP+ Cells
100
80
60
40
20
0
*
mm-duplex
Let7a duplex
F
+control duplex
+let-7a duplex
GFP
ruby
merge

**Figure III – 4 Endogenous Etv2 is repressed by *let-7a*.** (A) Two photon images of *Tg(fli1a:egfp)*$^{y1}$ embryos injected with 2 nl of 50µM solution of either control or *let-7a* duplex and immunostained with Etv2 polyclonal serum and Alexa-568 secondary antibody. Lateral view, dorsal is up, anterior to the left. Arrows denote Etv2/GFP-positive cells (left panels) or Etv2-negative/GFP-positive cells in the forming dorsal aorta (right panels). (B) Histogram showing fold change in expression of indicated genes at 15ss in embryos injected with 50 µM *let-7a* compared to those injected with control duplex measured by the nCounter system. Genes normalized to *actb2* (*beta-actin*) and *eef1a1l1* (*ef1alpha*). *p<0.05. (C) Histogram of relative nCounter expression counts normalized as in (B) for indicated genes following injection with mRNA encoding Etv2 (+Etv2) or Etv2 lacking the DNA binding domain (no Etv2) and mismatch (no let-7) or *let-7a* duplex (+ let-7). (D) Whole mount in situ hybridization using riboprobes against *etv2* (left) or *gata1a* (right) at 15 ss in embryos injected with 2 nl of a 50 µM solution of *mis-match let7a* (control) or *let-7a* duplex RNA. Angioblasts that have migrated to the midline, or lack thereof, are indicated by arrows. Dorsal view of flat mounted embryo, anterior is up. (E) Histogram showing percentage of successfully transplanted wild type host embryos (miniRuby-positive) that display contribution to vascular tissue, as indicated by presence of *Tg(fli1a:egfp)*$^{y1}$-positive cells. Donors were injected with control or *let-7a* duplex as above. Data are from three independent experiments and significance was calculated using the Fisher's exact test; *p < 0.05. (F) Confocal micrographs showing contribution of *Tg(fli1a:egfp)*$^{y1}$ positive cells (green channel) from donors that were injected with control or *let-7* RNA duplex. miniRuby-positive cells (red channel) indicate overall contribution of donor cells in the trunk.

*Lin28a overexpression reduces let-7 expression with no effect on Etv2 expression*

We next wanted to investigate Etv2 expression in the context of *let-7* loss of function. The zebrafish expresses 18 *Let-7* family precursor RNAs and 10 mature miRNAs forms, all of which have identical seed sequences (mirBASE, release 19), making loss of function studies difficult. However, the *lin28* gene binds to and inhibits *let-7s* biogenesis and negatively regulates mature forms by terminal uridylation [180-183]. We hypothesized that *lin28a* overexpression would cause a decrease in *let-7* expression manifesting in a measurable affect on *etv2* expression. Therefore we overexpressed zebrafish *lin28a* mRNA and assessed *let-7* and Etv2 expression. Lin28a is highly expressed in the developing nervous system at 24hpf (Fig. III-5A). Lin28a staining in the pronephric duct is clearly visible in the trunk at 24hpf but no expression is detected in the vasculature at earlier or later stages (Fig III-5A, J.M. unpublished observation). The lack of *lin28a* expression in the axial vasculature is consistent with the evidence of multiple *Let-7* family members being enriched in the vasculature at this time point [37]. Northern analysis on 24hpf zebrafish RNA after global *lin28a* mRNA over expression results in a significant decrease in *let-7a* expression (Fig. III-5B). *Let-7a* expression was down-regulated approximately 80% of control when assessed by Northern (unpublished quantification of Northern by densitometry). Furthermore, qPCR revealed significant down-regulation of multiple *let-7* family members by

*lin28a* overexpression (Fig. III-5C), while *mir-126,* an unrelated miRNA expressed specifically in endothelial cells, was not significantly reduced [184]. Interestingly *etv2* transcript levels as well as several of its induced genes remained relatively unaffected by *lin28a* overexpression (Fig. III-5D), in contrast to when *let-7a* is overexpressed (Fig. III-4B). The same is observed when analyzing Etv2 protein expression at the same time point (Fig. III-5E).

Our results suggest that there is negative regulation of *etv2* through the 3'UTR and that this is mediated through let-7 family members (Fig. III1-4), although our lin28 results do not support this (Fig. 5B-F). It is possible that we are unable to overexpress *lin28a* at high enough levels to sufficiently deplete the *let-7* family to observe a corresponding increase in *etv2* transcript levels or that *let-7* mainly represses translation inhibition and not transcript degradation. Alternatively, other miRNAs could possibly contribute to *etv2* down-regulation. We looked at Etv2 expression in MZ *dicer1* embryos. *Dicer1* encodes an essential nuclease required for microRNA maturation, therefore MZ *dicer1* embryos are devoid of mature miRNAs [166, 167]. Wild type embryos at 48 hpf did not exhibit Etv2 expression in endothelial cells (Fig. III-5F). By contrast, Etv2 protein expression was apparent at this stage in MZ *dicer1* embryos (Fig. III-5F) that had been injected with *miR-430* to rescue some developmental defects associated with a lack of *dicer1* function [185].

**Figure III – 5**

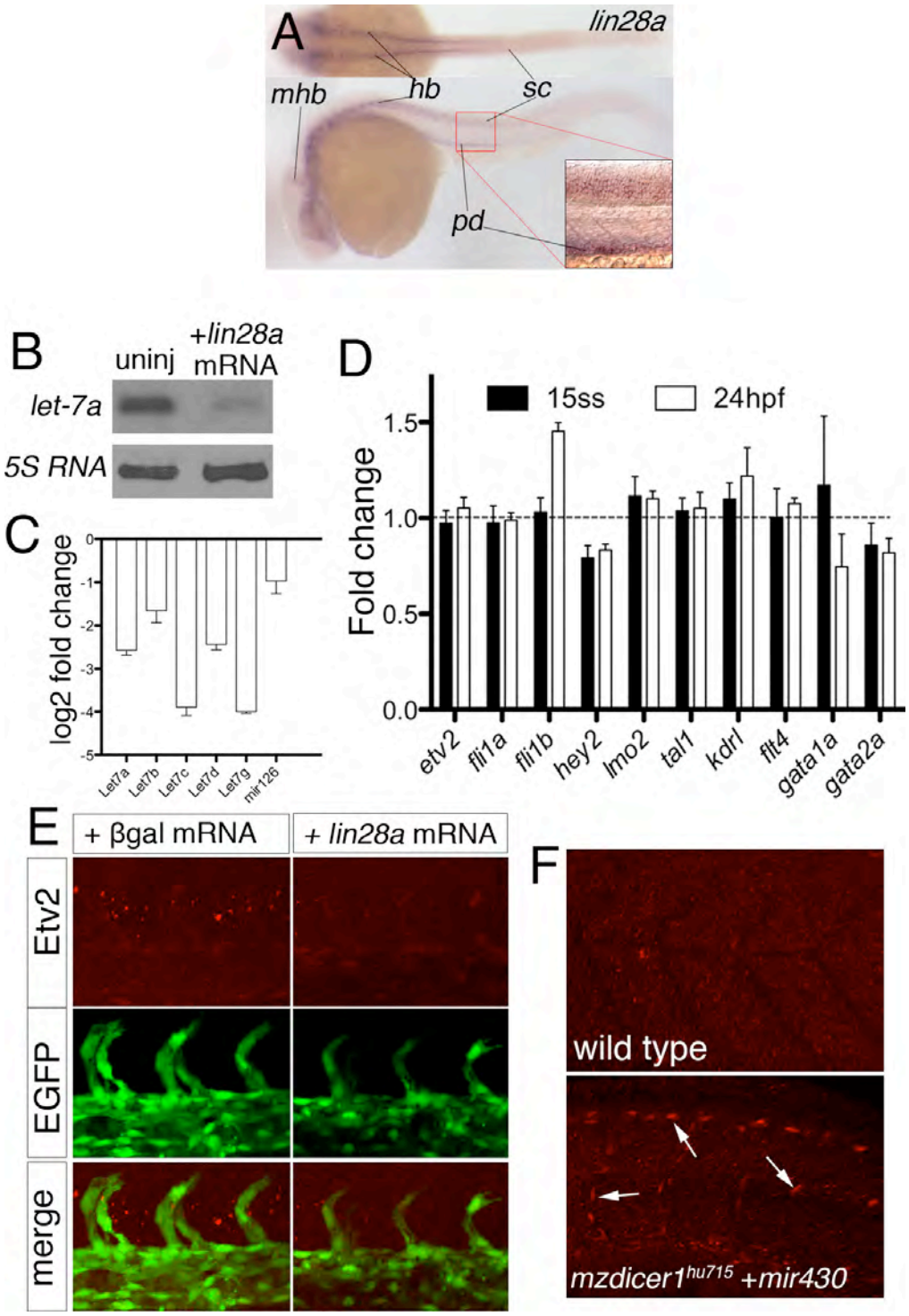

A
lin28a
mhb
hb
sc
pd
B
uninj
+lin28a mRNA
let-7a
5S RNA
C
log2 fold change
Let7a
Let7b
Let7c
Let7d
Let7g
mir126
D
15ss
24hpf
Fold change
etv2
fli1a
fli1b
hey2
lmo2
tal1
kdrl
flt4
gata1a
gata2a
E
+ βgal mRNA
+ lin28a mRNA
Etv2
EGFP
merge
F
wild type
mzdicer1hu715 +mir430

**Figure III – 5: Contribution of *let-7a* and other microRNAs to Etv2 repression.** (A) Bright field image of 24hpf embryo in situ hybridized for *lin28a* transcript, lateral (lower panel) and dorsal view (upper panel) anterior to the left. *Lin28a* is expressed in the midbrain hindbrain boundary (mhb), hindbrain (hb), spinal cord (sc) and in the pronephric duct (pd, insert). (B) Northern analysis of RNA isolated from 24 hpf embryos left uninjected or injected with 1 ng *lin28a* mRNA. Blots were hybridized with DIG labeled probes against *let-7a* and *5s* RNA. (C) Histogram showing log2 fold change comparison of *let-7* family members at 15ss assessed by miScript qPCR quantification between embryos injected with 1 ng *lin28a* mRNA and those left uninjected, quantification from triplicate experiments . (D) Histogram showing fold change comparison of indicated genes assessed by nCounter quantification between embryos injected with 1 ng *lin28a* and 1 ng bgalactosidase mRNA. Genes normalized to *actb2* (*beta-actin*) and *eef1a1l1*(*ef1alpha*). Significance was calculated using the student t-test, quantification from triplicate experiments (E) Two-photon micrographs of trunk blood vessels in *Tg(fli1a:egfp)*$^{y1}$ embryos immunostained with Etv2 polyclonal antiserum and Alexa-568 secondary antibody at 24 hpf following injection with 1ng of *β-galactosidase* (left panels) or *lin28a* mRNA (right panels). (F) Wild type (top) and *mzdicer1*$^{hu715}$ mutant embryos injected with 2nl of 10µM-amount of *mir-430* duplex RNA (bottom) immnuostained with Etv2 polyclonal antiserum and Alexa-568 secondary antibody at 48hpf. Etv2-positive nuclei in the endothelial cells of trunk blood vessels are denoted by arrows (bottom).

## Discussion

*Etv2* is expressed and functions during early vertebrate development to specify the angioblast from the lateral plate mesoderm and is down-regulated for proper endothelial differentiation and vasculature maturity. To date no negative regulatory mechanism has been described that causes the down-regulation of Etv2 expression. Here we present a novel post-transcriptional negative regulatory mechanism mediated in part by the *let-7* family of miRNAs using the zebrafish as a model organism. The 3'UTR of *etv2* is post-transcriptionally regulated in a *let-7* binding site dependent manner. Additionally, endogenous *etv2* transcript and protein are inhibited upon *let-7a* overexpression with a concomitant reduction in endothelial cell number and gene expression. Furthermore, Etv2 protein expression persists in MZ *dicer1* mutant zebrafish that lack all miRNAs. Therefore, the *let-7* family of miRNAs post-transcriptionally regulates Etv2 for proper vascular development.

We found that *etv2* transcript is expressed with three varying length 3'UTRs due to alternative polyadenylation. The two longer 3'UTR isoforms are post-transcriptionally-regulated *in vivo* and can be inhibited by the addition of *let-7*. Although, the shortest *etv2* 3'UTR did not repress reporter expression in our endothelial autonomous sensor assay, it contains two *let-7* binding sites and can be repressed upon the overexpression of *let-7a.* Importantly, zebrafish *etv2* post-transcriptional regulation is *let-7* binding site

number dependent and *let-7* overexpression causes a significant reduction of endogenous *etv2* transcript and protein. Additionally, Etv2 protein expression persists in MZ *dicer1* embryos emphasizing the role of miRNA repression in the regulation of *etv2* expression. However, the role of alternative polyadenylation and how it modulates *etv2* expression is unknown at this time. Interestingly, studies have shown that 3'UTRs are shortened in proliferating or transformed cells to escape miRNA regulation [186, 187]. Conversely, 3'UTRs are lengthened during zebrafish development as differentiation proceeds [188]. These findings suggest that *etv2* may use alternative polyadenylation of its 3'UTR to modulate its expression during vascular development. Following this logic, *etv2* may be expressed with the short 3'UTR during angioblast emergence and proliferation from the lateral mesoderm. Subsequently when *etv2* function is no required the longer 3'UTRs are expressed to allow greater negative regulation by *let-7.* However, more careful quantification of each 3'UTR isoforms during distinct developmental time points would need to be conducted to verify this hypothesis.

Human and mouse *Etv2* 3'UTRs also contain *let-7* binding sites (data not shown). Moreover, zebrafish and human endothelial cells highly express several *let-7* family members and the differentiated endothelium of adult mammals do not express ETV2 [37, 175-177, 189]. Combined, this data suggests that *let-7* regulation of *etv2* is a conserved developmental

mechanism. However, *let-7* mediated transcript degradation alone does not explain the loss of *etv2* expression in our studies, as *etv2* transcript is not downregulated in MZ *dicer1* embryos. Gene expression analysis after global *etv2* overexpression in the zebrafish suggests the existence of an *etv2* positive auto-regulatory loop [129]. Consequently, loss of *etv2* transcript after *let-7* overexpression is likely caused by a combination of mechanisms. *Let-7* mediated degradation of *etv2* expression likely starts with translational repression [190], reducing protein numbers to ultimately result in a concomitant reduction in *etv2* transcription. This multi-tiered regulatory mechanism may also explain why *etv2* translation blocking morpholino and *let-7* overexpression give similar phenotypes. Both inhibitory methods reduce the number of Etv2 expressing angioblasts at mid-somitogenesis by *in situ* hybridization, indicating a shared common mechanism is at work. Consistent with this theory is the identification of an enhancer element in the zebrafish *etv2* locus that recapitulates endogenous *etv2* expression. The transgene is expressed during early stages of development in the vasculature but not later [119], indicating transcriptional regulation of Etv2 also plays an important role in its expression dynamics.

Overexpression of *lin28a* causes a striking reduction in the expression of *let-7* family members enriched in endothelial cells. However, we do not detect a change in *etv2* transcript or protein expression and vascular development is unperturbed. This could be for several reasons. Considering

the number and diversity of *let-7* miRNAs expressed in the zebrafish endothelium [37], *lin28a* overexpression may not reduce *let*-7 below a functional threshold required to elicit upregulation of its target genes and consequently disrupt development. This is consistent with *Lin28a* overexpressing mice being viable and fertile, all thought slightly larger [191]. Alternatively, Etv2 may be negatively regulated by other miRNAs in the absense of *let-7*, considering Etv2 protein persists in *dicer1* null zebrafish embryos. However, we are currently unable to distinguish between these possibilities. The combined mechanisms of alternative polyadenylation and *let-7* regulation could explain why *etv2* expression is rapidly degraded during development. Despite our *lin28a* overexpression results, we demonstrate *let-7* negatively regulates *etv2* by binding its 3' UTR and this mechanism is likely responsible for the down-regulation of *etv2* during vascular development.

Our results demonstrate that *etv2* is negatively regulated by the *let-7* family of miRNAs through its 3'UTR to allow for proper vascular development. *Etv2* has the functional characteristics of an early acting pluripotency determinant. *Etv2* is required for and can induce the specification of the hemangioblast, a bi-potential cell type that gives rise to hematopoietic and endothelial cells [128, 192]. Furthermore Etv2 is required to be down-regulated for endothelial differentiation to proceed. Etv2 plays an early functional role in the expansion and maintenance of endothelial progenitors, as suggested by analysis of Etv2 overexpressing murine hematopoietic cells

[122]. Etv2 up regulates the pluripotent-specific reprogramming factor *zfp296* in this context*. Zfp296* enhances iPSC reprogramming by inducing expression of pluripotency determinants *Oct4* and *Nanog,* indicating Etv2 expression may functions to ensure endothelial progenitor self-renewal during early development [193]. This potential role is further supported by studies demonstrating that Etv2 is required for the maintenance of hematopoietic stem cells in mice [152]. Although a similar role for endothelial cell progenitors can only be speculated. Therefore our findings that the *let-7* gene family post-transcriptionally regulates Etv2 are consistent with its conserved role in promoting differentiated cell fates or blocking transformation by negatively regulating genes necessary for growth and proliferation [194-196]. Considering Etv2 is a strong transcriptional activator and its proper expression during development essential for life, miRNA mediated repression is likely not the only negative regulatory mechanisms acting on *etv2* during development. Additional studies are required to understand the full range of mechanisms controlling *etv2* expression during vascular development.

# CHAPTER IV: POST-TRANSLATIONAL REGULATON OF ETV2 BY UBIQUITIN-DEPENDENT PROTEOLYSIS

## Introduction

Formation of a fully functional, patent vascular system requires the proper spatial and temporal expression of endothelial lineage determinants. Etv2 is considered the master regulator of the vascular system because in its absence angioblasts fail to be specified from the lateral mesoderm, resulting in severe vascular morphogenesis defects and a loss of circulation [7, 8, 111, 116]. Conversely, Etv2 overexpression can precociously and ectopically activate endothelial cell gene programs, and autonomously enhances the commitment to endothelial lineages (Chapter II; [6]). Etv2 is only expressed in a short developmental window to specify the angioblasts through the initiation of a transcriptional network (Chapter II). Interestingly, Etv2 expression must decrease in order for endothelial differentiation to proceed, as persistent expression of Etv2 is deleterious to vascular development [122]. An identified enhancer element located within the zebrafish *etv2* locus is capable of driving transgene expression in a manner that recapitulates endogenous *etv2* expression. Transgene expression occurs early during vasculogenesis but not at later stages, indicating that transcriptional regulation accounts at least in part for the loss of etv2 expression [119]. Additionally, we've identified a post-transcriptional mechanism capable of inhibiting *etv2* expression in the zebrafish. Etv2 transcript is negatively regulated by miRNA-mediated repression through its 3'UTR by the *let-7* family (Chapter III). Considering Etv2 is such a strong transcriptional

activator, and proper expression is essential for normal development, we sought to identify additional negative regulatory mechanisms contributing to Etv2 downregulation.

The proper differentiation of cellular lineages requires the precise control of transcription factor protein levels and localization. Ubiquitin-mediated proteolysis mechanisms control the stability of various proteins that are essential for cellular function and transcriptional activation. For example, canonical Wnt signal activation inhibits the *glycogen synthase kinase-3* (*GSK-3)* dependent phosphorylation of *β-catenin,* thereby preventing its ubiquitination and proteasomal degradation. Subsequently, *β-catenin* accumulates in the cytoplasm, translocates to the nucleus and interacts with the TCF/LEF family to activate transcription of target genes [197]. Degradation of a protein by the ubiquitin-mediated proteolysis pathway involves two distinct and consecutive steps: (1) covalent attachment of multiple ubiquitin molecules to the target protein; and (2) degradation of the tagged protein by the 26S Proteasome complex [198]. Ubiquitin is a protein of 76 amino acids, and is covalently bound to lysines in target proteins, forming poly-ubiquitinated chains on proteins destined for proteasomal degradation. Ubiquitination is mediated by a multi-step process catalyzed by three enzymes working successively: E1 (Ubiquitin-Activating Enzyme), E2 (Ubiquitin-Conjugating Enzymes), and E3 (Ubiquitin-Ligating Enzymes). The E3 enzyme confers substrate specificity to the ubiquitin-mediated proteolysis

pathway and recognizes degrons (amino acid sequences that mark proteins for degradation) in target protein sequences. Often proteins with a short intracellular half-life have degrons with a PEST motif, a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) [199].

The ETS family of transcription factors plays a major role in the proper differentiation of endothelial cells and the formation of the vasculature system [200]. Transcriptional activity and proteasomal degradation are intrinsically linked, often the more transcriptionally active a protein is, the more quickly it is turned over [201]. Ets1 is the most closely related ETS transcription factor to Etv2 and is expressed in the developing and differentiated endothelium [7, 60]. Ets1 phosphorylation controls transcriptional activity and K47-linked poly ubiquitination controls its proteasomal degradation [79, 202]. Furthermore, the ETS transcription factors *ETV1*, *ETV4* and *ETV5* are all targets for the E3 ubiquitin-ligase *COP1*, which causes proteasomal degradation by ubiquitination [203]. Therefore proteasomal degradation of the ETS family of transcription factors is a common mechanism of negative regulation. Thus, we sought to determine if Etv2 is post-translationally regulated in a similar manner.

In this work we find that Etv2 is post-translationally regulated, highly unstable and degraded by the proteasome. Bioinformatic analysis of the zebrafish Etv2 protein revealed the presence of several putative PEST motifs encoded in the primary sequence. In HEK293T cells, overexpressed

zebrafish and mouse Etv2 are highly unstable in the presence of the translational inhibitor cycloheximide. The cycloheximide-induced instability of Etv2 is rescued by the addition of the proteasome inhibitor MG132, indicating Etv2 is degraded by the proteasome. We establish that Etv2 is co-immunoprecipitated with ubiquitin. A non-polymerizable form of ubiquitin is unable to illicit proteasomal degradation and stabilizes Etv2 when they are co-expressed. Finally, we demonstrate that endogenous zebrafish Etv2 is highly unstable *in vivo* and quickly degraded. Together our work shows that Etv2 is post-translationally ubiquitinated and degraded by the proteasome, revealing another mechanism that endothelial cells use to downregulate Etv2 during vascular development.

## Materials and Methods

### Zebrafish Handling and Maintenance

Zebrafish and their embryos were handled according to standard protocols [141] and in accordance with the University of Massachusetts Medical School IACUC guidelines. The *Tg(fli1a:egfp)*$^{y1}$ transgenic has been described [12, 142, 143].

### Plasmid Construction

All primers used for the amplification of open reading frames are listed in Appendix II. Plasmids pCS-*etv2* and pCSMT-*etv2* have been described in Chapter II. The zebrafish *ets1a* open reading frame was amplified from 24 hpf whole embryo cDNA and used in a BP recombination reaction with plasmid pDONR221 (Invitrogen) to make pME-*ets1a*. The mouse *etv2* open reading frame was amplified from a full-length mouse ORFeome collection (Clone ID: 100015809; Thermo Scientific) and used in a BP recombination reaction with plasmid pDONR221 (Invitrogen) to make pME-Mm *etv2*. The zebrafish *bozozok and lnx1* open reading frames were amplified from a full-length Zebrafish Gene Collection (ZGC) clone (Clone ID: 8158462 and Clone ID: 8001145, respectively; Thermo Scientific) and used in a BP recombination reaction with plasmid pDONR221 (Invitrogen) to make pME-*bozozok and* pME-*lnx1*. pME-*ets1a*, pME-Mm *etv2,* pME-*bozozok* or pME-*lnx1* were used

in LR reactions with pCSMTDest [145] to generate pCSMT-*ets1a*, pCSMT-Mm *etv2,* pCSMT-*bozozok* and pCSMT-*lnx1*.

**Cell culture**

HEK293T cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37$^{o}$C in an environment of 5% $CO_2$. Cultures were split into 6-well plates and transfected overnight with 2µg of mammalian expression vectors at 50-60% confluence using Lipofectamine 2000 per manufactures recommendation (Invitrogen), before harvesting or chemical treatment. Cells were lysed using 500 - 1,000µL RIPA buffer (150mM NaCl, 50mM Tris-HCl pH8.0, 1% NP-40, 0.1% SDS) containing cOmplete Mini EDTA-Free Protease Inhibitors (Roche). Lysates were spun down at 10,000 x G to remove insoluble material and mixed in Laemmli buffer to run on SDS-PAGE gels for immunoblot detection or immunoprecipitation with subsequent immunoblot detection. Cycloheximide (Sigma) was used at a final concentration of 50µg/mL and MG132 (Calbiochem) at a final concentration of 40µM. Immunoprecipitations were performed by incubating 1mL of cell lysates suspended in RIPA buffer with 30µL of monoclonal anti-c-myc antibodies conjugated to protein A agarose beads (Clonetech) and spun end over end overnight. Beads were washed four times in fresh RIPA buffer, boiled in Laemmli buffer and then run on SDS-PAGE gels. Ubiquitin co-immunoprecipitation was detected using a

polyclonal anti-FLAG antibody (Sigma-Aldrich; Cat#: F7425). Vectors used for Co-immunoprecipitation studies are pCSMT-Bozozok, pCSMT-lnxI, pCSMT-*Etv2*, pCSMT-*Ets1a*, pcDNA-Flag-UBB (Gift from Dr. Fumi Urano). Vectors used for proteasomal degradation and stability studies are, pCS-*Etv2*, pCSMT-Mm-*Etv2*, pCSMT-RNF6(Gift from Dr. Ingolf Bach) and pRK5-HA-Ubiquitin-KO (Addgene plasmid 17603). C-Myc-tagged proteins were detected using a 1:10,000 dilution of monoclonal anti-c-myc(9E10) antibody (Sigma). Exogenously expressed zebrafish Etv2 protein was detected in Westerns using a 1:5,000 dilution of anti-Etv2 polyclonal antibody serum (same as Chapter II). The α-tubulin and nucleolin monoclonal antibodies where used at a dilution of 1:10,000 and were a kind gift from the lab of Dr. Michael Green of the University of Massachusetts Medical School.

**Zebrafish cycloheximide treatment**

*Tg(Fli1:egfp)*$^{y1}$ embryos at 15 ss with chorions intact were incubated in an egg-water cycloheximide (50µg/mL) solution at 28.5$^{0}$C for three hours. DMSO was used as a control. Embryos were fixed at ~18ss and then subjected to Etv2 polyclonal antibody staining. The experiment was done in triplicate and 5 embryos from each experimental treatment were imaged. Z-Stacks where assembled in Image-J and average red pixel intensity measured in the trunk vasculature. The average of red pixel intensity for all

samples where plotted and significance determined by student T-test. $p < 0.05$ was deemed significant.

**PEST domain identification**

The full Etv2 ORF protein sequence (accession: NM 001037375) was entered into the ePESTfind analysis tool at SWAMI: The next generation biology workbench (www.ngbw.org). Default parameters were maintained for all settings. PEST domain identification was accomplished using published methods [204].

**Antibody production and whole mount immunohistochemistry**

The methods and antibodies are the same as described in Chapter II.

## Results

*Etv2 is unstable and degraded by the proteasome*

Etv2 downregulation is required for proper vascular development and we have identified post-transcriptional regulation by the Let-7 family of miRNAs as one negative regulatory mechanism (Chapter III). However, *Let-7* overexpression cannot completely repress Etv2 expression (Fig. III-4) and Lin28, a Let-7 family inhibitor, has no effect on Etv2 expression (Fig. III-5), revealing that an additional negative regulatory mechanism(s) must exist. Ets1 is the most closely related ETS factor to Etv2, and Ets1 is degraded by the proteasome after ubiquitination, indicating that Etv2 could be regulated in a similar manner [53, 202].

Analysis of the Etv2 protein sequence revealed several putative proteasomal degradation signals and an intrinsically unstructured region (Fig. IV-1). Accordingly, we found that zebrafish Etv2 proteins are rapidly degraded when exogenously expressed in HEK293T cells treated with cycloheximide, compared to DMSO control (Fig. IV-2 upper panels, compare lanes 1-4 with 5-7). Etv2 was stabilized in the presence of the proteasome inhibitor, MG132 (Fig. IV-2 upper panels, compare lanes 1-4 with 8-10). Additionally, the rapid degradation of Etv2 in the presence of cycloheximide can be blocked by the addition of the proteasome inhibitor MG132 (Fig. IV-2 upper panels, compare lanes 5-7 with 11-13). The mouse ortholog of Etv2

exhibits the same behavior under the same cell culture conditions as zebrafish Etv2 (Fig IV-2 lower panels), suggesting a conserved negative regulatory mechanism.

**Figure IV - 1**

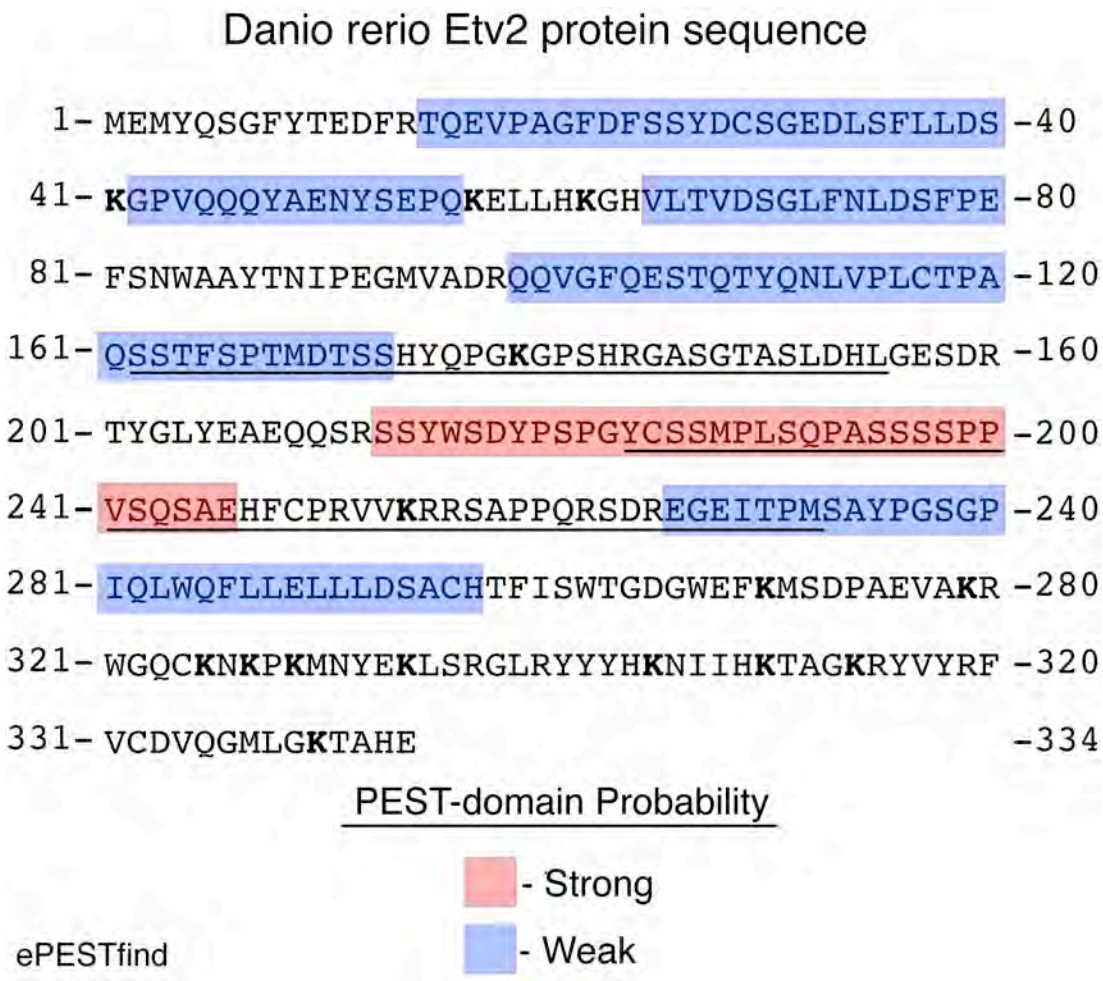



**Figure IV – 1: The Etv2 protein contains putative proteolytic cleavage sequences.** Full-length zebrafish Etv2 protein sequence highlighting sequences that have a high (red) or weak (blue) probability of being a PEST proteolytic cleavage domain. Lysines in bold are sites of possible ubiquitination. Underlined amino acids are intrinsically unstructured sequence segments as determined by IUPred. PEST domain analysis performed using ePESTfind.

**Figure IV - 2**

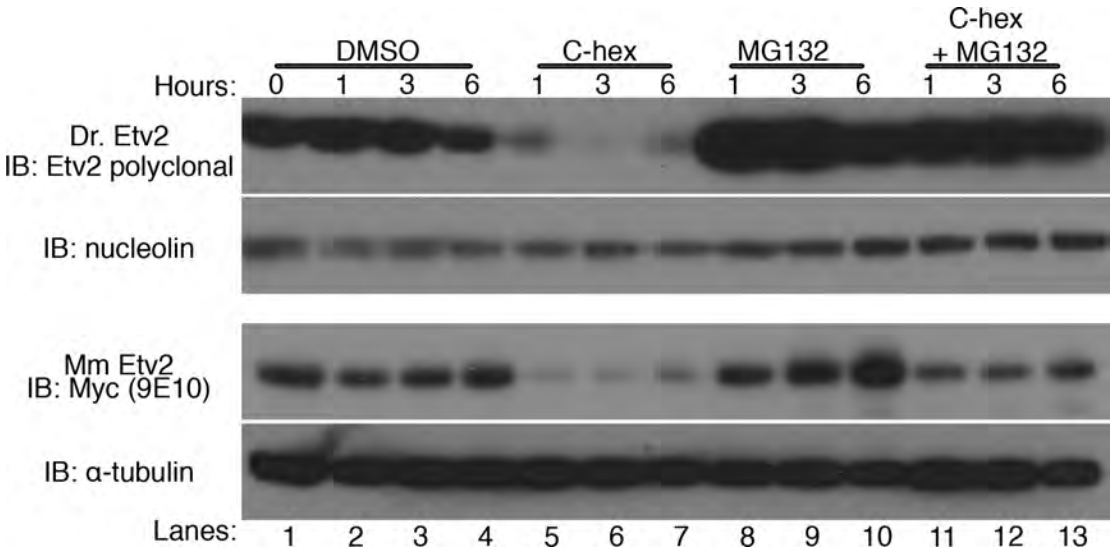



**Figure IV – 2: Etv2 is degraded by the proteasome.** HEK293T cells were transfected with mammalian expression vectors for zebrafish Etv2 (pCS-*etv2,* top) or myc-tagged mouse Etv2 (pCSMT-Mm *etv2,* bottom) and then treated with DMSO, cyclohexmide (50µg/mL), the proteasome inibitor MG132 (40µM) or a combination of both for the indicated times. Immunoblots were probed with Etv2 polyclonal antiserum or a monoclonal against the myc epitope (9E10). Blots were stripped and reprobed with monoclonals for nucleolin or α-tubulin.

Proteasomal degradation is often mediated by poly-ubiquitination of the target protein. Appropriately, we found that myc-tagged zebrafish Etv2 can be co-immunoprecipitated with flag-tagged ubiquitin when co-transfected into HEK293T cells (Fig. IV-3 lane 5). As positive controls we did pull-downs in HEK293T cells cotransfected with flag-ubiquitin, *bozozok* (a zebrafish protein known to be ubiquitinated), and the E3 ubiquitin ligase for *bozozok*, *lnx1* (Fig. IV-3 lane 4) [205]. The zebrafish *ets1a* gene is also ubiquitinated, in agreement with published data on the mammalian Ets1 homolog (Fig. IV-3, lane 6) [202]. Additionally, Etv2 is stabilized in HEK293T cells when cotransfected with a non-polymerizable form of ubiquitin (all lysines mutated to arginines) that prevents proteasomal degradation (Fig. IV-4 upper panels, compare lanes 1-3 to 4-6) [206]. The non polymerizable from of ubiquitin also prevents the rapid degradation of Etv2 in cycloheximide-treated cells (Fig. IV-4 upper panels, compare lanes 7-9 to 10-12). When performed in parallel as a positive control, we obtained similar results with the E3 ubiquitin-protein ligase RNF6, which was previously demonstrated to ubiquitinate itself (Fig. IV-4 lower panels) [207]. In agreement with the cell culture data, we observed that 15 ss zebrafish embryos treated for 3 hours with cycloheximide exhibit rapid downregulation of Etv2 protein, losing more than half of the signal intensity after Etv2 antibody staining (Fig. IV-5). Levels of an endothelial cell specific nuclear localized form of EGFP (*Tg(fli1a:negfp)*$^{y7}$) were only mildly

affected in the same time frame (Fig. IV-5). These results demonstrate that Etv2 protein is regulated by ubiquitin-dependent proteasomal degradation, and that the Etv2 protein is rapidly turned over *in vivo* during vascular development

**Figure IV – 3**

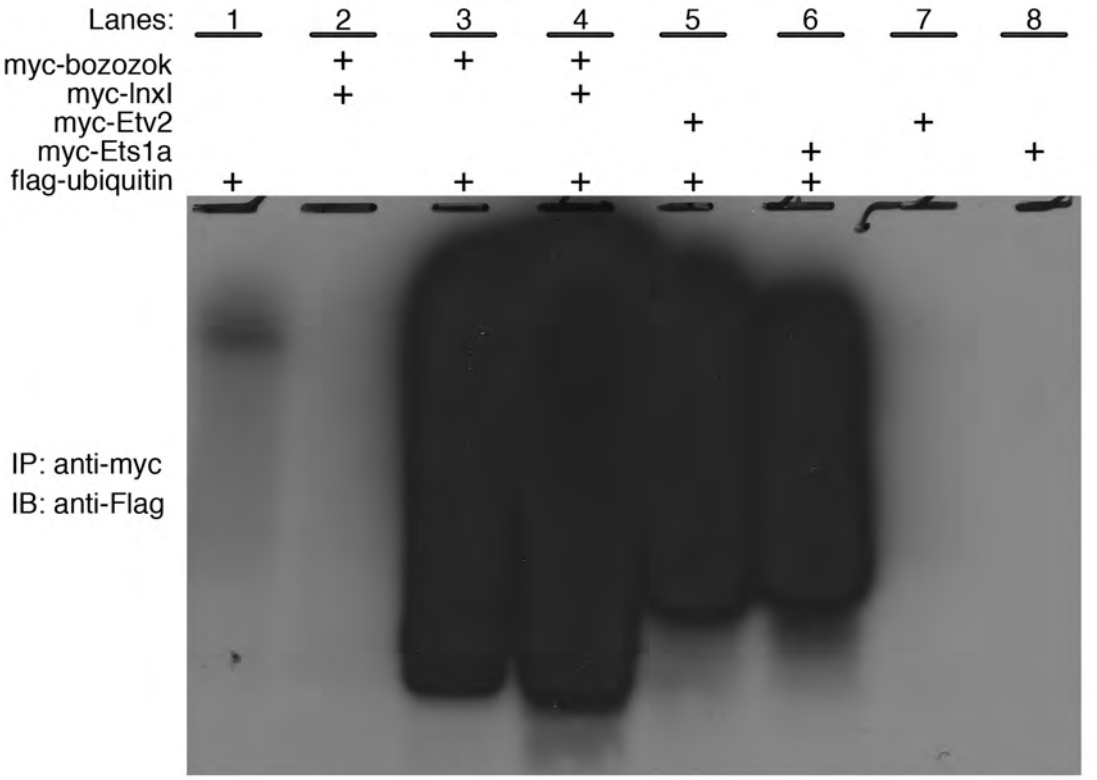



**Figure IV – 3: Etv2 is ubiquitinated.** HEK293T cells were transfected with indicated combinations of mammalian expression vectors for zebrafish myc-tagged *bozozok*, *lnx1*, *etv2*, and *ets1a* (pCSMT-*bozozok*, pCSMT-*lnx1*, pCSMT-*etv2*, and pCSMT-*ets1a*, respectively) and FLAG-tagged ubiquitin (pcDNA-Flag-UBB). Lysates were immunoprecipitated with c-myc monocolonal antibody (9E10) and ubiquitinated proteins were detected by immunoblotting with polyclonal Flag antibody.

**Figure IV - 4**

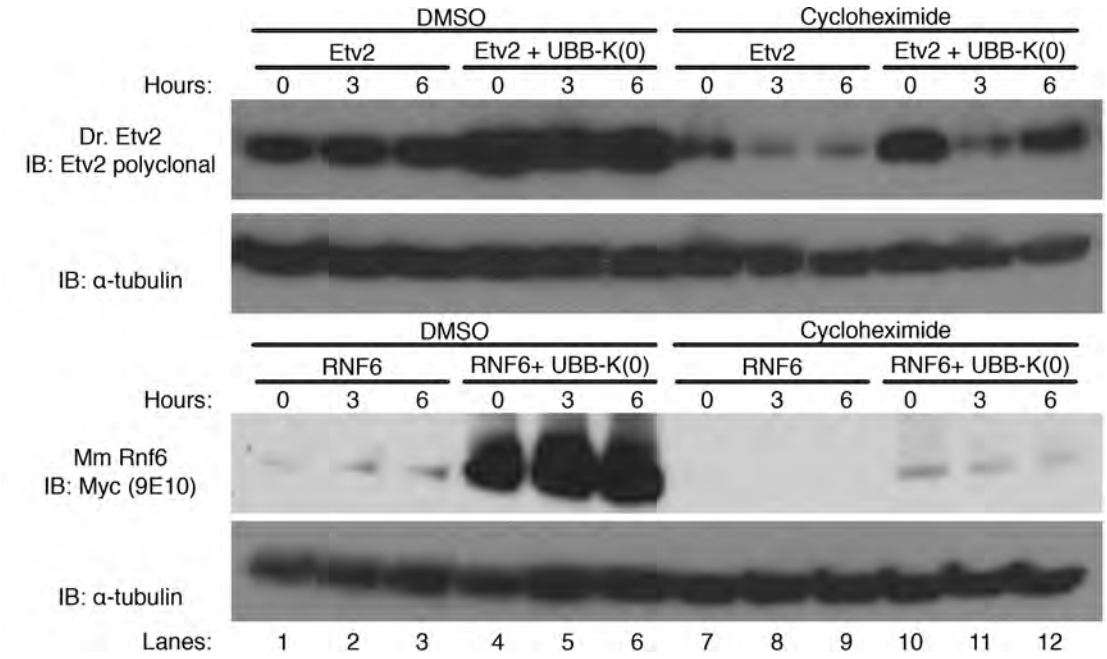



**Figure IV – 4: Etv2 is stabilized in the presence of a non-polymerizable form of ubiquitin.** HEK293T cells were transfected with mammalian expression vectors for zebrafish Etv2 (pCS-*etv2,* top) or myc-tagged mouse rnf6 (pCSMT-*rnf6,* bottom) with and without co-transfection of a vector expressing a non-polymerizable form of ubiquitin (pRK5-HA-Ubiquitin-KO). Transfected cells were treated for the indicated times with cycloheximide (50µg/mL) or DMSO as a control. Immunoblots of cell lysates were probed with Etv2 polyclonal antiserum or a monoclonal against the myc epitope (9E10). Blots were stripped and reprobed with a monoclonal antibody for α-tubulin.

**Figure IV – 5**

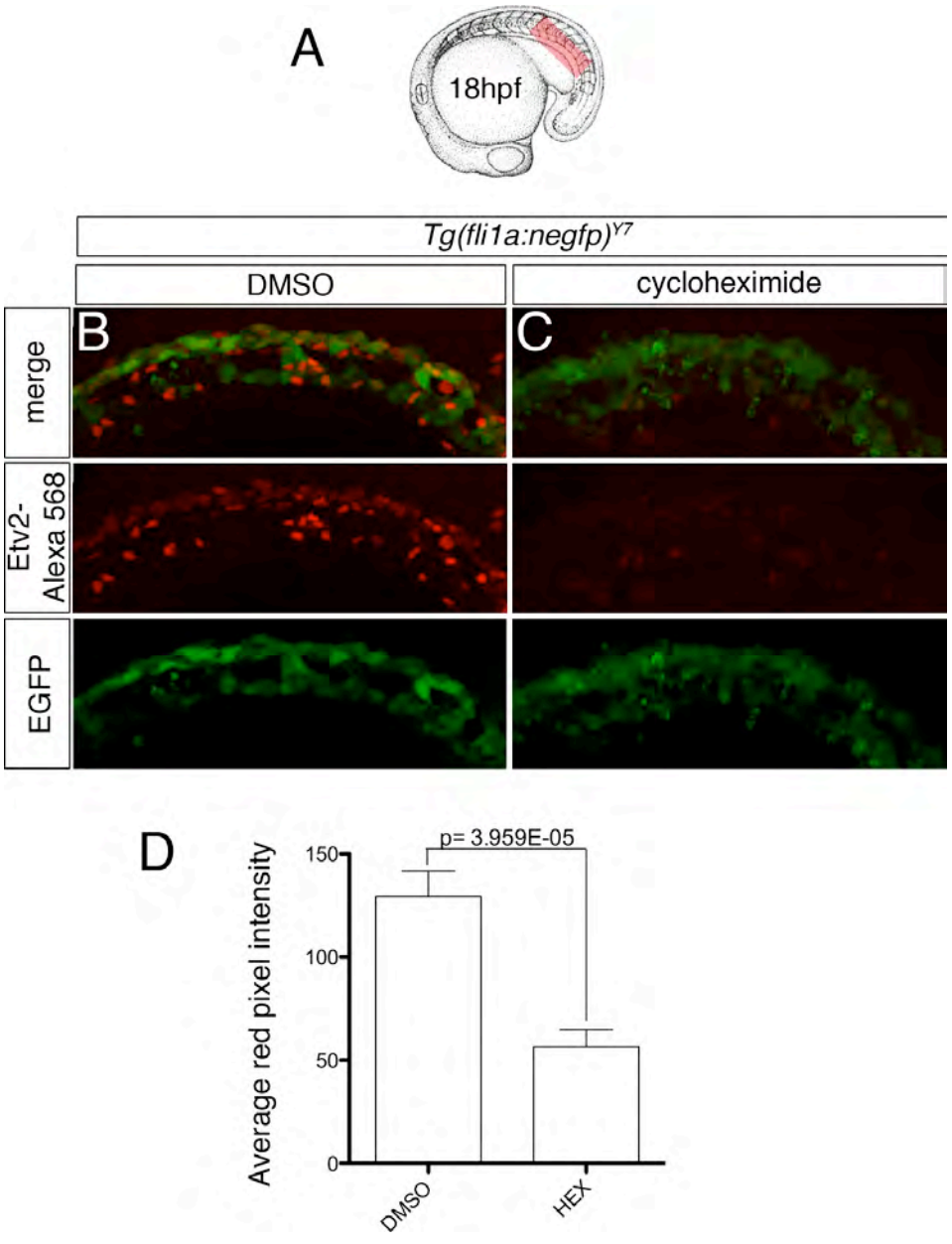

A
18hpf
Tg(fli1a:negfp)Y7
DMSO
cycloheximide
merge
Etv2-Alexa 568
EGFP
B
C
D
p= 3.959E-05
Average red pixel intensity
150
100
50
0
DMSO
HEX

**Figure IV – 5: Etv2 is rapidly turned over *in vivo*.** (A) Camera lucida drawings depicting the developmental stage and area (red box) of embryos that were imaged in B-D. (B,C) Representative Two-photon micrographs of Etv2 immunostaining in 18ss *Tg(fli1a:negfp)*$^{y7}$ treated wth DMSO or Cycloheximide (50µg/mL) for three hours at 15ss. Top panels, overlay of Alexa-568 and EGFP signals. Middle panels, immunostained Etv2 protein was detected with Alexa-568 secondary antibody. Bottom panels, transgenic expression of nuclear-localized EGFP. (D) Quantification of average Etv2 fluorescence pixel intensity from DMSO and cycloheximide-treated embryos.

## Discussion

*Etv2* down-regulation is required for the proper formation of the vasculature system. We have previously described a mechanism whereby post-transcriptional miRNA mediated repression by the *let-7* family regulates *etv2* expression. In this work we have added post-translational ubiquitin-dependent proteolysis as a new mechanism controlling Etv2 expression. We demonstrated that zebrafish Etv2 is ubiquitinated and degraded by the proteasome in HEK293T cells. Additionally, Etv2 is stabilized in the presence of a non-polymerizable form of ubiquitin. Our *in vitro* cell culture results led us to look at protein stability *in vivo*. Etv2 is highly unstable during zebrafish mid-somitogenesis, as demonstrated by rapid degradation in the presence of cycloheximide. Altogether, our results demonstrate that both post-transcriptional and post-translational mechanisms ensure correct temporal *etv2* expression leading to the proper formation of the vasculature.

Etv2 is highly unstable and is degraded by the proteasome in an ubiquitin-dependent manner. Etv2 protein sequence analysis revealed the presence of a potential PEST domain immediately upstream of the DNA binding domain. PEST domains have only been found in less than 10% of mammalian proteins in SWISS-Prot, and the majority of PEST-sequence containing proteins are rapidly degraded [204]. The presence of an unstructured region as an initiating site of proteolysis is another requirement for proteasomal degradation [208, 209]. The potential strong PEST domain overlaps with an unstructured region making this protein segment the mostly likely degron signal for Etv2.

Consistent with PEST domains conferring instability, we found that in the presence of the translation inhibitor cycloheximide, both zebrafish and mouse Etv2 are rapidly degraded. Etv2 degradation is proteasome dependent because the proteasome inhibitor MG132 both stabilizes exogenous Etv2 and rescues its cycloheximide-induced instability. Proteasomal degradation is likely initiated by ubiquitination because Etv2 is coimmunoprecipitated with ubiquitin, and Etv2 is stabilized when co-expressed with a non-polymerizable form of ubiquitin [206]. All these effects are likely mediated by the internal degron we identified but more biochemical analysis is needed to confirm this. However, loss of the majority of the N-terminal domain of Etv2 including the potential degron stabilized the C-terminal DNA binding domain in HEK293T cells even in the presence of cycloheximide. Similarly, fusing mCherry to the N-terminal region of the Etv2 protein (minus the DNA binding domain) destabilized the chimeric fusion protein leading to a reduction in red fluorescence (unpublished observations). Sequential segmental deletion of the Etv2 protein and further analysis is needed to definitively identify the degradation signal encoded in the Etv2 protein.

Consistent with our cell culture results, we found that endogenous zebrafish Etv2 protein is highly unstable and quickly degraded in the presence of cycloheximide. This result adds support to our previous analysis of the post-transcriptional regulation of *etv2* by the *let-7* family and the developmental timing of *etv2's* functional requirement. We previously speculated that the phenotypic similarity of *let-7* repression and translation inhibition by morpholino was caused first by translational inhibition and then a concomitant reduction in etv2

transcription due to the disruption of a positive auto-regulatory mechanism. Our finding that Etv2 is rapidly turned over supports this model, because even minor decreases in Etv2 translation would reduce the overall amount of Etv2 protein, which is rapidly degraded. This would then lead to exacerbated decreases in the transcriptional activity of the *etv2* promoter. Furthermore, the instability of Etv2 helps alleviate concerns *etv2* is actually required significantly later than suggested from our conditional knockdown experiments in Chapter II, because the knockdown from uncaging may be too slow and/or partially effective. After uncaging, the morpholino blocks translation of Etv2, which is rapidly cleared from endothelial cell progenitors. The morpholino works because we get the reported phenotype when the *etv2* cMO is uncaged at early time points. Therefore, it is not likely slow or partially effective and the auto regulatory mechanism described above would likely compensate for any inefficiency. Therefore Etv2's developmental functional window and auto-regulation mechanism is strengthened by the revelation of Etv2 instability.

Our results demonstrate for the first time that Etv2 is ubiquitinated and degraded by the proteasome, which leads to additional questions [202]. Is there a specific E3 ubiquitin ligase that mediates Etv2 ubiquitination specifically in endothelial cells, or is a more general mechanism used? Both mouse and zebrafish Etv2 display robust down-regulation when overexpressed in HEK293T cells, which are not an endothelial cell line, suggesting a more general mechanism is utilized. Additionally, are other post-translational modifications, such as acetylation, sumoylation or phosphorylation acting on Etv2, and do these

additional modifications affect protein stability? Ets1 sumoylation represses its transcriptional activation, whereas phosphorylation can either activate or repress Ets1 transcriptional activity [71, 79, 202, 210]. Phosphorylation of the ETS transcription factor ESE-1 prevents its ubiquitin-dependent proteasomal degradation [211]. In contrast, the ETS transcription factor MEF (ELF4), is regulated by phosphorylation-dependent proteolysis via the general class of Skp2-Cul1/Cdc53-F-box (SCF) poly-ubiquitinating protein complex [212]. The post-translational regulation of ETS transcription factors is a complex and multidimensional process, and Etv2 is likely to be regulated by additional mechanisms beyond ubiquitination.

# CHAPTER V: PERSPECTIVES AND FUTURE DIRECTIONS

**Figure V-1**

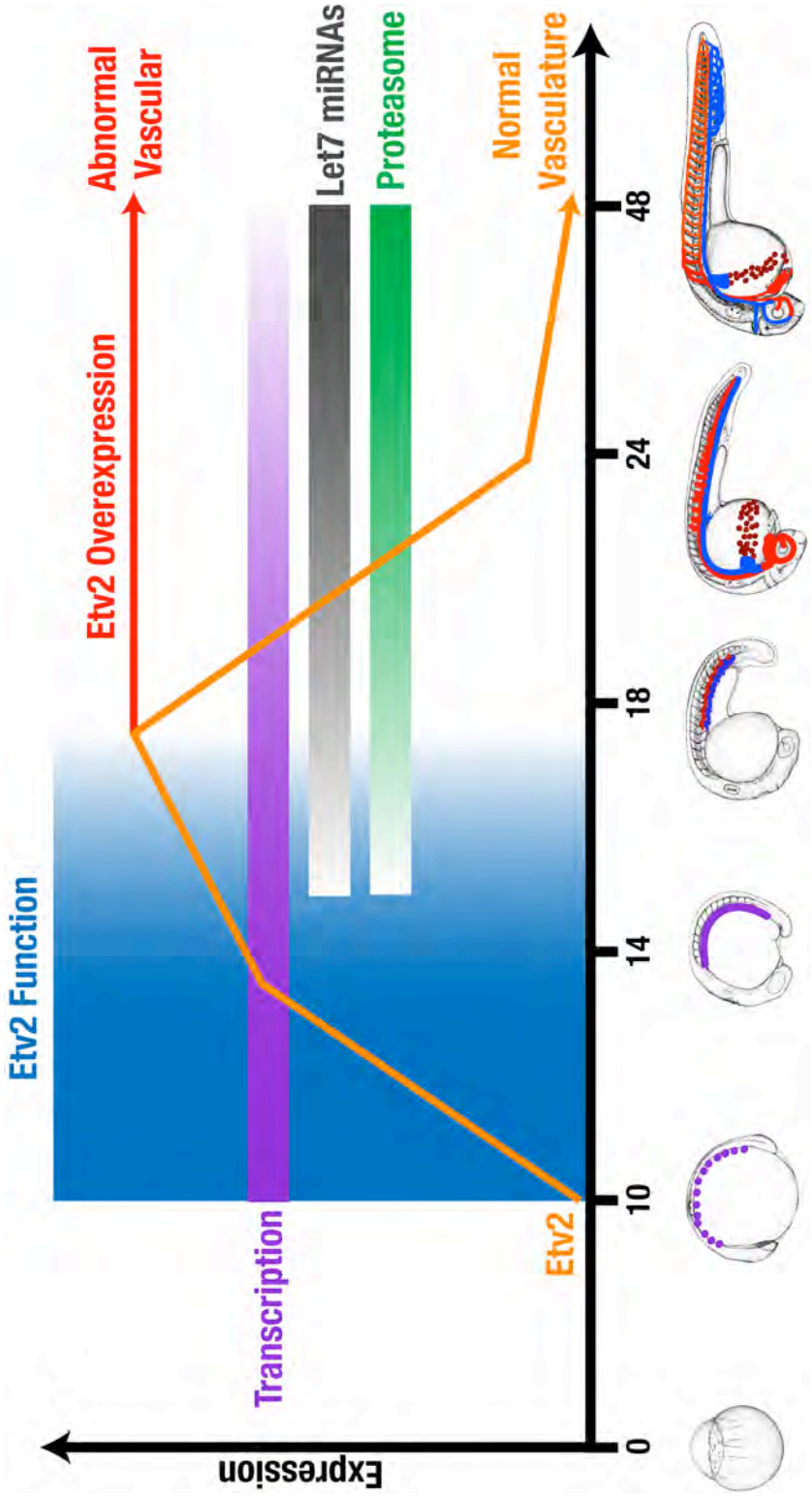

Etv2 Function
Expression
Transcription
Etv2 Overexpression
Abnormal
Vascular
Let7 miRNAs
Proteasome
Normal
Vasculature
Etv2
0
10
14
18
24
48

**Figure V-1: Etv2 expression and regulation summary.** Etv2 is expressed early during somitogenesis, with expression peaking around 18 ss and then it is rapidly down-regulated (orange line); Etv2 down-regulation is required for normal vascular development. Etv2 function is required early but not as long as Etv2 is expressed (blue box). Etv2 is transcribed at the start of its expression and is eventually turned off sometime before 48 hpf (purple box; [119]). The *Let-7* family of miRNAs (gray box) and ubiquitin-dependent proteasomal degradation (green box) starts negatively regulating Etv2 expression sometime after its function is no longer required. If Etv2 expression persists (red line) then the vasculature is unable to form properly [122, 139]. The camera lucida drawings below the x-axis represent the stage of vascular development at each time point depicted. Endothelial precursors (purple), arterial (red) and venous (blue) endothelial cells or vessels are illustrated.

The preceding work makes a significant contribution in understanding the role and regulation of *etv2* during vascular development in the zebrafish. The expression, function and regulation of *etv2* during zebrafish vascular development are summarized in (figure V-1). In combination with previous published studies in other animals, our results in the zebrafish demonstrate the Etv2 mode of action and mechanisms of regulation are conserved across species.

**Expression and function of Etv2**

*Etv2* is a transiently expressed autonomous master regulator of endothelial cell lineage fate and is down regulated by multiple mechanisms to insure vascular development occurs normally. Through carefully staged and quantified expression studies we found that *etv2* transcripts are expressed early during zebrafish development in angioblasts concomitant with their emergence from the lateral mesoderm. However, *etv2* transcript expression is transient and is nearly undetectable in the differentiated endothelium by 24 hpf, consistent with previous reports in mice, *Xenopus* and differentiating embryonic stem cells (Figure V-1, orange line; [8, 116, 120]). Visualization of Etv2 protein expression demonstrates Etv2 protein mirrors its RNA expression profile. Our results tighten the window of expression previously reported for Etv2 in the zebrafish [6], and presents the first known antibody raised to Etv2. Furthermore our conditional expression experiments show that Etv2 expression is transient, as well as its functional requirement. Etv2 functions in a short developmental time window

beginning with the start of angioblasts specification during early segmentation stages and prior to the completion of the primitive vascular cord (Figure V-1; blue box). *Etv2* is expressed in endothelial progenitors and its early conditional ablation prevents the formation of the vascular system, highlighting its requirement for endothelial lineage commitment. We demonstrate this requirment is cell autonomous through mosaic transplants analysis. *Etv2* specifies endothelial cells autonomously by activating endothelial gene programs. We identify through a combination of gene expression studies using microarrays that *etv2* induces a multitude of endothelial cell enriched genes consistent with previously published studies [129-131]. Our microarrays revealed that some of the most highly induced transcriptional targets of *etv2* are transcription factors essential for normal vascular development. We hypothesize that *etv2* initiates angioblast specification by inducing a transcription factor cascade that is subsequently responsible for the continued differentiation of endothelial cell lineages. We know from overexpression studies in mice and in endothelial cell transdifferentiation studies that Etv2 downregulation is a requirement for terminal endothelial cell differentiation or endothelial cells retain a progenitor like state (Figure V-1, red line; [122, 139]), but what caused *etv2* down-regulation was previously unknown.

### N**egative regulation of Etv2**

*Etv2* is negatively regulated by multiple mechanisms to allow the proper formation of the vasculature. Identification of post-transcriptional and post-

translational Etv2 negative regulatory mechanisms is the most novel contribution our work brings to the field of developmental vascular biology and illuminates why *etv2* is transiently expressed. Transcriptional regulation is another mechanism that can be gleaned from the literature as regulating *etv2* expression [119, 129]. Analysis of the *etv2* promoter in zebrafish has identified an enhancer that recapitulates the transient expression of *etv2* during development. The transgene is expressed in the developing zebrafish endothelium until 2 days at which time expression is lost suggesting *etv2* is transcriptionally repressed (Figure V-1, purple box; [119]). Interestingly, *etv2* also activates its own transcription [129]. Thus far we are unsure if *etv2* transcriptional repression is caused by active repression complex recruitment, passive loss of activation or a combination of both. The self-activating positive feed back mechanism used by *etv2* suggests that it acts a bi-modal switch for its own expression, suggesting a more passive loss of transcriptional activators controls *etv2* transcriptional regulation. When Etv2 protein is negatively regulated, transcription can be turned off quickly by depleting the available pool of factors activating transcription at the promoter. However, the initial *etv2* transcriptional activator has yet to be identified. We have identified two mechanisms that work to limit the Etv2 protein pool during development and hence turn off *etv2* expression. We found that the 3'UTR of *etv2* is able to post-transcriptionally repress exogenous transcripts and the *let-7* family of miRNAs mediates this repression. Over-expression of *let-7* duplex RNAs represses exogenously expressed *etv2* 3'UTRs and endogenous transcripts with a concomitant reduction in Etv2 protein expression. Additionally,

mutation of *Let-7* binding sites in the *etv2* 3'UTR suppresses its post-transcriptional repression indicating there sufficiency in mediating repression. Therefore, the *let-7* family is able to block translation from the *etv2* transcript inhibiting Etv2 protein accumulation (Figure V-1, gray box). Importantly, *let-7* overexpressing cells are prevented from committing to endothelial cell lineages when transplanted into Wt zebrafish because *etv2* is repressed disrupting angioblast specification. *Let-7* mediates post transcriptional repression by binding 3'UTRs and deadenylating transcripts disrupting translation [213]. The second mechanism, post-translational degradation, which insures Etv2 protein down-regulation in endothelial cells, has a more direct effect. Exogenously expressed Etv2 protein is ubiquitinated and degraded by the proteasome in HEK293T cells. We demonstrate the instability of Etv2 protein in culture was also happening *in vivo*. Therefore, Etv2 is post-translationally down-regulated by ubiquitin-dependent proteasomal degradation (Figure V-1, green box). Unfortunately our work was unable to identify the negative actor in Etv2 post-translational degradation un-like our ability to identify *Let-7* as a post-transcriptional regulator. We have thus far been unable to single out a particular E3 ubiquitin ligase that recognizes Etv2 marking it for destruction. However, Etv2 degradation is probably caused by a general mechanism as it happens in non-endothelial cells and is likely mediated through its overlapping strong PEST motif and inherently unstructured region, which make up its most likely degron. Altogether, this work has demonstrated that Etv2 is required early to autonomously specify angioblast from the mesoderm and does not function later

during endothelial cell differentiation. Furthermore, the transient nature of Etv2 expression is caused by a combination of post-transcriptional and post-translational mechanism that ultimately act to reduced the transcription of *etv2*. However, a number of important biological and mechanistic questions remain surrounding the function and regulation of Etv2 during vascular development.

**Figure V-2**

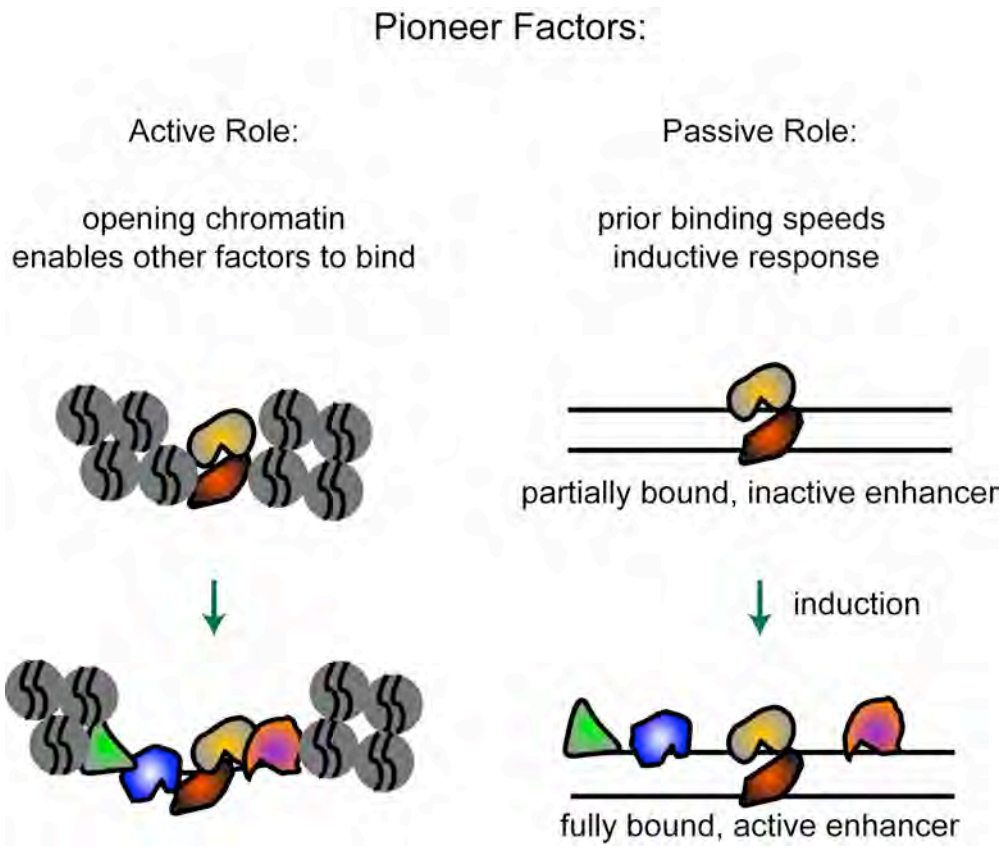



**Figure V-2: Mechanistic Roles of Pioneer Transcription Factors.** In an active role, pioneer factors can directly facilitate other factors binding to regulatory regions by opening up the local chromatin. In the passive role, prior binding of pioneer factors to regulatory sequences, such as an enhancer or promoter, reduces the number of additional factors that are needed to bind at a later time point to create an active enhancer. Modified from [214].

**Etv2 as a pioneer transcription factor**

How does Etv2, a single transcription factor, execute an entire program of endothelial cell differentiation? It is interesting to speculate that Etv2 is a "pioneer transcription factor" because it is one of the earliest known genes expressed in endothelial cell progenitors and is required for endothelial gene expression [6-8, 116, 120]. Additionally, it is sufficient to activate endothelial gene expression on its own and in non-mesodermal tissues [6, 127, 129-131]. A pioneer transcription factor must perform a genetic function early in the activation of transcription in a particular lineage but they must also physically bind to the genome prior to activation and prior to other factors binding and therefore imparting competence for activation. One of the first established pioneer transcription factors to be identified was the bHLH transcription factor family member MyoD1, which is required for skeletal muscle differentiation from the mesoderm. Expression of MyoD1 on its own can induce skeletal muscle differentiation in multiple cell types [215]. Another example of a pioneer transcription factor is the forkhead transcription factor family member Foxa1, which is required for the induction of the liver from the endoderm [216]. Both MyoD1 and Foxa1 impart competence for transcriptional activation by binding to the genome and opening compacted chromatin, thereby opening binding sites for other trans-acting factors. Myod can initiate access of genes in repressive chromatin by initiating chromatin remodeling through the recruitment of histone acetyltransferases (HATs) and the Swi/Snf chromatin-remodeling complex [217-219]. Alternatively, Foxa1 has an inherent ability to disrupt local internucleosomal

interactions that are known to stabilize chromatin higher order structures [220, 221]. Nearly nothing is known about how Etv2 activates gene transcription except that it contains an N-terminal activation domain [107], which bears no motif homology to any other known proteins. Etv2 is required early to initiate a transcriptional cascade leading to endothelial cell differentiation but we don't know if it does so by opening up compacted chromatin (Chapter II). However, chromatin-remodeling is essential for the proper formation of the vascular system. Endothelial specific knockout of brahma-related gene 1 (BRG1), one of the catalytic ATPases of the Swi/Snf complex is required for endothelial cell differentiation. The extraembryonic yolk sac vasculature in these conditional mutants is dilated and fails to remodel [222]. Although chromatin remodeling plays a role in endothelial cell differentiation we do not know if Etv2 is able to recruit factors able to open chromatin. However, there is evidence that endothelial expressed ETS proteins can. Ets1 and Ets2 are capable of recruiting p300, which acts as both a bridge between DNA bound transcription factors and the basal transcriptional machinery and as a HAT, therefore linking chromatin remodeling with transcriptional activation [223-225]. This example indicates ETS transcription factors can have an active role in the opening and organization of the local chromatin structure around a gene (Figure V-2). However, Etv2 may play a more passive role as transcriptional activator. Pioneer factor binding may not alter chromatin structure and/or allow other factors to bind but instead the mere presence at gene regulatory sequences can reduce the number of subsequent factor-binding events needed for transcriptional activation after an

inductive signal (Figure V-2, [214]). This passive model of activation is supported by the finding that that the majority of promoters require binding by a combination of transcription factors [226-228]. Etv2 has been shown to synergistically activate endothelial cell genes in combination with the foxc1/2 genes in mouse and ETS/Fox composite binding sites are strong computational predictors of endothelial gene expression [49, 61]. Therefore it is possible that Etv2 binding to gene regulatory regions primes these genes for activation but it is not until the Foxc1/2 binds, that transcription is activated. Furthermore, it is possible that the BMP, Wnt, and Notch pathways responsible for angioblast induction from the mesoderm provide the signal necessary for additional co-activators to bind with Etv2 initiating endothelial gene expression [8]. Currently, we cannot distinguish between the active and passive activation of endothelial cell gene expression by Etv2 and the reality is it likely uses a combination of both. Etv2 is a master endothelial lineage determinant and is expressed and functions like a pioneer transcription factor. Studies analyzing the capacity of Etv2 to modulate chromatin structure will be of great interest and will define the mechanistic nature of its transcriptional activity.

**Etv2 in disease**

What if any role does Etv2 play in the pathogenesis of vascular dysfunction and human disease? Excessive blood vessel growth is a significant cause of age related macular degeneration and underlies proliferative diabetic retinopathy [229, 230]. Additionally, vascularization of sold tumors is essential for their growth and eventual metastasis [4]. However, blood vessel growth in all these

cases forms through angiogenic processes; Etv2 is not expressed in the differentiated endothelium and is down-regulated even before the start of developmental angiogenesis, therefore making Etv2 a poor etiological agent for these diseases (Chapter II, [60]). However, hemangiomas and angiosarcomas are benign and malignant neoplasms, respectively, of endothelial cells characterized by uncontrolled vascular growth [231, 232]. Is it possible that continued Etv2 overexpression in endothelial cells causes them to retain a progenitor like state causing continued proliferation and a failure to differentiate? Several endothelial enriched ETS transcription factors are causative agents of cellular transformation, metastasis and poor clinical prognosis [55]. Overexpresion of Ets1 and Fli leads to leukemia, and Erg overexpression can cause prostate cancer. Thus far we do not know if Etv2 functions in the formation of hemangiomas. However, hemangioma-derived endothelial cells are clonal and exhibit abnormal properties [233]. A hemangioma derived multi-potent stem cell that can recapitulate hemangiomad in immune-deficient mice has been identified [234], suggesting that hemangiomas are initiated from a single abnormal progenitor cell. It is intriguing to think that continual Etv2 activation could cause the abnormal retention of endothelial cell progenitor characteristics. Etv2 activation coul result from diminished *let-7* expression levels, because Etv2 expression and Let-7 expression are inversely correlated (Chapter III). Etv2 activation could also occur by mutation or loss of its degron preventing its ubiquitin-dependent proteasomal degradation. This is all highly speculative and

a great deal more work is needed to define the role of Etv2 in disease if there is one.

# APPENDICES

# APPENDIX I: ZEBRAFISH TRANSGENIC ALLOWS FOR TEMPORAL AND SPATIAL ANALYSIS OF DEVELOPMENTAL NOTCH ACTIVATION.

## Introduction

The Notch gene was discovered and named due to the identification of a *Drosphila melanogaster* mutant with a serrated wing phenotype in the early 20th century [235]. However, the gene responsible for this phenotype was not cloned until 1985, making it one of the oldest studied signaling pathways [236]. The Notch pathway is an evolutionarily conserved signaling mechanism that regulates cell fate decisions in early embryonic development in a wide variety of tissues in metazoans [237]. Notch receptors are large transmembrane receptors that interact with cell surface ligands of the Delta and Serrate/Jagged gene family [236, 238]. Upon ligand binding the Notch receptor is proteolytically cleaved, freeing the Notch intracellular domain (NICD) from the plasma membrane [239]. NICD translocates to the nucleus where it participates in a core transcriptional complexes with the DNA-binding domain protein CSL (CBF-1/RBP-Jk/KBF2 in mammals, Suppressor of Hairless [Su(H)] in *Drosophila* and *Danio rerio*, and Lag-2 in *Caenorhabditis elegans*), the nuclear effector Mastermind (Mam) and other transcriptional activators (Fig. AI-1 Top; [240, 241]). The NICD/CSL complex acts as a transcription factor to turn on Notch target genes[237]. CSL is known to bind the consensus site “YGTGRGAAM” and this cis element is sufficient to bestow Notch responsiveness to a transgene [242, 243]. Thus, the Notch signaling pathway affects cellular function mainly through the direct induction of Notch target genes.

In the vascular systezm, Notch plays a significant role in endothelial cell differentiation during blood vessel development. In zebrafish, expression of Notch components, including the receptors *notch1b*, *notch5*, and the Notch ligands *delta-like 4* (*dli4*) and *deltac* (*dlc*) are all restricted to arterial endothelial cells and excluded from venous endothelial cells [146, 244]. Disruption of Notch signaling within the zebrafish causes loss of artery marker gene expression and expansion of venous endothelial cell marker into arteries [94, 146, 245]. Conversely, over-expression of a constitutively active form of the intracellular domain of notch1 represses vein gene expression and causes ectopic expansion of the artery marker efnb2a into the posterior cardinal vein [146]. Similar to the zebrafish, arterial endothelial cells of mice express multiple Notch receptors as well as several Notch ligands [246]. Notch1 and Dll4 knockout mice are also embryonic lethal due to defects in endothelial cell differentiation and angiogenesis [247, 248]. In addition, mouse embryos with targeted deletion of the CSL locus specifically in endothelial cells display abnormal vascular development including arteriovenous malformations and loss of artery marker gene expression[249]. Zebrafish global CSL knockdown also causes arteriovenous malformations and an absence of trunk circulation phenotype [244]. Taken together these results reveal a conserved role for Notch signaling in establishing arterial endothelial cell fate during embryonic vascular development.

In addition to its role in arterial endothelial cell differentiation, Notch also plays an important role in specifying different cell types and coordinating cell behaviors during angiogenic sprouting. Notch deficient mouse and zebrafish embryos exhibit a “non-productive angiogenesis” phenotype, characterized by excessive branching and blood vessel density and failure of these excess vessels to carry blood flow [12, 244, 248]. Conversely, Notch activation blocks angiogenic sprouting [44, 244, 250]. Interestingly, the Notch ligand *dll4* is most highly expressed in the tip-cell of a sprouting vessel, and is induced by vascular endothelial growth factor (VEGF) [251]. It is thought that *dll4* activates *notch1* in the trailing cell behind the Dll4 expressing tip-cell [44]. Subsequent Notch activation reduces the angiogenic behavior in the trailing cell enabling it to maintain its connection to the patent vasculature, in part through the down regulation of *vegf receptor-3* (*flt4*) expression [44]. Consistent with this model, tip cell marker gene expression is up-regulated in Notch or Dll4 deficient zebrafish or mouse embryos and this is associated with excessive endothelial cell proliferation and migration [44, 244]. Despite the significant amount of work concerning the role of Notch in blood vessel development, little is known about the temporal dynamics of Notch activation, the vascular cell types that activate notch and the ligands responsible for Notch activation.

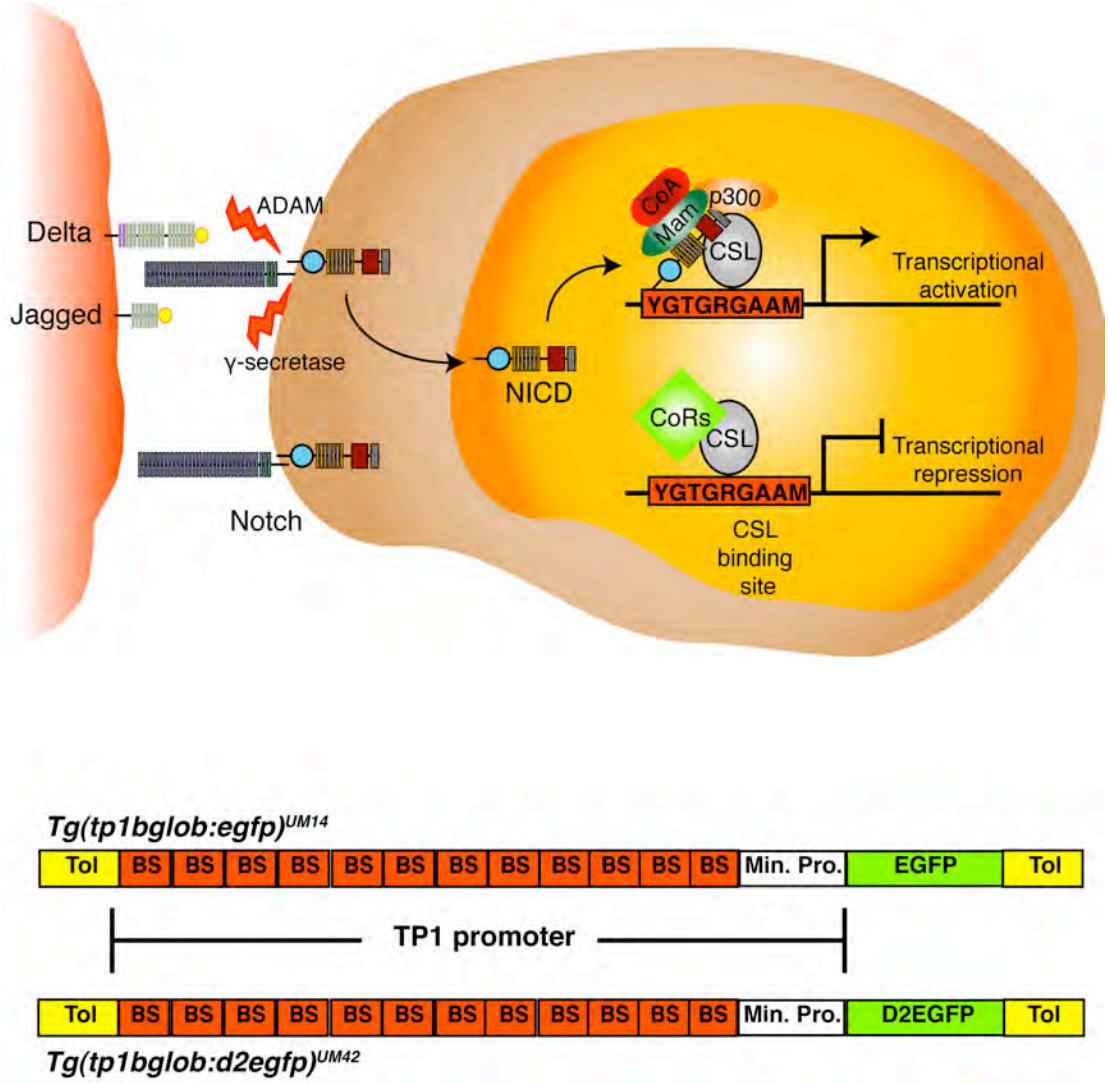
To date there has been few mechanisms to visualize Notch activation in the vascular system in real time in the zebrafish. Antibodies have been

designed to the -secretase-cleaved form of Notch and hence the activated form of Notch, but it is only useful in fixed tissues or lysates [252, 253]. In-situ hybridization for a Notch target genes will tell you that Notch was activated in a particular cell type but suffers from the same limitations, in that it cannot be followed in real time [146]. A transgenic zebrafish line that drives red fluorescent protein driven by the Notch target gene promoter *her4* has been developed and allows for *in vivo* analysis of Notch activation. However, transgene expression is restricted to the nervous system.

In an effort to view global Notch activation during development in the zebrafish embryo and to help inform its role during vascular development we generated transgenic Notch indicator lines (NIL). These NILs allow the temporal and spatial visualization of notch activity in multiple tissue types, but most interestingly for our studies the developing vasculature. A murine Notch Activity Sensor (NAS) transgenic line as previously been generated [242] and we have adapted their approach to generate a similar zebrafish transgenic. To visualize Notch activation we utilized the well-characterized TP1 promoter, which consists of 12 multimerized CSL binding motifs upstream of a rabbit β-globin minimal promoter [254, 255]. The CSL binding motifs are derived from the Epstein-Barr Virus terminal protein (TP1) gene promoter [256, 257]. The TP1 promoter was cloned upstream of enhanced green fluorescent protein (EGFP) or a destabilized version containing a C-terminal PEST domain from the mouse Oaz gene (D2EGFP; [258]), and flanked by Tol2 transposable

elements for efficient transgenesis. [259]. The constructs were injected into wild type zebrafish and stable transgenic raised, generating NILs *Tg(tp1bglob:egfp)*$^{UM14}$ and *Tg(tp1bglob:d2egfp)*$^{UM42}$ (Fig. AI-1 lower panel).

**Figure AI - 1**


Delta
Jagged
ADAM
γ-secretase
Notch
NICD
CoA
Mam
p300
CSL
YGTGRGAAM
Transcriptional activation
CoRs
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YGTGRGAAM
Transcriptional repression
CSL binding site
Tg(tp1bglob:egfp)UM14
Tol
BS
Min. Pro.
EGFP
TP1 promoter
D2EGFP
Tg(tp1bglob:d2egfp)UM42

**Figure AI-1: Notch activation and intracellular domain nuclear translocation activates Notch indicator transgene expression.** CSL, the Notch receptors cognate nuclear binding partner binds its conserved DNA binding sequence and represses gene expression in the absence of Notch signaling. Notch receptor binding to either the Jagged or Delta family of ligands causes two proteolytic processing events; as a result the Notch intracellular domain (NICD) is freed from the membrane and translocates to the nucleus. NICD binds to CSL displacing co-repressors and recruits co-activators to initiate transcription of Notch target genes (upper panel). Diagram of injection constructs used to make Notch indicator lines (lower panel). Twelve CSL binding sites were concatemerized upstream of a rabbit β-globin minimal promoter driving expression of enhanced green fluorescent protein [EGFP; *Tg(tp1blgob:egfp)*$^{UM14}$] or a destabilized version of EGFP [D2EGFP; *Tg(TP1:d2egfp)*$^{UM42}$] in a Notch dependant manner flanked by Tol2 transposable elements for efficient genomic integration.

## Materials and Methods

### Zebrafish Handling and Maintenance

Zebrafish and their embryos were handled according to standard protocols [141] and in accordance with the University of Massachusetts Medical School IACUC guidelines. The *Tg(flia.ep:DsRedex)*$^{UM13}$ transgenic has been described previously [16]. The *Tg(tp1bglob:egfp)*$^{UM14}$ and *Tg(tp1blob:d2egpf)*$^{UM82}$ line where made by injecting 25pg of purified pToltp1blglob:egfp or pToltp1bglob:d2egfp plasmid, respectively with 25pg of Tol2 transposase mRNA [259] into one-cell *wildtype*$^{CF}$ embryos to make mosaic F0 fish. Founders where identified and stable transgenic F1 fish were screened for transgene expression in the expected tissues and raised.

### Plasmids and Riboprobes

The Notch Indicator promoter element (12 CSL binding sites and β-globin minimal promoter) was PCR amplified off of plasmid pGA981-6 [254] using primers containing gateway attB4 and attB1r linkers (primers are in appendix II). The size-selected amplicon was BP cloned into pdonrP4P1r (Invitrogen) to yield p5E-TP1. The p5E-TP1 plasmid was used in a multisite gateway LR reaction with pME-egfp and pDestTOL2pA [145] to yield pToltp1blglob:egfp. The pToltp1bglob:d2egfp was made in a similar manner by substituting pME-egfp with pME-d2egfp. pME-d2egfp was made by PCR amplifying the destabilized version of EGFP (d2egfp) coding sequence from pGFP–PEST

[258] using primers containing gateway attB1 and attB2 linkers (Invitrogen; appendix II), and was BP cloned into pdonr221.

**Morpholino Injections**

Morpholinos have all been previously described and were injected into one cell *Tg(flia.ep:DsRedex)*$^{UM13}$; *Tg(tp1bglob:egfp)*$^{UM14}$ double transgenic embryos at the following concentrations: dll4(15ng) [244], dlc(8ng) [260], vegfA(10ng) [261], and 15ng control MO(standard scrambled control). Morphant phenotypes were imaged at 30 hpf.

**Chemical Notch inhibition**

*Tg(tp1bglob:egfp)*$^{UM14}$ were crossed and progeny collected. Embryos where raised at 28.5$^{o}$C until shield stage where embryos where manually dechorinated and placed in 1% agar-egg water coated 6 well plates. Half where were incubated in 50µM DAPT (N-[N-(3,5-Difluorophenace- tyl)-L-alanyl]-S-phenylglycine t-butyl ester; Sigma-Aldrich) (stock solution of 100 mM in DMSO) and the other with equal volumes of DMSO. EGFP expression was analyzed at 30hpf.

**In situ hybridization**

Fluorescein-labeled anti-EGFP and biotin-labeled anti-etv2 riboprobes where made off of pCS-EGFP [146] and pCS-Etv2 (described in Chapter II) respectively, using T7 polymerase, appropriately labeled nucleotides and

purified by mini Quick Spin RNA columns (Roche). Whole mount in situ hybridization was performed according to standard protocols [149].

**Double in situ hybridization**

Fluorescein labeled antisense EGFP probe and biotin labeled antisense Etv2 probe were generated for double fluorescent color whole mount in situ hybridization staining and described above. *Tg(tp1bglob:egfp)*$^{UM14}$ embryos were fixed at 12ss with 4% - paraformaldehyde (PFA) in PBS overnight at 4$^{o}$C. Embryos were then placed in 100% MetOH, and bleached with 3% - H2O2 in 100% MetOH for 1hr and washed with 100% MetOH. Embryos were then stored at -20$^{o}$C until hybridized. The double fluorescent hybridization was performed as described[262].

**Microscopy**

*Tg(tp1:blgob:egfp)*$^{UM14}$ embryos were imaged using an MZFLIII fluorescent dissection microscope under transmitted light or epi-fluorescence. *Tg(flia.ep:DsRedex)*$^{UM13}$; *Tg(tp1bglob:egfp)*$^{UM14}$ double transgenics were imaged using a using a Leica DMIRE2 confocal microscope (Objective: HC PL APO 20x/0.70CS).

## Results

*Visualization of Notch activation in zebrafish transgenic*

Characterization of the *Tg(tp1bglob:egfp)*$^{UM14}$ line EGFP expression by in situ hybridization or transgene visualization by epi-fluorescence microscopy demonstrates EGFP expression is dynamically expressed throughout development (Fig. A1-2A,B). Importantly, EGFP is expressed in tissues know to utilize Notch signaling including the developing somites (Fig. AI-2A, arrow heads) [260], central nervous system [263] and fin fold (Fig. A1-2B; [264]). Consistent with studies demonstrating a requirement for Notch in arterial endothelial cell differentiation, *Tg(tp1bglob:egfp)*$^{UM14}$ embryos express EGFP in the dorsal aorta (DA) but not in endothelial cells of the posterior cardinal vein (PCV; Fig. AI-2A, 24hpf insert; [146]). EGFP expression is Notch dependant as *Tg(tp1bglob:egfp)*$^{UM14}$ embryos treated with DAPT, a γ-secretase inhibitor that prevents Notch receptor cleavage and activation [265], eliminates EGFP expression (Fig. AI-2C). Together these data demonstrate that the NILs faithfully recapitulate Notch expression during development and transgene expression requires Notch activation.

*Notch activation in Endothelial cells*

Studies suggest that Notch signaling is dynamic and acts at multiple stages during vascular development, and the visualization of Notch is now possible thanks to the NILs we established [146, 244]. Although the NIL can inform the Notch activity in multiple tissue types [266-272], we have focused on the

endothelium because of our interest in better understanding endothelial differentiation and the availability of endothelial specific transgenic lines in which to do co-expression studies. Double in situ hybridization in Tg(tp1bglob:egfp)$^{UM14}$ transgenics for EGFP and etv2, an early marker of endothelial cells (Chapter I), indicate Notch is first expressed in endothelial cells around 12ss (Fig. AI-3A, arrows). Due to the dynamic nature of Notch activation in the endothelium we switched to using the *Tg(tp1bglob:d2egfp)$^{UM42}$* line, because the D2EGFP transgene has a relatively short half-life compared to EGFP [258] and allows Notch visualization *in vivo* without having to fix or process embryos. Standard EGFP is highly stable and long lasting; therefore some visualized EGFP positive cells in the *Tg(tp1bglob:egfp)$^{UM14}$* line may have activated Notch early in development but no longer require it. To co-visualize Notch activation at later time points more easily in the endothelium we crossed the the *Tg(tp1bglob:d2egfp)$^{UM42}$* line to the *Tg(fli1a.ep:DsRedex)$^{UM13}$* line, in which the fli1a gene promoter drives red fluorescent protein expression specifically in the endothelial cells[16]. The *Tg(tp1bglob:d2egfp)$^{UM42}$* transgenic demonstrates dynamic Notch expression during angiogenesis. Notch is barely detectable in the primitive vascular cord of *Tg(tp1bglob:d2egfp)$^{UM42}$*; *Tg(fli1a.ep:DsRedex)$^{UM13}$* embryos at 18ss (Fig AI-3B). We have previously demonstrated Notch positive endothelial cells as 12ss (Fig. AI-3A), therefore the lack of D2EGFP expression in endothelium may indicate notch was required earlier. However, it is more likely that the D2EGFP is expression at low levels and it is rapidly turned over making fluorescence hard to detect in the endothelium at this time point.

**Figure AI - 2**

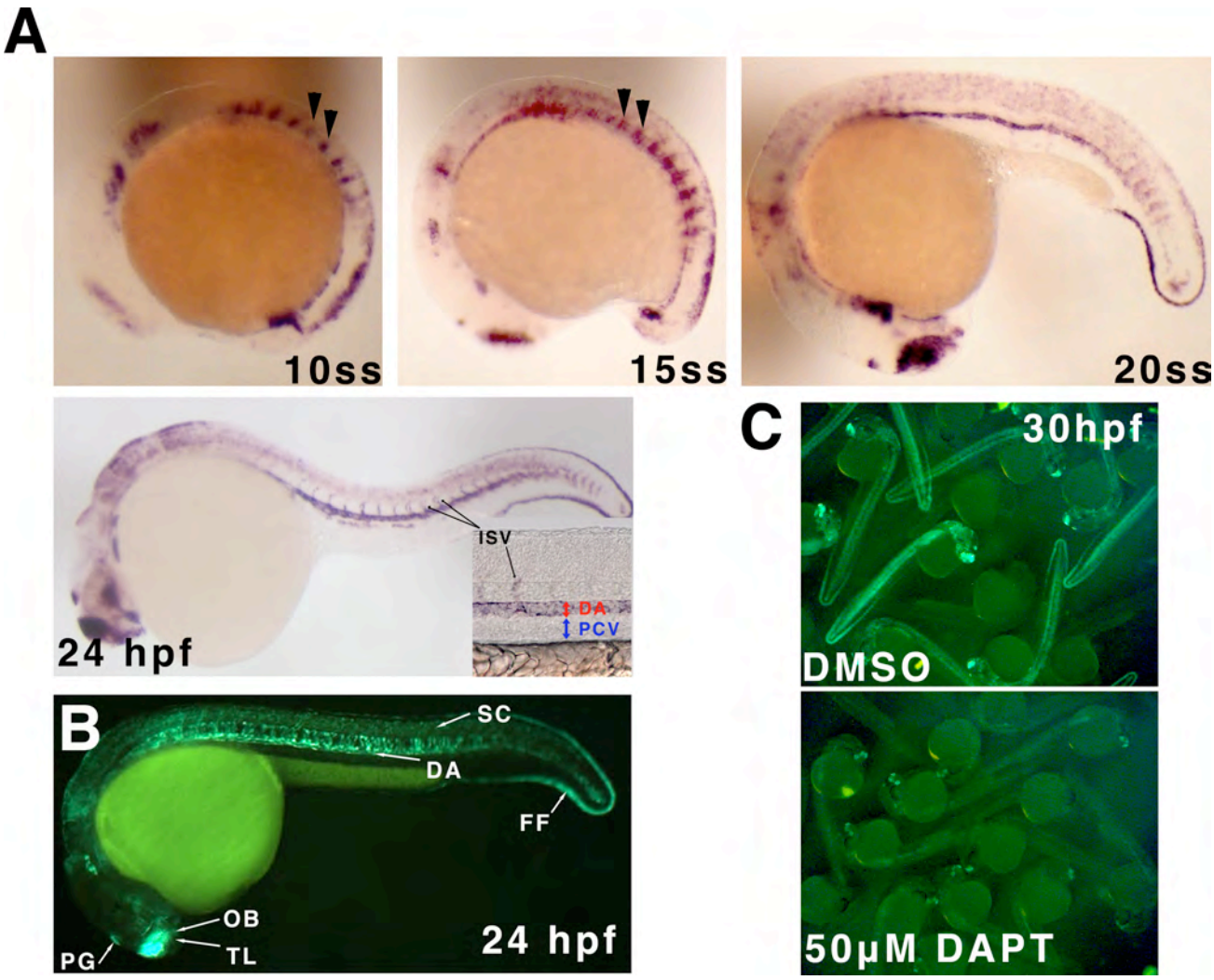


**Figure AI-2: The TP1 promoter recapitulates notch expression during development**. (A-C) *Tg(tp1bglob:egfp)*$^{UM14}$ transgenic zebrafish embryos at indicated developmental stages, lateral view, anterior to the left (A, B) . (A) Transmitted light images of in situ hybridizations for egfp transcript in transgenic embryos. Egfp is detectable in the somites (arrow heads), the intersegmental vessels (ISV) and in the dorsal aorta (DA) but not the posterior cardinal vein (PCV). (B, C) Epi-fluorescent images of transgenic embryos. (B) Transgene expression can be observed in the olfactory bulb (OB), spinal chord (SC), telencephalon (TL), pineal gland (PG), fin fold (FF) and DA. (C) Grouped transgenic embryos demonstrate strong transgene expression when treated with DMSO control (upper panel) compared to low or absent expression of egfp in embryos treated with 50µM DAPT at shield stage (lower panel).

**Figure AI - 3**

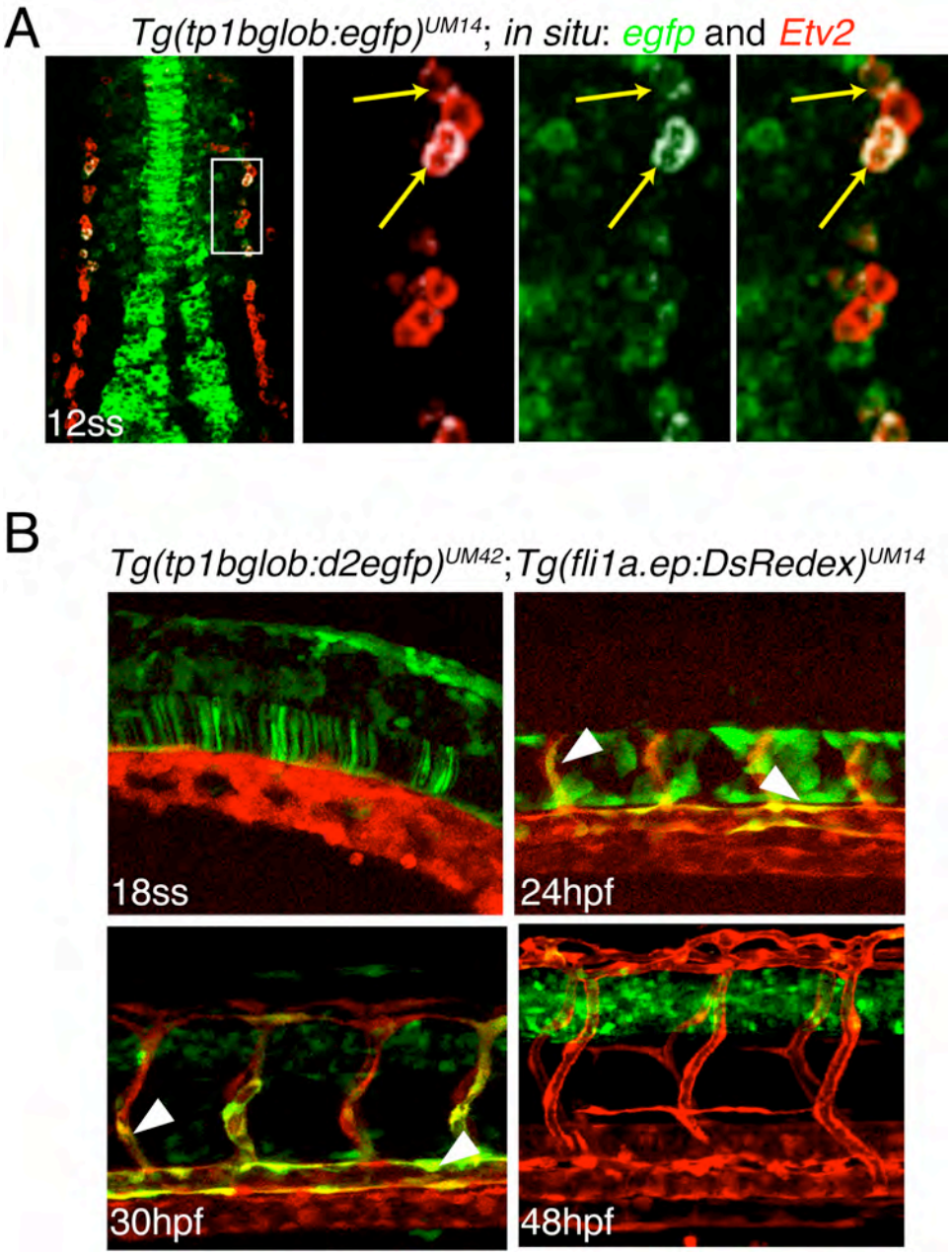


**Figure AI-3: Notch is dynamically expressed in the developing zebrafish vasculature.** (A) Epi-fluorescent images of flat mounted 12ss *Tg(tp1:egfp)*$^{UM14}$ embryos in situ hybridized for egfp (green) and etv2 (red) transcripts, anterior is up. Three right panels are magnified views of boxed area on left, arrows indicate cells with co-expression. (B) Confocal micrographs of developing trunk vasculature at indicated time points in double transgenic *Tg(tp1blgob:d2egfp)*$^{UM42}$; *Tg(fli1a.ep:DsRedex)*$^{UM13}$ zebrafish embryos, lateral view anterior to the left. Arrow heads indicate Notch expressing endothelial cells.

Accordingly, Notch positive endothelial cells are present at 24hpf and 30hpf, specifically in the DA and intersegmental vessels (ISV) consistent with Notch's role in artery development (Fig. AI-3B). Interestingly, D2EGFP expression is no longer expressed in the vasculature by 48hpf, indicating Notch is no longer activated in the endothelium at this stage. Therefore the NILs allow for temporal dissection of Notch activation in a tissue type specific manner through out development. Additionally, the NILs demonstrates that Notch is activated early but is subsequently inactivated in the endothelium of the DA as vascular development proceeds.

*Notch modulation by genetic manipulation*

To further validate Notch responsiveness in NILs, and to inform the use of particular Notch ligands in the vasculature we knocked down the function of several endothelial expressed Notch ligands and an endothelial inducer of Notch using morpholino anti-sense oligonucleotides and analyzed EGFP expression by epi-fluorescence microscopy at 30hpf in *Tg(tp1bglob:d2egfp)*$^{UM42}$; *Tg(fli1a.ep:DsRedex)*$^{UM13}$ embryos. Double transgenic embryos injected with scrambled control morpholino display positive endothelial cells located in the DA (Fig. AI-4B, arrow heads) and the ISVs (Fig. AI-4B, arrow). *Dlc* and *dll4* are two Notch ligands expressed specifically in arterial endothelial cells within the vasculature [244, 273]. Knockdown of *dll4* causes the loss of EGFP expression in the ISVs but not in the DA, consistent with the known role of *dll4* in ISV morphogenesis (Fig. AI-4C; [244]). *dlc* morpholino injection causes the loss of

EGFP expression in endothelial cells of both the DA and the ISVs, indicating dlc may work earlier than dll4 to specify arterial endothelial cells (Fig. AI-4D). Accordingly, the injection of both the dlc and the dll4 morpholinos completely eliminates EGFP expression in the vasculature. (Fig. AI-4E). The VEGF signaling pathway works up stream of Notch in a common genetic pathway to specify arterial endothelial cells and is required for ISV sprouting from the DA [18, 274]. Consequently, the knockdown of the vascular endothelial growth factor receptor-2 (VEGFR2/Kdrl) ligand, vascular endothelial growth factor-A (vegfa) causes a complete loss of EGFP expression in endothelial cells (Fig. AI-4F). In addition the ISVs fail to sprout as previously reported [274]. Our results demonstrate that the NIL is responsive to loss of Notch and can serve as a system to observe Notch expression upon genetic perturbation of Notch ligands or upstream activators.

**Figure AI - 4**

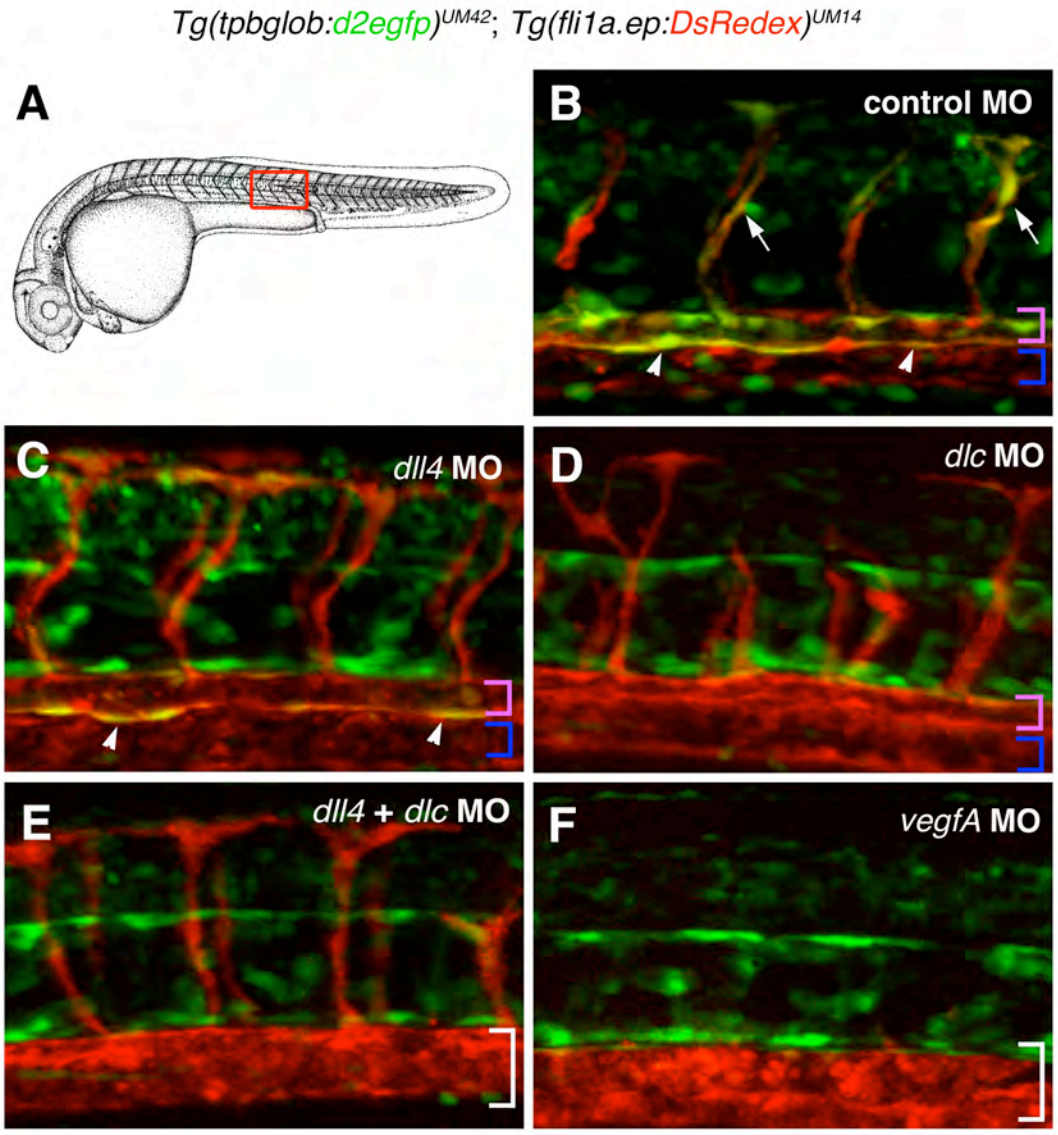


**Figure AI-4: Differential Notch indicator line expression in the vascular in response to Notch pathway disruption.** (A) Camera Lucida drawing of 30hpf zebrafish with box indicating views in (B-). (B-F) Confocal micrographs of trunk vasculature in 30hpf *Tg(tp1blgob:d2egfp)*$^{UM42}$; *Tg(fli1a.ep:DsRedex)*$^{UM13}$ embryos injected with 15ng of control MO (B), 15ng of dll4 MO (C), 8ng dlc MO (D), 15ng dll4 plus 8ng dlc MO (E) and 10ng of vegfA MO (F), lateral view anterior to the left. Red brackets demarcate the dorsal aorta, blue brackets demarcate the posteior cardinal vien and white brackets indicate an abnormal larger single vessel.

## Discussion

The NILs allow the *in vivo* visualization of notch activation in multiple tissue types throughout development. Importantly we know that the NIL transgene is a bona fide indicator of Notch activation because it is expressed in cell types at developmental time points known to require Notch activation, for example in the somites, nervous system and dorsal aorta. Further substantiating the NILs faithful recapitulation of Notch activation, treatment with the -secretase (DAPT) inhibitor which prevents notch receptor cleavage and translocation to the nucleus abolishes transgene expression. Additionally, the NIL has been out-crossed to a sable transgenic line expressing an inducible NICD overexpression cassette driven by the heat-shock promoter [267]. Upon heat shock ectopic upregulation of the Notch indicator line is observed further demonstrating the NILs ability to respond to Notch signaling.

The method of multimerizing transcription factor binding sites joined to a minimal promoter has proven effective to study other pathways including the Wnt/b-catenin/LEF pathway [275, 276] or signaling through NF-kB [277]. The CSL binding sites used in the NIL constructs are derived from the Epstein-Barr virus terminal protein (TP1) promoter and have successfully been used to visualize Notch activation in mammalian systems. Interestingly, the same promoter elements work in the zebrafish and are a testament to the conservancy of the Notch pathway and the DNA binding sequence of the CSL gene. The artificial nature of the TP1 promoter would make mis-expression of

the transgene highly likely. However, transgene expression is seen in Notch required tissues and we are unaware of any ectopic transgene expression not related to Notch expression.

Our main interest in the Notch pathway way stems from its role in endothelial cell differentiation and the NILs have been useful in helping inform our understanding of the role of Notch during vascular development. Notch activation is detected in the early angioblasts at a time where notch is actively involved in the segmentation of the somites. This dual utilization of Notch in separate cell types at the same developmental time points highlights the context dependant function of the pathway. This can further be appreciated by our findings of dynamic Notch activation in endothelial cells. The *Tg(tp1bglob:d2egfp)*$^{UM42}$ NIL has a fluorophore that is much less stable and therefore allows for the visualization of fast acting Notch utilization. Analysis of *Tg(tp1bglob:d2egfp)*$^{UM42}$ line demonstrates that even during the relatively short developmental process of angiogenesis the Notch pathway is activated and then no necessarily maintained. Notch expression can be visualized in the dorsal aorta at the start of angiogenesis at 24hpf but by 48hpf most of the notch activation is absent in the fully formed patent vasculature.

Notch ligand Knockdown by morpholino further validated the Notch responsiveness of the transgenic line while also informing our understanding of Notch ligand usage during vascular development. Differential cellular activation of the Notch transgene upon notch ligand and upstream effecter

knock down indicates the NIL is responsive to genetic perturbation of Notch signaling. In addition we found that knock down of *dlc* has a much greater effect on vascular morphology and Notch activation then does knockdown of *dll4* alone. *Dlc* knockdown prevented Notch activation in all endothelial cells of the trunk indicating it is used much earlier in the vascular differentiation process. *Dll4* knockdown only affected Notch activation in the ISV's, consistent with it's known role in modulating "tip-cell" behavior [244]. Loss of Notch activation in the vascular system after *vegfa* knockdown acts as another example that the ablation of up-stream Notch activators can be visualized using this transgene further validating its usefulness as a genetic tool.

Recognizing the benefit of the NILs and the Notch response promoter to the zebrafish community we freely shared the line with several collaborators by either shipping them NIL embryos or the gateway cloneable promoter plasmid. Several of these collaborators have published papers using these lines adding to our understanding of Notch regulation in several other tissues and developmental processes. The NIL has been used to identify progenitor endocrine cells responsible for the secondary transition in zebrafish pancreas development[267]. Additionally it has been used to track the development of canaliculi and intrahepatic billiary networks by time-lapse confocal microscopy [268] Not only has it been used as a marker to track notch positive cells but it has also been used as a genetic marker for notch

activation in adult neural stem cells, lymphatic progenitors, the pineal gland and hematopoietic stem cells [266, 269, 270, 272] More extensive analysis of NIL expression might reveal other sites of Notch activity and enhance the discovery of Notch function in novel developmental processes. There are currently two additional notch reporter lines that have been developed. One uses the photo-convertible *kaede* fluorophore, which aids in tracking notch activation in a temporal manner. The other has a nuclear localized red (*mCherry*) fluorophore. Having the ability to chose between a red or green fluorophore for Notch activation studies will allow researchers multitude of combinations for crossing with cell type specific transgenic lines for co-visualization studies. Generation and characterization of other Notch reporter transgenic lines carrying various reporter genes configurations shall also prove to be useful for extensive visualization of Notch activity in vivo. The NIL have proven a powerful tool to analyze Notch activation in a myriad of tissue types however it has limitations. The current TP1 promoter based NILs do not allow the differentiation between individual Notch receptor activation. All the Notch receptors complex with CSL making individual receptor signaling impossible to determine. Designing a transgenic line similar to those used in *Drosophila [278]* to determine that Notch is a membrane bound transcription factor will allow individual receptor activation analysis. These hypothetical lines will further enhance the study of the Notch signaling pathway and should be developed for the zebrafish in the future.

# APPENDIX II: OLIGONUCLEOTIDE SEQUENCES

| PCR Primers | | |
|---|---|---|
| **Primer #** | **Primer Name** | **Primer Sequence (5'-3')** |
| 906 | for GST-Etv2 F | gatcggatccgaaatgtaccaatctggatt |
| 907 | for GST-Etv2 R | gatcctcgagcgctgcgtcttttgacca |
| 964 | attB1 etv2 F | ggggacaagtttgtacaaaaaagcaggcttaaccatggaaatgtaccaatctg |
| 824 | attB2 etv2 R | ggggaccactttgtacaagaaagctgggtctaatgtgtccaggactctgt |
| 1974 | attB2 etv2(-dbd) R | ggggaccactttgtacaagaaagctgggtcctaagatcc |
| 587 | attB1 ets1a F | ggggacaagtttgtacaaaaaagcaggctgcgtgaccatgacggcagct |
| 953 | attB2 ets1a R | ggggaccactttgtacaagaaagctgggtcagactttactcgtccgtgtc |
| 2085 | attB1 lnx1 F | ggggacaagtttgtacaaaaaagcaggctcaaccatgacggagtctaagacg |
| 2086 | attB2 lnx1 R | ggggaccactttgtacaagaaagctgggtattaaaccagactgccaggcc |
| 3026 | attB1 bozozok F | ggggacaagtttgtacaaaaaagcaggctcaaccatggcaactcagaagttttcaaac |
| 3027 | attB2 bozozok R | ggggaccactttgtacaagaaagctgggtgctaatctgattcctgatgatcctcc |
| 3332 | attB1 mm Etv2 F | ggggacaagtttgtacaaaaaagcaggcttaaccatggacctgtggaactgggatgagg |
| 3333 | attB2 mm Etv2 R | ggggaccactttgtacaagaaagctgggtcttattggccttctgcacctggcagatgcc |
| 4173 | etv2 3'RACE F | catcattcacaaaacggcgggaaagcgctacg |
| 4174 | etv2 3'RACE nested F | ccgctttgtctgtgacgtgcagggcatgcttg |
| 4053 | attB1 lin28 F | ggggacaagtttgtacaaaaaagcaggctgcgccaccatgcccccggcaaatccgc |
| 4054 | attB2 lin28 R | ggggaccactttgtacaagaaagctgggtcctaatcagtgctctctggc |
| 1751 | etsrp 3'UTR short F attB2 | ggggacagctttcttgtacaaagtggcctggacacattagaggagga |
| 1752 | etsrp 3'UTR short R attB3 | ggggacaactttgtataataaagttgtgtaatcgtccgtcttcaaca |
| 1753 | etsrp 3'UTR long F attB2 | ggggacagctttcttgtacaaagtggtgttgaagacggacgattaca |
| 1754 | etsrp 3'UTR long R attB3 | ggggacaactttgtataataaagttgtctgttgaagcttttggagag |
| 4219 | attB2-etv2 3'utrF | ggggacagctttcttgtacaaagtggaggaggaattctcgaaggat |
| 4278 | attB3 etv2 peak3 3'utr R | gggacaactttgtataataaagttgatgccacaacaacagttttattgtaaataa |
| 1793 | F attB2 miR sensor control | ggggacagctttcttgtacaaagtggggcgcgcctacgtaactagt |
| 1794 | R attB3 miR sensor control | ggggacaactttgtataataaagttgctcgagactagttacgtagg |
| 1029 | TP1 attB4 F | ggggacaactttgtatagaaaagttgtgcaggtcgactctagag |
| 1194 | TP1 attB1R R | ggggactgcttttttgtacaaacttgtgatccttgaattcgaatcg |
| 1025 | D2EGFP attB1 F | ggggacaagtttgtacaaaaaagcaggctggtcgccaccatggtgagcaa |
| 1026 | D2EGFP attB2 R | ggggaccactttgtacaagaaagctgggttctacacattgatcctagc |
| 1763 | Etsrp 3'UTR short RT-F | cctggacacattagaggagga |
| 1764 | Etsrp 3'UTR short RT-R | tcactatctgatgtcaaaccatc |
| 4126 | Etsrp 3'UTR EST RT-F | catgttagctacctcttttcac |
| 4127 | Etsrp 3'UTR EST RT-R | acaccattctttactagagaaaat |
| 4130 | Etsrp 3'UTR Long RT-F | caacaacagatctgaagtca |
| 4131 | Etsrp 3'UTR Long RT-R | acgtgtgtttgtgtgtgcttgtct |
| **miRNA Duplexes** | | |
| | dre-Let-7a mature | rUrGrArGrGrUrArGrUrArGrGrUrUrGrUgArUrArGrUrU |
| | dre-Let-7a anti-sense | rArArCrUrArUrArCrArArCrCrUrArCrCrUrCrA |
| | dre-Let-7c mature | rUrGrArGrGrUrArGrUrArGrGrUrUrGrUrArUrGrGrUrU |
| | dre-Let-7c anti-sense | rArArCrCrArUrArCrArArCrCrUrArCrUrArCrA |
| | dre-Let-7f mature | rUrGrArGrGrUrArGrUrArGrArUrUrGrUgArUrArGrUrU |
| | dre-Let-7f anti-sense | rArArCrUrArUrArCrArArUrCrUrArCrUrArCrCrUrCrA |
| | dre-Let-7g mature | rUrGrArGrGrUrArGrUrArGrUrUrUrGrUrArUrArGrUrU |
| | dre-Let-7g anti-sense | rArArCrUrArUrArCrArArArCrUrArCrUrArCrCrUrCrA |
| | mutant-Let7-sense | rUrCrArCrCrUrUrGrUrArGrGrArUrGrUrArUrArGrUrU |
| | mutant-Let-7 anti-sense | rArArCrUrArUrArCrArUrCrCrUrArCrArArGrGrUrGrA |
| **Northern Probes** | | |
| | let-7a LNA | dig-AACTATACAACCTACTACCTCA-dig |
| | 5S DIG-oligo probe | N(dig)ATCGGACGAGATCGGGCGTA |

# APPENDIX III: NANOSTRING PROBESET

| Gene | Accession | Targeted Region | Target Sequence | PN(CP; RP) |
|---|---|---|---|---|
| etv2 | NM_001037375.1 | 790-890 | CTTTGGCAGTTTCTGCTAGAACTCCTGCTGGATTCTGCTTGCCACACTTTTATAAGTTGGACTGGTGATGGCTGGGAGTTTAAAATGTCAGATCCCGCTG | 340352; 240352 |
| kdrl | NM_131472.1 | 455-555 | AACATACCCAAACCAAAACGTTATCCTTGAGACGCAGATGAATCCTATGGCAGATGATGTTAAAAGAGGGGTACAGTGGGATCCAAAAAAAGGTTTCACG | 340356; 240356 |
| flt4 | NM_130945.1 | 620-720 | TCCTGACCTAAAAGTCACTCTCTTCTCGTTAGTGCCGTATCCAGAGCCTGTGGATGGCAGTGTGGTCACCTGGAATAATAAAAAGGGTTGGTCGATTCCC | 340353; 240353 |
| fli1a | NM_131348.2 | 620-720 | ACTTCCTGAGACTCACCAGCGTTTATAACACCGAGGTCCTTCTCTCACATCTCAATTACCTCAGGGAAAGTAGCTCATCGATATCATACAACACGCCATC | 340355; 240355 |
| fli1b | NM_001008780.1 | 1365-1465 | GTAATTTCTTCACGCCTCAATCCACCTACTGGAACTCCGCAACCAGTGTGGTTTATCCCAGTTCACCGATGCCACGACATCCCAGCACTCACACTCACTT | 340350; 240350 |
| hey2 | NM_131622.2 | 990-1090 | CGCTGGATTCCCACTCTTCAGCCCCAGCGTTACAGCATCTTCAGTGGCTTCTTCCACCGTGAGCTCTTCCGTTTCCACATCCACCACATCCCAACAGAGC | 346488; 246488 |
| actb2 | NM_181601.3 | 1647-1747 | CCTGGGCATATTGTAAAAGCTGTGTGGAACGTGGCGGTGCCAGACATTTGGTGGGGCCAACCTGTACACTGACTAATTCAATTCCAATAAAAGTGCACAT | 328374; 228374 |
| eef1a1l1 | NM_131263.1 | 1455-1555 | CCAAGTGAATTTCCCTCAATCACACCGTTCCAAAGGTTGCGGCGTGTTCTTCCCAACCTCTTGGAATTTCTCTAAACCTGGGCACTCTACTTAAGGACTG | 328447; 228447 |
| gata2a | NM_131233.1 | 2030-2130 | ATTTACTGAGTCACTTTGGTACTGAAAGAGCGGACGCAGAATCACTGTGTGGTAGTCAAAACGGCCACCTCAAAACTCTCATAAAGGACTCGCTTTGAGC | 328441; 228441 |
| tal1 | NM_213237.1 | 635-735 | TAGCAATCGAGTCAAGCGCAGACCTGCACCTTATGAGGTTGAAATCAACGATGGTTCGCAGCCCAAAATTGTGCGACGGATTTTCACGAACAGTCGCGAG | 340349; 240349 |
| lmo2 | NM_131111.1 | 215-315 | GCGTACACAATGTGTGCTGGATGTTTCTGACCTTTGATACACTTGCTAAGACAGCAGAACAGGTGCATCTCTGAAGCGTTTTGTGCGGCAGATGGTCTTT | 340354; 240354 |
| gata1a | NM_131234.1 | 175-275 | ACAGACTCTGGTTTACTGCCACCCGTTGATGTAGATGAACCTTTCTACTCAAGCTCTGAGACTGACCTACTGCCATCGTATTATTCCACCAGCGTCCAGA | 328442; 228442 |

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