University of Denver

Digital Commons @ DU

Electronic Theses and Dissertations

Graduate Studies

2020

EHP1 and EHD2 Regulate Dll4 Caveolin-Mediated Endocytosis During Blood Vessel Development

Amelia Margaret Webb

Follow this and additional works at: <https://digitalcommons.du.edu/etd>

Part of the **Cell Biology Commons**

EHBP1 and EHD2 regulate Dll4 caveolin-mediated endocytosis during blood vessel
development

A Thesis

Presented to

the Faculty of the College of Natural Sciences and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Amelia Webb

August 2020

Advisor: Erich Kushner

©Copyright by Amelia Webb 2020

All Rights Reserved

Author: Amelia Webb

Title: EHBP1 and EHD2 regulate Dll4 caveolin-mediated endocytosis during blood vessel development

Advisor: Erich Kushner

Degree Date: August 2020

Abstract

During angiogenesis, the development of new blood vessels from existing vessels, there must be tight regulation between a leading tip cell and trailing stalk cells during vascular patterning. Lateral inhibition produced by Notch signaling is central to the specification and maintenance of tip and stalk cells in growing angiogenic sprouts. The Notch pathway is activated by the ligand Delta at adherens junctions between tip and stalk cells. Despite the centrality of Delta/Notch signaling to angiogenesis, relatively little is known about the mechanism of this signaling event. It has been proposed that the force generated by the Notch bound Delta ligand exposes a cleavage site necessary for Notch activation. Here, we show for the first time in endothelial cells that the proteins Epsin homology domain protein 2 (EHD2) and EHD2 binding partner 1 (EHBP1) regulate Delta like ligand 4 (Dll4) endocytosis by tethering its caveolar endocytic pit to the actin cytoskeleton.

Acknowledgements

I am very appreciative of all the mentoring and support I have received from Dr. Kushner throughout my time in his lab. He inspired me as an undergraduate to embrace my curiosity, pursue research, and gave me the extra push to go to graduate school. I'd also like to thank everyone in Dr. Kushner's lab for being so supportive and helpful during my research. Thank you to my family for always being there for me, I could not have done this without any of you. Special thanks to my dad for the long hours at the dining room table helping me code. Thank you to Jennifer Bourne at University of Colorado Anschutz for her contribution in TEM imaging and to Stryder Meadows at Tulane University for collaborating with us and sharing his investigations into EHD2.

Table of Contents

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
List of Figures	v
 Chapter One: Introduction	 1
 Chapter Two: Materials and Methods.....	 9
Zebrafish Handling and Experimental Methods	9
Cell Culture	11
pHrodo internalization assay.....	13
Microscopy	14
Immunohistochemistry	15
Western Blot	15
Pharmacological Treatment	16
RNA-Sequencing	16
Quantification and Statistical Analysis	17
 Chapter Three: Results.....	 19
Loss of EHBP1 and EHD2 produces vascular defects in zebrafish.....	19
EHBP1 and EHD2 phenocopy Notch loss of function	22
EHBP1 and EHD2 organize around membranous Dll4 independent of clathrin....	27
EHBP1, EHD2, and caveolin localize to adherens junctions in 3D sprouts	31
Loss of EHBP1 and EHD2 blunts Dll4 endocytosis and caveolar integrity	34
RNA-seq analysis of EHBP1 deficient cells.....	38
 Chapter Four: Conclusions	 44
 References.....	 50
 Appendix A.....	 58
Appendix B	61

List of Figures

Figure 1. Notch signaling regulates lateral inhibition during angiogenesis.....	2
Figure 2. Domains of Notch1 and Dll4.....	3
Figure 3. Domain architecture of EHD2 and EHBP1.....	7
Figure 4. Global deletion of EHBP1 and EHD2a/b produces vascular defects in zebrafish	20
Figure 5. Vascular excision of EHBP1 produces vascular defects in zebrafish	21
Figure 6. γ -secretase inhibitor produces vascular defects in zebrafish.	23
Figure 7. Notch loss of function in EHBP1 and EHD2 KO zebrafish.....	24
Figure 8. EHBP1 and EHD2 preferentially express in tip cells during angiogenesis.....	26
Figure 9. EHBP1 and EHD2 organize around Dll4 independent of clathrin.....	28
Figure 10. EHBP1 and EHD2 organize around Dll4 dependent on actin.....	30
Figure 11 EHBP1 and EHD2 organize around Dll4 dependent on caveolin.....	32
Figure 12. EHBP1, EHD2, and Caveolin localize to adherens junctions in 3D vascular sprouts	33
Figure 13. Endocytosis of Dll4 and NECD are dependent on EHBP1 and EHD2.	36
Figure 14. Loss of EHBP1 and EHD2 result in Notch downregulation and loss of caveolar structure.	38
Figure 15. RNA-Seq analysis of loss of EHBP1 in HUVEC.	40
Figure 16. Gene ontology analysis of differentially regulated genes reveals cell-wide signaling disruptions.	42
Figure 17. Model of EHBP1 and EHD2 generating force on caveolar pit	47

Chapter One: Introduction

Angiogenesis is the process in which a new blood vessel develops from an existing vascular bed. During this process, in response to a chemotactic gradient of vascular endothelial growth factor (VEGF), a single endothelial cell in a group must identify itself as a tip cell. This tip cell will then lead the charge towards the VEGF-A signal, pulling behind it a trail of stalk cells in the growing vascular sprout (Figure 1A). Tip and stalk each have distinct morphological and functional identities: the tip is defined by its spiny, branching filopodia reaching forward as the tip migrates; the stalk is defined by its smoothened appearance and suppressive stability (Gerhardt et al. 2009). In order for proper angiogenic growth to proceed, the maintenance of tip/stalk cell specification is paramount.

Central to tip/stalk cell specification is the Notch signaling pathway. There are 4 Notch receptors found in mammals (Notch1-4) (Quillard et al. 2013). Differences between the 4 receptors are mainly in the number of EGF repeats (Sander, Krysinska, and Powell 2006). Although each Notch receptor is present in the vasculature, Notch1 is the predominant receptor involved in angiogenesis (Liu et al. 2003). Notch is a transmembrane protein composed of an extracellular domain (NECD) and an intracellular domain (NICD). Cells enriched in Notch with high Notch activation will adopt a stalk cell identity, whereas a cell deficient in Notch or Notch signaling will adopt a tip cell identity (Figure 1, Benedito et al. 2009, Blanco et al. 2013).

Figure 1. Notch signaling regulates lateral inhibition during angiogenesis. (A) A growing angiogenic sprout migrates towards a chemotactic gradient, with tip cell (blue) forming spiny, reaching branches and stalk cell (grey) appearing smooth. **(B)** With increased Delta and reduced Notch signaling, more cells will adopt a tip cell fate; there is low stability in the stalk and increased sprouting. Conversely, with high Notch signaling increased lateral inhibition will lead to low sprouting.

Importantly, the NECD is composed of 36 Epidermal Growth Factor (EGF) repeats and a Negative Regulatory Region (NRR) domain (Figure 2). The NRR contains three Lin-12-Notch (LNR) repeats that interact with a heterodimerization domain (HD). There is a cleavage site termed S1 on the NECD (Figure 2). The functional significance of this cleavage site is debated, as it has been found to be highly variable between the 4 mammalian Notch variants and is unnecessary for neither Notch binding to its ligand nor Notch activation (Gordon et al. 2009). Obscured within the interaction between LNR and HD at a resting state is a second cleavage site (termed S2) (Figure 2). When exposed, the

Figure 2. Domains of Notch1 and Dll4. (A) Notch extracellular domain (NECD) is composed of 36 Epidermal Growth Factor (EGF) repeats, 3 Lin12 -Notch Repeats (LNR), and the heterodimerization domain (HD). The S1 cleavage site is in the NECD. The S2 cleavage site is hidden by the interaction between the LNR and HD. The Notch intracellular domain (NICD) is composed of the transmembrane domain (TM), RBP-J/CBF1-associated module (RAM), Ankyrin (ANK) repeats, nuclear localization sequence (NLS), and a proline/glutamic acid/serine/threonine (PEST) rich domain. The S3 site is located in the TM. (B) Dll4 is composed of a Delta-Serrate Lag (DSL) domain and 8 EGF repeats.

S2 cleavage site is cleaved by a disintegrin and metalloprotease (ADAM) complex. This cleavage event necessarily precedes cleavage by γ -secretase at the S3 cleavage site to release the NICD. Once free, the NICD translocates to the nucleus, binding the

transcription factor CSL, also known as RBP-J, to upregulate downstream genes that promote lateral inhibition (Gordon et al. 2008). *Activation* of Notch is *inhibitory*. Activation of lateral inhibition in the stalk cell results in a cascade of events to maintain stalk cell identity. The stalk cell acts to remove VEGF receptors from the membrane and repress the expression of Delta (Mack and Iruela-Arispe, 2018).

Conversely, in the tip cell, VEGF is enriched on the membrane and Delta is upregulated. While this describes a binary fate alternating in adjacent cell types, endothelial cells also undergo lateral induction through which the stalk cell fate is directionally propagated through all stalk cells. By this mechanism, a positive feedback loop is created in which each stalk cell both activates Notch signaling and promotes Notch signaling (Sjöqvist and Andersson, 2019).

The activity of Notch (or lack thereof) is first dictated by its binding to one of two families of proteins at the apical junction between two endothelial cells: Jagged and Delta. In structure, Jagged and Delta are seemingly very similar to one another; each bind Notch EGF repeats 11-13 through their own EGF repeats mediated by the Delta-Serrate Lag (DSL) domain on both Delta and Jagged (Fiúza and Arias 2007, Figure 2). Despite the structural similarity to one another, binding of Notch to Jagged and Delta have opposite signaling consequences (Benedito et al. 2009). Binding of Notch to Jagged inhibits Notch signaling; binding of Notch to Delta activates Notch signaling. This opposing role of signaling of Delta and Jagged is not well defined in literature, but has been proposed to be a result of differential Notch downstream targets (e.g. “non-canonical” pathway activation) or differential Fringe glycosylation of Delta and Jagged (Le Gall et al, 2008; Panin et al, 2002).

It has been shown that Delta endocytosis regulates Notch activation (Chintis, 2006). One proposed mechanism for this activation is the application of a mechanical force generated by Delta/NECD transendocytosis into the ligand-containing cell. This force has been shown to be on the order of 19 pN per single bond (Shergill et al. 2012) and is necessary to force apart the LNR/HD interaction, thus exposing the S2 site for cleavage and subsequent S3 cleavage. Studies on the mechanical force generated by Notch/Delta to date have only focused on Delta like ligand 1 (Dll1) (Shergill et al. 2012, Meloty-Kapella et al. 2012). However, Dll1 and Delta like ligand 4 (Dll4) are functionally different; For instance, Dll4 mutant mice die embryonically while Dll1 mutant mice survive until birth (Gessler, 2009). Despite the prominence of Delta like ligand 4 (Dll4) in angiogenesis, the endocytic force provided by the Dll4 endocytic vesicle and the precise mechanism of Dll4 endocytosis remains unknown.

Clathrin mediated endocytosis is the process by which cargo is packaged into a coated pit. First, cargo bound to an AP-2 receptor recruits coat proteins to the site of endocytosis. The coat that surrounds the incoming cargo is composed of clathrin—a triskelion protein that complexes into a lattice to mold the shape of the incoming vesicle to a canonical spherical shape (Morris et al 2019). Also recruited to AP-2 is Eps15, an EH domain binding protein that interacts with Epsin to regulate the endocytosis of ubiquitinated proteins (Benmerah et al 1998). As the coated pit becomes fully formed, two important mechanisms regulate its release from the plasma membrane. First, the internalized pit is pushed inward by the active polymerization of actin (Kaksonen and Roux, 2018). Next, dynamin winds around the neck of the endocytic vesicle, constricting to pinch off the vesicle from the plasma membrane through its GTPase activity

(Kaksonen and Roux, 2018). After internalization, the clathrin coated vesicle is uncoated to release its cargo into the cell.

Caveolin mediated endocytosis displays many similarities to clathrin mediated endocytosis. As clathrin pits are shaped by clathrin, so too are caveolae shaped by caveolins and cavins. Caveolins are hairpin shaped proteins embedded into the plasma membrane (Kovtun et al. 2015). The hairpin shape bends the membrane around them to form a distinct invagination and are stabilized by cavins. Again, similar to clathrin mediated endocytosis, caveolar endocytosis also requires the action of actin and dynamin. Dynamin is required for the scission of the vesicle from the plasma membrane. The caveolar endocytic vesicle can then interact with actin through the protein Filamin A bound to caveolin 1 (Cav1) to regulate its intracellular trafficking, such as in the MAPK signaling cascade (Samaj et al. 2004). However, Echarri and Del Roza (2015) propose that there remain yet many unidentified linkers between Cav1 and the cytoskeleton.

In mammals, there are 4 Epsin15 homology domain (EHD) proteins that serve as dynamin-related GTPases and ATPases. EHD1-4 are each involved in endocytic processes, although EHD2 stands alone from this group in being the only with a solved crystal structure and the only to interact with caveolae (Moren et al. 2012). EHD2 contains 4 main functional domains: the N-terminal p-loop, the GTPase(G)-domain, the helical domain, and the Epsin15 homology (EH) domain. In the cell, EHD2 multimerizes through an interaction between the G-domain and the EH domain (Hoernke et al. 2017, Moren et al. 2012, Figure 3). This interaction serves a dual purpose. First, the binding of

Figure 3. Domain architecture of EHD2 and EHB1. EHD2 is composed of an N-terminal p-loop, GTPase(G)-domain, helical domain, and Epsin15 homology (EH) domain. EHB1 contains 3 NPF motifs which bind the EH domain, a CH domain capable of tethering actin, and a C-terminal CAAX box.

the G-domain to the EH domain converts the function of the domain from a GTPase to an ATPase (Melo et al. 2017). Secondly, the multimerization of EHD2 allows it to form a circular ring around an endocytic vesicle to mediate cleavage (Moren et al. 2012). This complex has also been shown to localize to Cav1, assisting in caveolin endocytosis through stabilization of the caveolae pit structure (Torrino et al. 2018).

EHD2's binding partner, EHB1, has been much less well characterized. EHB1 consists of 3 primary functional domains: the NPF motif, the calponin homology (CH) domain, and the C-terminal CAAX box (Figure 3). The NPF motif is what binds the EH domain of EHD2. The CH domain tethers the EHD2/EHB1 complex to the cytoskeleton. EHB1 and EHD2 have been found to regulate the clathrin mediated endocytosis of Transferrin and Glut4 (Guilherme et al. 2003). A similarly strong interaction between a Dll4 endocytic vesicle complexed to the actin cytoskeleton through EHB1 and EHD2 would be required to provide the appropriate mechanical force to expose the S2 domain on Notch.

While Notch signaling has a wide array of functions throughout development and in different tissues, its role in vascular development cannot be understated. Improper

regulation of Notch can contribute to a wide array of vascular disease processes, and complete deletion of Notch1 leads to embryonic lethality in mice following severe vascular defects (Krebs et al. 2000). Among the vascular diseases formed from improper Notch signaling is hereditary hemorrhagic telangiectasia (HHT). Affecting up to 30/100,000 people, the consequences of arteriovenous malformations (AVMs) in HHT can range in severity from nosebleeds to fatal stroke (Peacock et al. 2015). Given the magnitude of Notch signaling in the medical community, the critical lack of understanding regarding the mechanism and regulators of Dll4 endocytosis represents an unfortunate blind spot in the community. Equally lacking is a holistic understanding of the functional relationship between EHD2 and EHBP1 during endocytosis. We seek here to elucidate the role of EHD2 and EHBP1 in relation to one another, their role in Dll4 endocytosis, and the downstream consequences they pose to Notch signaling as a whole. We propose that EHBP1 and EHD2 act on caveolar endocytic machinery to tether the Dll4 endocytic pit to the cytoskeleton and it is through this bridge that the force required to expose the S2 cleavage site is generated.

In this thesis, we will show that loss of EHBP1 and EHD2 resulted in Notch loss of function both *in vivo* and *in vitro*. EHBP1 and EHD2 organize themselves around Dll4 puncta on the membrane dependent on actin and caveolin-1 and independently of clathrin. Endocytosis of a Dll4 antibody and soluble NECD ligand are impaired in the absence of EHBP1 and EHD2 and caveolar pits lose their structure, supporting the role of EHBP1 and EHD2 to mediate caveolin-mediated endocytosis through tethering to the actin cytoskeleton. Finally, loss of EHBP1 leads to have cell-wide consequences to signaling pathways in endothelial cells.

Chapter Two: Materials and Methods

Zebrafish Handling and Experimental Methods

All zebrafish used in this study were AB strain. Zebrafish housing and protocols were all approved by the Institutional Animal Care and Use Committee (IACUC, number 946125-1).

Tg(kdrl:GFP) strain previously published by Choi et al. 2007. Tg(fli:LifeAct-GFP) strain previously published by Hen et al. 2015. Tg(kdrl:mCherry) strain previously published by Proulx et al. 2010. Tg(kdrl:cre) strain previously published by Bertrand et al. 2010. Tg(cdh5:gal4FF) strain previously published by Bussmann et al. 2011.

Tol2 transposase RNA was synthesized from pT3TS-Tol2 (Addgene, #31831) using the MEGAscript™ T3 Transcription Kit (Thermo Fisher Scientific, AM1338) and stored at -80°C at a dilution of 100 ng/μl. Injection mixture was prepared on ice containing 300 ng Tol2 transposase RNA and 500 ng recombinant plasmid and was brought to 10 μl total volume with 0.1% phenol-red (VWR, 470301-974) in water. 1-4 cell embryos were injected directly into cell with 2 pL injection mixture.

4-guide CRISPR/Cas9 targeted gene KO was performed as outlined by Wu et al 2018. In brief, 4 single guide RNA templates fused to a scaffold were synthesized for each target gene using HiScribe™ SP6 RNA Synthesis Kit (New England BioLabs, E2070S). Injection mixture was prepared on ice containing 5 μM Cas9 (PNA Bio, CP02), 1 μg/μL sgRNA, and brought to 6 μL with 0.1% phenol-red in water. Cas9 and

sgRNA guides were pre-complexed at 37°C for 5 mins. 1-4 cell embryos were injected directly into yolk with 2 pL injection mixture.

Scaffold primer sequence: 5' –AAAAGCACCGACTCGGTGCCACTTTTTCAA GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC– 3'.

Scramble guide sequences: 5' –TAATACGACTCACTATAGGCAGGCAAAGAATCC CTGCCGTTTTAGAGCTAGAAATAGC- 3', 5' –TAATACGACTCACTATAGGTAC AGTGGACCTCGGTGTCGTTTTAGAGCTAGAAATAGC- 3', 5' –TAATACGACTC ACTATAGGCTTCATACAATAGACGATGGTTTTAGAGCTAGAAATAGC- 3', 5' – TAATACGACTCACTATAGGTCGTTTTGCAGTAGGATCGGTTTTAGAGCTAGAA ATAGC- 3'. EHBPl guide sequences: 5' – TAATACGACTCACTATAGGGAGTCCCC ATCGGGTCGTGTTTTAGAGCTAGAAATAGC- 3', 5' –TAATACGACTCACTATA GGCTCATCCGGGTCACCAAAGTTTTAGAGCTAGAAATAGC- 3', 5' –TAATACG ACTCACTATAGGCGACCTATCAGCAGGCCGGTTTTAGAG CTAGAAATAGC- 3', 5' – TAATACGACTCACTATAGGAGATGCCGCAATGAAGGCGTTTTAGAGCT AGAAATAGC- 3'. EHD2a guide sequences: 5' –TAATACGACTCACTATAGGCGGT TCATACAGTAACCGGTTTTAGAGCTAGAAATAGC- 3', TAATACGACTCACTA TAGGTTTGCGGAGCACGTAGACGTTTTAGAGCTAGAAATAGC- 3', 5' –TAATA CGACTCACTATAGGAAACGAGCACGCCTTGTAGTTTTAGAGCTAGAAATAGC- 3', 5' -TAATACGACTCACTATAGGCCTTTAACTTGAAGTCGGTTTTAGAGCTA GAAATAGC-3'. EHD2b guide sequences: 5' –TAATACGACTCACTATAGGTCACT GTCCTGATCACCTGTTTTAGAGCTAGAAATAGC- 3', 5' -TAATACGACTCACTA TAGGTGCTCTCCAGGACCTGGTGTGTTTTAGAGCTAGAAATAGC- 3', 5' –TAATA CGACTCACTATAGGCGAACAGCTTGCGGTTGTGTTTTAGAGCTAGAAATAGC-

3', 5' –TAATACGACTCACTATAGGTTCTTAGGAGCAACTAAGTTTTAGAGCT
AGAAATAGC- 3'.

EHBP1^{lox} fish genotyped using forward primer 5' –GATGTGTAGAGGAATAC
CGTTCGTATAGC- 3' and reverse primer 5' –GTCAGGCTTCACATTGCAGAGTTC-
3'. EHBP1^{lox} sequence was incorporated to a pME empty backbone for sequencing using
the forward primer 5' – CGACGGTATCGATAAGCTTGATATCTCTGTGACAGAAT
TGGTCAGTTTGACA- 3' and reverse primer 5' –GCGGCCGCTCTAGAACTAGTGG
ATCGCAGCGCAGCTCAGGCAAG- 3'.

Cell Culture

Glass-bottomed imaging dishes were exposed to UV light for 6 minutes and
coated with Poly-D-Lysine (Thermo Fisher Scientific, A3890401) for a minimum of 20
minutes. Dishes were then coated with 15 µg/mL laminin mouse protein (Thermo Fisher
Scientific, 23017015) overnight at 37°C. Cells were plated onto laminin coated dishes for
4-6 hours prior to imaging or fixation. 0.9 µM siRNA (Thermo Fisher Scientific,
s225944, s26959, am4611) was introduced to primary human umbilical vein endothelial
cells (HUVEC) using the Neon® transfection system (Thermo Fisher Scientific).

Adenovirus constructs (tagRFP-EHD2, tagRFP-EHBP1, Emerald-Clathrin) were
created as previously described (He et al. 1998). In brief, constructs were introduced via
Gibson Assembly® (New England BioLabs, E2611) into pShuttle-CMV (Addgene,
#16403). PShuttle-CMV plasmids were then digested overnight with MssI (Thermo
Fisher Scientific, IVGN0244) and purified using E.N.Z.A.® Gel Extraction Kit (Omega

Bio-Tek, D2500-01). Linearized pShuttle-CMV plasmids were transformed into the final viral backbone using electrocompetent AdEasier-1 cells (Addgene, #16399). Successful incorporation of pShuttle-CMV construct into AdEasier-1 cells confirmed via digestion with PacI (Thermo Fisher Scientific, IVGN0184). 5000 ng plasmid was then digested at 37°C overnight, then 85°C for 10 minutes and transfected in a 3:1 polyethylenimine (PEI, Sigma Aldrich, 408747):DNA ratio into 70% confluent HEK 293A cells (Thermo Fisher Scientific, R70507) in a T-25 flask (Genesee Scientific, 25-207).

Over the course of approximately 2-4 weeks, fluorescent cells became swollen and burst or budded off of the plate. Once approximately 50% of the cells had lifted off of the plate, cells were scraped off and spun down at 2000 rpm for 5 minutes in a 15 mL conical tube. The supernatant was aspirated, and cells were resuspended in 1 mL DPBS (Genesee Scientific, 25-508B). Cells were then lysed by 3 consecutive quick freeze-thaw cycles in liquid nitrogen, spun down for 5 minutes at 2000 rpm, and supernatant was added to 2 70% confluent T-75 flasks (Genesee Scientific, 25-209). Propagation continued and collection repeated for infection of 10 15 cm dishes. After collection and 4 freeze thaw cycles of virus collected from 10 15 cm dishes, 8 mL viral supernatant was collected and combined with 4.4 g CsCl (Sigma Aldrich, 289329) in 10 mL DPBS. Solution was overlaid with mineral oil and spun at 32,000 rpm at 10°C for 18 hours. Viral fraction was collected with a syringe and stored in a 1:1 ratio with a storage buffer containing 10 mM Tris, pH 8.0, 100 mM NaCl, 0.1 percent BSA, and 50% glycerol. Primary Human Umbilical Vein Endothelial Cells (Promocell, C-12253) were treated with virus for 16 hours at a 1/1000 final dilution in all cell culture experiments.

Fibrin Bead Assay performed according to protocol as outlined by Nakatsu et al. 2007.

pHrodo internalization assay

pHrodo™ iFL Red STP Ester (Thermo Fisher Scientific, P36010) was brought to 10 mM with DMSO. On the day of antibody labeling, pHrodo™ iFL Red STP Ester was diluted to 2 mM in DMSO (VWR Life Science, 97063-136). Antibody was brought up in DPBS (2 mg/mL final concentration for Dll4 polyclonal antibody [Thermo Fisher Scientific, PA5-46974]; 1 mg/mL final concentration for recombinant human Notch-1 Fc Chimera [R&D Systems, P46531]) and added to 1/10 volume 1 M sodium bicarbonate, pH 8.5. 3.3 µL of 2 mM pHrodo™ dye was added to the antibody and allowed to react in the dark for 1 hour with gentle flicking every 15 minutes. While this reaction is occurring, Zeba™ Spin Desalting Column (Thermo Fisher Scientific, 89882) was washed 3 times with 300 µL DPBS at 1500 x g for 1 minute. After 1 hour, the labeled antibody solution was loaded into the desalting column and allowed to absorb. DPBS was overlaid on top of labeled antibody to bring total column load volume to 70 µL and spun at 1500 x g for 2 minutes. Flow-through stored at 4 °C.

For bead tethered pHrodo, 6 µL of pHrodo-conjugated antibody brought to 200 µL TBST and added to 10 µL of either Dynabeads™ Protein G (Thermo Fisher Scientific, 10004D) or Protein A Agarose Resin (Gold Biotechnology, P-400-5). Beads and antibody were incubated and rotated at room temperature for 10 minutes. Conjugated

beads were washed 3 times with 200 μ L TBST then stored in a final volume of 10 μ L TBST at 4°C.

3 μ L pHrodo labeled antibody and 1 μ L Hoechst 33342 trihydrochloride, trihydrate (Thermo Fisher Scientific, H3570) was added to 70-80 % confluent HUVECs plated on laminin coated dishes in 1 mL media and incubated at 37°C for 10 minutes. After 10 minutes, the cells were washed 3 times with 1 mL DPBS and then placed in 2 mL media. 10 z-stack images were taken for each condition, this marks time point 0 minutes. After images were taken of each group, cells were returned to 37°C for 10 minutes and imaged again.

Microscopy

Images were taken on a Nikon Eclipse Ti inverted microscope equipped with a CSU-X1 Yokogawa spinning disk field scanning confocal system and a Hamamatsu EM-CCD digital camera. Cell culture images were captured using a Nikon Plan Apo 60x NA 1.40 oil objective using Olympus type F immersion oil NA 1.518 (ThorLabs, MOIL-30). Fish images were taken using either Nikon Apo LWD 20x NA 0.95 or Nikon Apo LWD 40x NA 1.15 water objectives. For transmission electron microscopy images, primary HUVEC of specified treatment were fixed in 2% glutaraldehyde, 2% PFA, 0.2 M Cacodylate buffer and sent to Jennifer Bourne at the University of Colorado Anschutz Medical Campus.

Immunohistochemistry

2D cell culture was fixed in 4% paraformaldehyde in DPBS for 10 minutes. Cells were then washed 3 times for 5 minutes in TBST and permeabilized in 0.1% Triton-X 100 for 10 minutes. Cells were then washed 3 times for 5 minutes and blocked for 1 hour in 2% bovine serum albumin (BSA). Primary antibodies were applied at specified dilutions in Key Resources Table (Appendix A) overnight. Cells were washed 3 times for 10 minutes in TBST and then moved to secondary for 2 hours at specified dilutions in Key Resources Table. Cells were washed again 3 times for 15 minutes in TBST before imaging.

Western Blot

Primary HUVEC culture was trypsinized and lysed using Ripa buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/mL leupeptin) containing 1x ProBlock™ Protease Inhibitor Cocktail -50, Plus EDTA (GoldBio, GB-334-20). Total concentration of protein in lysate was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) measured at 562 nm and compared to a standard curve. 20-50 μ g protein was prepared in 0.52 M SDS, 1.2 mM bromothymol blue, 58.6% glycerol, 75 mM Tris pH 6.8, and 0.17 M DTT. Samples were boiled for 10 minutes, then 35 μ L was loaded in a 7-12% SDS gel and run at 170 V. Protein was then transferred to Immun-Blot PVDF Membrane (BioRad, 1620177) at 4°C, 100 V for 1 hour 10 minutes. Blots were

blocked in 2% milk for 1 hour, then put in primary antibody at specified concentrations overnight. After 3 10-minute washes with TBST, secondary antibodies at specified concentrations were applied for 4 hours. After 3 additional TBST washes, blots were developed with ProSignal® Pico ECL Spray (Genesee Scientific, 20-300S).

Pharmacological Treatment

DAPT (Sigma Aldrich, D5942) was applied to cells for 3 days at a final concentration of 5 μ M. Dynasore hydrate (Sigma Aldrich, D7693) was applied to cells for 30 minutes at a final concentration of 100 μ M. LY-411575 (Sigma Aldrich, SML0506) was diluted in egg water to a final dilution of 2 μ M from 30-48 hpf. Latrunculin A (Sigma Aldrich, 428021-100UG) was applied to cells for 1 hour at a final concentration of 5 μ M. Cytochalasin B (Sigma Aldrich, C6762-5MG) was applied to cells for 1 hour at a final concentration of 10 μ M. Methyl- β -Cyclodextrin (Sigma Aldrich, M7439-1G) was applied to cell for 10 minutes at a final concentration of 10 mM.

RNA-Sequencing

RNA collected from siRNA treated HUVEC was collected in triplicate and sent to Novogene for RNA-sequencing. Sequence files received from Novogene were processed using the Bioconductor package in R-Studio as described by Love et al. (2015) using Homo Sapiens GRCh38 version 98 as a reference genome.

Quantification and Statistical Analysis

Ectopic vessels were defined by a sprout emerging out of or separate from the defined ISV and quantified in 48 hpf embryos expressing tg(kdrl:GFP). Number of filopodia per ISV was defined by filopodial extensions (observed with zebrafish line tg(fli:LifeAct-GFP)) emerging from a fully formed ISV that has connected with the dorsal longitudinal anastomotic vessel. RT-PCR was quantified using the gel analysis function in Fiji image analysis software (Schindelin et al., 2012). In sum, rectangular sections were drawn around individual lanes in gray-scale, high-quality gel image using the pathway *Analyze > Gel > Select First Lane, Analyze > Gel > Select Next Lane*. After all lanes are selected, the pathway *Analyze > Gel > Plot Lanes* was used. The peaks of each lane were then segmented using the Straight Line selection tool, and highlighted with the Wand tool. Selection of the area inside the peak generates a Results window with the area and percent of each peak. The percent value of each sample was divided by the percent value of the control to obtain a relative density. Relative densities of the gene of interest (e.g. Hey2) were divided by the relative density of the housekeeping gene (GAPDH) to obtain a final adjusted density value.

Cellular uptake of pHrodo-labeled antibody was also quantified using Fiji image analysis software. Stack files were z-stacked at maximum intensity, and each color channel was adjusted so that the background was zero. Each individual cell was outlined with the Freehand Selections tool. The color channels were then separated and any background fluorescence (488 channel) was subtracted from the pHrodo fluorescent intensity (561 channel) using pathway *Process > Image Calculator*. The Integrated

Density of pHrodo fluorescent intensity within each cell boundary was then recorded for every cell at each time point. Each experiment was performed in duplicate. All statistical analysis was performed in GraphPad Prism8.

Chapter Three: Results

Loss of EHBP1 and EHD2 produces vascular defects in zebrafish

To investigate whether EHBP1 and EHD2 have a role in angiogenesis, we first sought to characterize how the loss of EHBP1 or EHD2 affects intersomitic vessel (ISV) development in *Danio rerio* (zebrafish). ISVs are used as a hallmark of normal blood vessel patterning as in normal development, they form highly stereotyped chevron shapes. During angiogenesis, the ISVs sprout from the dorsal aorta and migrate towards the dorsal longitudinal anastomotic vessel roughly between 20-36 hours post fertilization (hpf). In this vessel bed, specification between tip cell and stalk cell through the Dll4/Notch1 signaling pathway must be tightly regulated and under normal signaling dynamics will form highly stereotyped patterns. Aberrations in normal ISV growth, such as ectopic vessels, are morphodynamically obvious and indicate a potential disruption to the Notch signaling pathway.

A 4-guide KO approach (Wu et al. 2018) was employed targeting EHBP1 and EHD2 in zebrafish to create F0 knockouts (KO) at these loci (Figure 4A). All injections were done into a *tg(kdrl:GFP)^{+/+}* line, GFP under a vascular promoter, for visualization of blood vessels. Due to multiple gene duplication events in teleosts, EHD2 has two isoforms in zebrafish: EHD2a and EHD2b. While previous *in situ* hybridizations suggest that EHD2b is the dominant isoform (Thisse and Thisse, 2007), each isoform was targeted individually as well as in combination. Quantification of the proportion of

Figure 4. Global deletion of EHBP1 and EHD2a/b produces vascular defects in zebrafish. (A) Red dashed box denotes area of imaging. 4-guide knockout (KO) fish were imaged on $tg(kdr:l:GFP)^{+/+}$ background at 48 hpf. Yellow arrows denote abnormal vascular growth. Teal arrows point to spiny projections. (B) Quantification of the proportion of 4-guide KO fish with ectopic ISVs. Ectopic was defined as a vessel projection emerging from the ISV distinct from the central stalk. T-test analysis used between EHBP1 and Scram. One-way ANOVA analysis between EHD2a, EHD2b, EHD2a/b and Scram. Error bars represent SEM. n= number of fish quantified.

fish with ectopic ISVs in each condition revealed a significant increase in EHBP1 knockout fish (18.15%), as well as a significant increase in EHD2a/b knockout fish (21.76%) compared to a scramble control (Figure 4B). These results suggest that EHBP1 and EHD2a/b are necessary for normal sprouting behaviors in endothelial cells (ECs).

Focusing on EHBP1, as this protein is less well characterized, we sought to determine the endogenous expression of EHBP1 in zebrafish. However, antibodies that detect EHBP1 in zebrafish are not available. To circumvent this, we fused a FLAG epitope to the n-terminal domain of the endogenous EHBP1 locus using CRISPR/Cas9 targeting (Hoshijima et al. 2016) (Figure 5A). Staining for FLAG-EHBP1 endogenous expression revealed that EHBP1 strongly localized to neuronal projections (Figure 5A). To determine if EHBP1 was being expressed in ECs we dissociated embryos expressing

Figure 5. Vascular excision of EHBP1 produces vascular defects in zebrafish. (A) Staining against FLAG epitope in various stages of development highlighting expression in the neural tube (NT) in indicated genotypes. **(B)** Ex vivo validation of expression of FLAG-EHBP1 in endothelial cells. Yellow arrows denote areas of FLAG positivity. **(C)** Confirmation of in frame lox site incorporation into F1 generation EHBP1 floxed fish. Predicted sequence shown in first row, sequenced F1 fish shown in second row. Sanger sequencing peaks of small region in c-terminal lox site highlighted to the right. **(D)** Representative images of ISVs in indicated genotypes. Yellow arrows denote abnormal vascular growth. **(E)** Quantification of proportion of fish with ectopic ISVs. T-test analysis between groups. Error bars represent SEM. n= number of fish quantified.

both FLAG-EHBP1 and a GFP-endothelial reporter at 48 hours post fertilization (hpf) and plated individual ECs on a dish. We found there was indeed EHBP1 expression in ECs at this time point (Figure 5B). EHD2b detected using *in situ* hybridization by Thisse and Thisse (2007) demonstrated a similar expression pattern to EHBP1 along neuronal projections.

Given the potentially confounding effects of performing a global knockout, a floxed EHBP1 transgenic line was also created using CRISPR mediated recombination. Validation of the successful incorporation of these lox sites into an F1 generation fish is shown in Figure 5C. In line with our previous observations, excision of the floxed allele exclusively in blood vessels using a kdrl promoter [EHBP1^{lox/lox}; tg(kdrl:GFP)^{+/+}; tg(kdrl:Cre)^{+/+}] also showed a significant increase (12.56%) in the proportion of fish with ectopic ISVs (Figure 5D,E). This suggests that the blood vessel aberrations in EHBP1 and EHD2a/b knockout lines are not due to a confounding effect of global gene deletion. Together, the global and vascular specific knockouts suggest that EHBP1 and EHD2a/b mediate endothelial sprouting behaviors and are required for normal angiogenesis.

EHBP1 and EHD2 phenocopy Notch loss of function

We next sought to characterize the EHBP1 and EHD2a/b vascular abnormalities in the context of Notch activity, as loss of Notch signaling has been previously shown to promote hypersprouting (Therapontos and Vargesson 2010). As a reference, the tg(fli:LifeAct-GFP)^{+/+} line which overexpresses a GFP tagged F-actin in the vasculature was treated with 2 μ M of the γ -secretase inhibitor LY-411575 (Figure 6). Treatment

with this small molecule γ -secretase inhibitor prevents cleavage of the NICD at the S3 site and subsequent translocation of the NICD to the nucleus. In this Notch loss of function control, there was a significant increase in the number of ectopic sprouts per ISV in comparison with a DMSO treated control group, phenocopying the increase in ectopic sprouting observed in EHP1 and EHD2 KOs and supporting the potential role of EHP1 and EHD2 in Notch signaling (Figure 6B). We also observed a significant increase in the number of filopodia per ISV in the LY-411575 treated fish (Figure 6A,C). Given that spiny, filopodial projections are characteristic of a tip-identifying cell, as would occur in a low-Notch signaling environment, we used this as another measurement of Notch loss of function. The 4-guide KO injections were thus repeated using the F-Actin overexpression line (Figure 7A).

Figure 6. γ -secretase inhibitor produces vascular defects in zebrafish. (A) ISVs of fish treated with either DMSO or 2 μ M LY-411575 on *tg(fli:LifeAct)^{+/+}* background. Yellow arrows point to ectopic sprouts. Teal arrows point to filopodial projections. (B) Number of ectopic ISVs per fish between indicated groups. Error bars represent SEM. (C) Number of ISV filopodial extensions between indicated groups. T-test analysis performed between groups. Error bars represent SD n=number of fish.

Figure 7. Notch loss of function in EHB1 and EHD2 KO zebrafish. (A) Representative images of 4-guide KO of EHB1 and EHD2 on *tg(fli:LifeAct-GFP)^{+/+}* background. Yellow arrows point to ectopic sprouts. **(B)** Number of ISV filopodial extensions among indicated groups. One-way ANOVA analysis between groups. Error bars represent SD. **(C)** Relative expression of Hey2 transcript in 48hpf 4-guide KO fish compared to GAPDH control across indicated groups. One-way ANOVA analysis between groups. Error bars represent SEM. n=number of fish, ns=nonsignificant.

The number of filopodia per ISV were quantified and showed no significant difference in the filopodia per ISV in the 4-guide KO fish compared to a scramble control (Figure 7B). These 4-guide KO fish were collected at 48 hpf and their RNA was extracted and converted to cDNA library. The expression of

downstream Notch1 target Hey2, a marker of S3 cleavage and downstream Notch activation, was monitored in each KO sample and compared to a GAPDH control. We found a significant decrease in the expression of Hey2 in EHBP1, EHD2b, and EHD2a/b KO fish in comparison with the scramble control (Figure 7C), further evidence of a Notch loss of function in the absence of EHBP1 and EHD2.

In canonical tip/stalk cell specification, tip cells exhibit elevated Dll4 levels which act to activate lateral inhibition through Notch activation in the trailing stalk cells (Figure 1). Given the Notch loss of function phenotype in EHBP1 and EHD2a/b KO fish, we hypothesized that these proteins may influence hierarchical tip/stalk cell positioning. In order to do this, GFP tagged 5xUAS constructs of the human orthologs of EHBP1 and EHD2 were constructed. It should be noted that the zebrafish EHBP1 shares a 72.4% homology to its human ortholog; zebrafish EHD2a and EHD2b share a 69.3% and 81.9% homology respectively to their human ortholog. Therefore, we expect the human orthologs to be representative of the zebrafish orthologs and behave similarly. We predicted that if EHBP1 or EHD2 did not affect Notch activation there would be a largely equal EC contribution in tip or stalk cell positions.

Confirming this, we injected a control tg(5xuas:LifeAct-GFP) construct and observed mosaic integration with an even 50/50 distribution between tip and stalk ECs in growing ISVs at 24 hpf (Figure 8). However, ECs expressing GFP-EHD2 (tg(5xUAS:GFP-EHD2)) demonstrated a significant bias toward the tip cell position with 76.9% of ISVs exhibiting EHD2-overexpressing ECs in the tip position (Figure 8)

Similarly, GFP-EHBP1-expressing ECs showed a 61.5% bias to the tip cell position in ISVs at 24 hpf (Figure 8). These results support the role of EHBP1 and EHD2 in Notch

Figure 8. EHBP1 and EHD2 preferentially express in tip cells during angiogenesis. Representative images of GFP-EHD2, GFP-EHBP1, and LifeAct-GFP overexpression constructs in growing ISVs at 24 hpf. Vasculature marked by Tg(kdrl:mCherry) (Red). Quantification of the proportion of expressing endothelial cells in either the tip (T, blue) or stalk (S, red) cell position in the growing vascular sprouts shown to the right. T-test analysis between groups.

activation, suggesting that EHBP1 and EHD2 may play a role in tip cell-related signaling and support the spatiotemporal role of EHBP1 and EHD2 in Notch activation in tip cell-related signaling.

EHBP1 and EHD2 organize around membranous Dll4 independent of clathrin

Given the effect of EHBP1 and EHD2 on Notch activation and tip cell preference shown thus far *in vivo*, we next investigated the precise cellular mechanism through which EHBP1 and EHD2 are acting on Dll4/Notch signaling. We hypothesized that EHBP1 and EHD2 were involved in Dll4 endocytosis. Literature to date on Dll4 endocytosis is inconclusive in identifying its endocytic machinery. In the same vein, there are few papers on EHBP1 in relation to endocytosis and even fewer on EHBP1 and EHD2 in combination. They have been shown to be involved in both clathrin-dependent and independent endocytic trafficking pathways. Thus, we first looked to a 2003 article by Guilherme et al. which identified EHBP1 and EHD2 in facilitating the clathrin mediated endocytosis of Transferrin and Glut4 through tethering to the actin cytoskeleton.

To address this, we chose to focus only on the extracellular pool of Dll4 present on the membrane and thus available for Notch binding and endocytosis using a pHluorin-Dll4 construct. The pH sensitive GFP variant pHluorin fluoresces on the surface of the cell at a neutral pH but is quenched when internalized into low pH acidic vesicles (Figure 9A, Sankaranarayanan et al. 2000). Therefore, overexpression of the pHluorin-Dll4 construct in cell culture shows only the membrane-presenting pool of Dll4. An additional

Figure 9. EHBP1 and EHD2 organize around Dll4 independent of clathrin. (A) Schematic of pH-dependent function of GFP variant pHluorin tag. pHluorin fluoresces on the membrane at neutral pH, but is quenched when internalized into acidic endosomes. **(B)** EHBP1 localizes around sites of pHluorin-Dll4 independent of clathrin. **(C)** EHD2 localizes around sites of pHluorin-Dll4 independent of clathrin. Boxed are magnified section on right. Yellow arrows show areas of colocalization.

consideration we took into account is that the cellular expression of Dll4 in 2D cell culture is highly mosaic. In order to both stimulate Dll4 expression and homogenize it in a single dish, all cells were plated onto laminin coated dishes, an integrin that stimulates Dll4 expression (Estrach et al 2011).

Co-expression of pHluorin-Dll4 and tagRFP-EHBP1 in HUVEC showed that EHBP1 clustered around sites of membranous Dll4 (Figure 9B). Likewise, Co-expression of pHluorin-Dll4 and tagRFP-EHD2 showed that EHD2 formed clusters surrounding Dll4 puncta on the membrane (Figure 9C). These results support the potential role of EHBP1 and EHD2 in Dll4 endocytosis. However, clathrin was largely absent at these

sites (Figure 9B,C). Instead, the clathrin expression was diffuse and non-specific, indicating that Dll4 is potentially endocytosed through a clathrin-independent pathway.

Emerald-clathrin and either tagRFP-EHBP1 or tagRFP-EHD2 were co-expressed in HUVEC and stained for F-actin (Figure 10 A,B). We observed that while pHluorin-Dll4 puncta localized to EHBP1 and EHD2 clustered at sites of large F-actin bundles as would be necessary to facilitate a coordinated endocytic event, clathrin was also absent from these sites (Figure 10 A-D). Cells were then treated with one of two actin inhibitors: cytochalasin B or latrunculin A. Cytochalasin B works by blocking de-novo actin polymerization; Latrunculin A works by actively degrading existing actin fibers as well as preventing de novo synthesis. In cells with either inhibitor, localization of EHBP1 and EHD2 became diffuse and non-specific (Figure 10 E,F). At select sites where actin junctions remained, there was a slight although noticeably blunted localization of EHBP1 and EHD2. Together, these results suggest that EHBP1 and EHD2 may act in the clathrin-independent endocytosis of Dll4 that is dependent on tethering to the actin cytoskeleton to function.

Localization of EHBP1 and EHD2 to Dll4 is dependent on Caveolin-1

Given the evidence of EHBP1 and EHD2 coordinating actin tethering to membranous Dll4 independent of clathrin, we turned next to the potential that EHBP1 and EHD2 are coordinating Dll4 endocytosis through caveolin endocytosis. EHD2 has previously been shown to act as dynamin-like ATPase driving the scission of caveolar endocytic pits from the membrane, although this role has not been linked to Dll4,

Figure 10. EHP1 and EHD2 organize around Dll4 dependent on actin. (A,B) EHP1 and EHD2 localized to sites of F-actin bundling independent of clathrin. **(C,D)** EHP1 and EHD2 cluster around Dll4 puncta at sites of F-actin bundling. **(E,F)** Actin inhibitors Cytochalasin B and Latrunculin A disrupt EHP1 and EHD2 localization. Boxed are magnified section on right. Yellow arrows show areas of colocalization.

EHP1, or actin-tethering (Morén et al. 2012). TagRFP-EHP1 and caveolin1 both formed around clusters of pHluorin-Dll4 on the membrane (Figure 11A). Equally, tagRFP-EHD2 and caveolin 1 also formed around pHluorin-Dll4 on the membrane in canonical clusters of rosettes characteristic of caveolae (Figure 11B).

To determine if this colocalization suggested that Dll4 is in fact endocytosed through a calthrin-independent, caveolin-mediated endocytosis, we used the cholesterol inhibitor Methyl- β -cyclodextrin (M β CD) which disrupts caveolae. Accordingly, caveolin-1 in cells treated with M β C failed to form canonical rosettes and instead was structurally indistinct throughout the cell (Figure 11C,D). EHP1 and EHD2 also lost much of the structural definition shown in the previous figures and failed to organize around Dll4 (Figure 11C,D). These results suggest that EHP1 and EHD2 facilitate endocytosis of Dll4 via caveolin-mediate endocytosis.

EHP1, EHD2, and caveolin localize to adherens junctions in 3D sprouts

Despite homogenization of Dll4 in 2D HUVEC assays, the localization of proteins may not accurately reflect the localization of the same proteins in a 3D vascular sprout. We therefore employed a 3D sprouting assay wherein beads coated with HUVEC are embedded into a matrix and form sprouts to assess the expression of EHP1 and EHD2 relative to Dll4 at adherens junctions (Figure 12A). As in 2D cell culture, tagRFP-EHP1 colocalized strongly with actin in 3D sprouts, supporting the role of EHP1 to tether actin through its CH domain (Figure 12B). This localization was severely disrupted in sprouts treated with EHD2si, supporting the cooperative nature of EHP1 and EHD2

Figure 11 EHP1 and EHD2 organize around Dll4 dependent on caveolin. (A,B) EHP1 and EHD2 localize to surface pHluorin-Dll4 and colocalize with caveolin-1. (C) EHP1 and (D) EHD2 fail to localize to Dll4 when treated with MβCD (cholesterol inhibitor). Boxed are magnified section on right. Yellow arrows show areas of colocalization.

working in concert at the Dll4 caveolar endocytic pit at the junction between tip and stalk cell (Figure 12C). It is possible that EHD2 is required for EHP1 to tether actin through its CH domain, potentially through some modification of CH domain availability. Alternatively, EHD2 may not be required for EHP1 to tether actin, but rather signals EHP1 to recruit it to an endocytic pit.

TagRFP-EHP1 also displayed a strong affinity to the adherens junction in the 3D sprout, which was severely disrupted in EHD2si treated cells (Figure 12D,E).

Figure 12. EHP1, EHD2, and Caveolin localize to adherens junctions in 3D vascular sprouts. (A) Schematic of 3-dimensional sprout growth in fibrin bead assay (FBA). (B,C) Representative EC sprout stained for actin expressing tagRFP-EHP1,

treated with indicated siRNA. **(D,E)** Representative EC sprout stained for VE-cadherin (VE-cad) expressing tagRFP-EHBP1, treated with indicated siRNA. **(F,G)** Representative EC sprout stained for VE-cadherin (VE-cad) expressing tagRFP-EHD2, treated with indicated siRNA. **(H)** Dll4 and caveolin-1 colocalize at adherens junction in P6 mouse retinal blood vessels. Boxed are magnified section on right. Yellow arrows show areas of colocalization. Teal arrows show areas of mislocalization.

Equally, tagRFP-EHD2 strongly localized to adherens junctions and this localization was disrupted in EHBP1si treated cells (Figure 12F,G). This localization supports the EHBP1 and EHD2 gain of function assay (Figure 8) to conclude that EHBP1 and EHD2 are present at sites of Dll4/Notch1 binding and therefore ready and able to trans-endocytose the Dll4/Notch1 complex at the adherens junction between tip and stalk cell. The dependence of EHBP1 and EHD2 on each other to localize to these sites paint a picture of interdependent proteins available, and required for, tethering to the cytoskeleton at adherens junctions. In P6 mouse eyes, caveolin strongly colocalized to Dll4 at adherens junctions (Figure 12H). Taken together, these results support the role of EHBP1 and EHD2 acting with caveolin to tether Dll4 endocytic pits to the actin cytoskeleton.

Loss of EHBP1 and EHD2 blunts Dll4 endocytosis and caveolar integrity

With evidence of EHBP1 and EHD2 working together to tether caveolin-mediated endocytosis of Dll4 to the cytoskeleton, we next sought to characterize any Dll4 endocytic defects resulting from loss of EHBP1 or EHD2 in cell culture. In order to do this, an anti-Dll4 antibody was linked to pHrodo™ iFL Red STP Ester and used in a pulse-chase internalization assay. Similar to pHluorin, pHrodo™ is a pH sensitive probe. However, in contrast to pHluorin, pHrodo™ is non-fluorescent on the membrane and

increases fluorescent intensity with decreasing endosomal pH (Figure 13A, Kamen et al. 2019). In an endosome targeted for lysosomal degradation, the intensity of the pHrodo will reach a peak and then be quenched once the tethered antibody and probe are degraded by the lysosome. This property allows us to track how internalization of a tagged antibody bound to Dll4 on the membrane is impacted by loss of EHBP1 and EHD2. As a control, a goat-anti-mouse IgG was also linked to the pHrodo tag.

We observed that pHrodoTM pulse-chase with a non-specific antibody does result in a low-level internalization over time (Figure 13B). In scramble treated cells in which the pHrodoTM linked anti-Dll4 antibody was used, there was a sharp peak in fluorescent intensity at the 10-minute time point. This suggests that antibody-bound Dll4 is rapidly internalized and trafficked to the lysosome. Notably, this internalization event is absent in EHBP1si, EHD2si, and combination EHBP1/EHD2si treated cells suggesting that EHBP1 and EHD2 are required for proper internalization of Dll4 to occur (Figure 13B). This absence of internalization is consistent in cells treated with the dynamin inhibitor Dynasore and cholesterol inhibitor M β CD which disrupts caveolae, suggesting that it is a caveolin endocytic defect (Figure 13C). As EHD2 is functionally similar to dynamin, these results also suggest that the endocytic defect caused by loss of EHD2 may be due to the failure of scission of the Dll4 endocytic pit from the membrane. To validate that this disruption of antibody bound Dll4 endocytosis was directly related to its role in Notch signaling, we then pHrodoTM labelled a recombinant soluble NECD protein which will bind and transendocytose Dll4. Because this recombinant NECD has no tension or force to apply to Dll4 as a native NECD bound to the cell would, we functionalized this

Figure 13. Endocytosis of Dll4 and NECD are dependent on EHBP1 and EHD2. (A) Schematic of pHrodo labeled Dll4 antibody. PHrodo gains fluorescent intensity with increasing endosomal pH, thus used as a metric of endocytosis. (B,C) Relative internalization of pHrodo-Dll4 pulse-chase over time between indicated siRNA groups. IgG was used a non-specific internalization control. Dynasore is pan-endocytosis inhibitor. M β CD is a cholesterol specific inhibitor that disrupts caveolin. (D) Schematic recombinant Notch intracellular domain (NECD) functionalized to microbead and labeled with pHrodo. (E,F) Relative internalization of NECD pulse-chase over time between indicated siRNA groups. Significance between scramble group and other groups listed in order of figure legend. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$. ns=non-significant.

labelled protein to an agarose bead (Figure 13D). The kinetics of internalization were different in scramble treated cells compared to the Dll4-antibody internalization (Figure 13E). However, this baseline internalization was also significantly impaired by treatment with EHBP1si, EHD2si, and EHBP1/EHD2si in combination (Figure 13E). The same internalization defect was also seen in Dll4si, Jag1si, and dual Dll4/Jag1si, again linking this defect directly to Dll4 endocytosis and the Notch signaling pathway (Figure 13F).

We considered that the disruptions in siRNA treated cells to Dll4 endocytosis may be indicative not of an endocytic defect, but rather a reduction of Dll4 due to a negative feedback loop. However, we observed that in whole cell lysate samples of EHBP1si and EHD2si treated cells with DAPT and dynasore controls that protein levels of Dll4 remained constant (Figure 14A,B). Therefore, the reduced internalization of Dll4 is a direct result of loss of EHBP1 and EHD2, supporting their role as endocytic mediators of Dll4/Notch1 transendocytosis in endothelial cells.

In order to visualize this endocytic impairment in the absence of EHBP1 and EHD2, we submitted treated samples to the University of Colorado Anschutz Medical campus for TEM imaging. We observed an increased number of small endocytic caveolar vacuoles near the plasma membrane, but failing to form large structures or fuse with the membrane (Figure 14C). This is consistent with TEM observations of EHD2si treated cells previously (Morén et al. 2012). We propose that this is a result of caveola unable to be stabilized at the plasma membrane by the structural machinery of EHBP1 and EHD2. In the absence of the structural support and mechanical force generated by EHBP1 and EHD2, Dll4 is unable to expose the S2 cleavage site of Notch1.

Figure 14. Loss of EHBP1 and EHD2 result in loss of caveolar structure. (A,B) Western blot of Dll4 levels across indicated treatment groups. **(C)** TEM analysis of caveolar structure in ECs in across indicated siRNA treatments. White inset boxes show area of magnification.

RNA-seq analysis of EHBP1 deficient cells

The purpose of our RNA-sequencing analysis was two-fold. First, comprehensive knowledge of EHBP1 is almost absent from the literature. Second, the evidence we have gathered thus far of the role of EHBP1 and EHD2 in caveolin-mediated endocytosis has broad reaching implications for the potential cell-wide roles of these two proteins. We therefore sent 3 replicates each of scramble siRNA treated cells and EHBP1si treated cells to Novogene for sequencing. Prior to sending the samples, confirmation of KD efficiency was validated by western blot (Figure 15A).

In the EHBP1si treated group, there were only 99 significantly differentially regulated genes compared to the control (Appendix B). A volcano plot shows the log-fold-change of gene expression between the scramble and EHBP1si treated ECs, with upregulation presented above the line and downregulation presented below the line

(Figure 15B). Of these 99 differentially regulated genes, approximately 45% either contained EGF repeats, analogous to Dll4 and Notch, or were indirectly related to EGF-containing protein signaling processes (Figure 15B, C). Further analysis revealed that 8 of the 99 genes were directly related to caveolin endocytosis (Figure 15B, D) and 4 of the 99 genes were actin remodeling genes (Figure 15B, E). This suggests that EHBP1's role in regulating caveolin-mediated endocytosis may not be specific to Dll4 but can accommodate a variety of cargo.

To see if this trend translated to an increased consequence to angiogenic signaling, we grouped the differentially regulated genes by gene ontology (Figure 16). 8 genes were directly attributed to either angiogenesis, blood vessel development, or response to hypoxia (Figure 16A). Another 11 genes were more broadly attributed to the trafficking ontologies SNARE binding, G protein-coupled receptor binding, exocyst, and MAPK cascade (Figure 16B). Beyond these, all differentially regulated gene ontologies are represented in a bar plot and show a dramatic impact on cellular processes as a whole (Figure. 16C).

Figure 15. RNA-Seq analysis of loss of EHP1 in HUVEC. (A) Confirmation of knockdown efficiency of EHP1 siRNA replicates. (B) Volcano plot distribution of all differentially regulated genes in EHP1si condition compared to control. Dots above the

line represent upregulation; dots below the line represent downregulation. Dark green dots represent significantly differentially regulated genes. Highlighted points are relevant to angiogenic signaling. **(C)** Number of significantly different transcripts that contain EGF repeat motifs, are **(D)** directly related to caveolin-mediated endocytosis or **(E)** directly related to actin remodeling.

Figure 16. Gene ontology analysis of differentially regulated genes reveals cell-wide signaling disruptions. (A) Significantly differentially regulated genes related to angiogenesis. **(B)** Significantly differentially regulated genes related to endo- or

exocytosis. **(C)** All gene ontology groupings of significantly differentially regulated genes. Bars shown in red were upregulated as a group. Bars shown in blue were downregulated as a group.

Chapter 4: Conclusions

The endocytosis of Delta ligands, particularly Dll4, to regulate Notch signaling in endothelial cells has previously remained uncharacterized. We have shown here for the first time that two proteins, EHBP1 and EHD2, act to regulate Notch signaling through the transendocytosis of the Dll4/Notch1 complex by caveolin-mediated endocytosis. This is the first characterization of Dll4 as being endocytosed by caveolin-mediated endocytosis in endothelial cells, and this knowledge can be used to further studies of Dll4/Notch1 endocytosis and signaling. Furthermore, this is the first report to show EHBP1 and EHD2 acting together to regulate clathrin-independent endocytosis through tethering to the actin cytoskeleton.

Loss of either EHBP1 or EHD2 in zebrafish produced a significant increase in vascular defects characteristic of Notch pathway inhibition. Normally smoothened, established ISVs with a stalk cell identity became branching, spiny, migratory cells characteristic of increased tip cell identity. This aberration to the Notch pathway was confirmed by RT-PCR, which revealed a significant downregulation of the downstream Notch target Hey2 in EHBP1 and EHD2 KOs. Overexpression of vascular driven EHBP1 and EHD2 injected into zebrafish showed a strong preference toward the tip cell position, as would be expected by proteins regulating the endocytosis of Dll4. While previous literature suggested that EHBP1 and EHD2 worked together in clathrin mediated endocytosis, we show here that EHBP1 and EHD2 regulate Dll4 endocytosis independent

of clathrin. Clathrin is largely absent from sites of EHBP1, EHD2, and actin. However, EHBP1 and EHD2 still localized to junctions of actin containing Dll4. This suggested that some other endocytic mechanism was at play. Clusters of EHBP1 and EHD2 colocalized strongly with caveolin-1 around Dll4 on the membrane. This localization was disrupted in cells treated with caveolin 1 inhibitor M β C, suggesting that EHD2 and EHBP1 are in fact binding to caveolin 1. The functional localization of these proteins was validated in cells with 3D structure, showing that EHBP1 and EHD2 localized to each other at actin bundles and adherens junctions and are dependent on one another to maintain this localization. In P6 mouse eyes stained for Dll4 and caveolin 1 there was a strong colocalization at adherens junctions.

We validated the functional requisite of EHBP1 and EHD2 to assist Dll4 in caveolin-mediated endocytosis through pulse chase internalization assays. In cells lacking EHBP1 or EHD2, a pHrodo labeled Dll4 antibody failed to internalize with the same dynamics as scramble siRNA treated cells. This failure to internalize was also observed in a pulse chase internalization assay with a bead-functionalized recombinant Notch extracellular domain. These results suggest that internalization of the Dll4/NECD complex is impaired in the absence of EHBP1 and EHD2. This failure to internalize was not due to a feedback loop downregulation of Dll4. In line with previous reports, TEM revealed that loss of EHBP1 and EHD2 compromised the structural integrity of the caveolar pit.

Taken together, we propose a model wherein EHD2 oligomerizes around the neck of the endocytic vesicle containing Dll4 bound to the NECD by binding to caveolin 1

(Figure 17). The EH domain of EHD2 also binds to the NPF motifs of EHBP1. EHBP1 is tethered to the actin cytoskeleton, generating the force necessary to expose the S2 cleavage site of Notch and allow for effective Notch signaling (Figure 17). In the absence of EHBP1, the caveolar pit will be unable to tether to the actin cytoskeleton. In this event, the caveolar pit will lose structure provided by the actin support and be unable to generate the force necessary for Dll4/NECD transendocytosis. In the absence of EHD2, the caveolar pit will lose the ability to perform scission from the membrane in addition to losing the bridge to the actin cytoskeleton through EHBP1.

The impairment of caveolin-mediated endocytosis in the absence of EHBP1 and EHD2 was also shown here to have broad reaching implications for endo- and exocytosis as a whole through the RNA sequencing analysis of EHBP1 siRNA treated endothelial cells. The exocyst complex, MAPK cascade, SNARE binding, and G protein coupled receptor binding are just some of the cellular signaling pathways affected by the loss of EHBP1 in the cell.

One future experiment should employ atomic force microscopy on Dll4 and Notch1 in both scramble treated, EHBP1si treated, and EHD2si treated endothelial cells to validate the force requirement of the EHBP1-EHD2 complex on exposing the S2 cleavage domain of Notch1. To further this aim of validating exposure of the S2 cleavage site on Notch1 we propose to use, and have begun to construct, a recombinant Notch1 protein tagged at the n-terminus with a Flag epitope and at the c-terminus with an HA

Figure 17. Model of EHBP1 and EHD2 generating force on caveolar pit. (A) Dll4 binds NECD in the budding caveolin pit. (B,C) EHD2 binds caveolin and oligomerizes around the neck of the endocytic pit. EHBP1 binds to EHD2 and tethers the actin skeleton through the CH domain. This generates the force necessary to expose the S2 cleavage site on Notch.

epitope. This would allow us to stain against both epitopes to observe whether cleavage of the S2 site has occurred.

If an insufficient force were applied, as we propose is occurring in the absence of EHBP1 and EHD2, staining against both epitopes should decorate the cell membrane where the intact Notch1 ligand remains. If a sufficient force were applied, staining for the

HA epitope should decorate the nucleus, where the cleaved NICD has traveled; the Flag epitope should decorate vesicles containing Dll4.

If, in fact, this direct demonstration of force holds showing that EHBP1 and EHD2 provide a necessary and sufficient force to expose the S2 cleavage domain of Notch1, this would have interesting implications for the opposing signaling effects between Dll4 and Jag1 after binding Notch1. As stated in the introduction, Dll4 and Jag1 are almost identical in domain composition, bind Notch1 at the same site, yet have opposite signaling consequences; the reasons for this signaling discrepancy are vague and incomplete.

Is it possible that the real signaling discrepancy between Dll4 and Jag1 are produced by an insufficient force after Jag1/Notch1 binding? In this case, Jag1 would occupy Notch1 EGF binding sites, effectively blocking binding by Dll4. But, without the force supplied by EHBP1 and EHD2, Jag1 would be unable to expose the S2 cleavage site and would therefore blunt Notch signaling. All experiments shown here could be repeated with Jag1 instead of Dll4 to demonstrate this possibility.

Overall, the data shown here demonstrate for the first time in endothelial cells that Dll4 is endocytosed via caveolin-mediated endocytosis using the proteins EHBP1 and EHD2. This is important as it contradicts the clathrin-mediated endocytic mechanism of other Delta-like ligands in other tissue types described in the literature and expands the field's knowledge of a signaling pathway absolutely critical to angiogenesis. Furthermore, our characterization of the broad trafficking role of EHBP1 in endothelial

cells suggests a vital function of a previously poorly characterized protein which can be explored more deeply in future studies.

References

- Benedito, Rui, et al. "The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis." *Cell*, vol. 137, no. 6, 2009, pp. 1124–1135., doi:10.1016/j.cell.2009.03.025.
- Benmerah, Alexandre, et al. "AP-2/Eps15 Interaction Is Required for Receptor-Mediated Endocytosis." *Journal of Cell Biology*, vol. 140, no. 5, 1998, pp. 1055–1062., doi:10.1083/jcb.140.5.1055.
- Bertrand, Julien Y., et al. "Haematopoietic Stem Cells Derive Directly from Aortic Endothelium during Development." *Nature*, vol. 464, no. 7285, 2010, pp. 108–111., doi:10.1038/nature08738.
- Blanco, R. and H. Gerhardt, *VEGF and Notch in tip and stalk cell selection*. Cold Spring Harb Perspect Med, 2013. 3(1): p. a006569.
- Bussmann, J., et al. "Arterial-Venous Network Formation during Brain Vascularization Involves Hemodynamic Regulation of Chemokine Signaling." *Development*, vol. 138, no. 9, 2011, pp. 1717–1726., doi:10.1242/dev.059881.
- Chitnis, Ajay. "Why Is Delta Endocytosis Required for Effective Activation of Notch?" *Developmental Dynamics*, vol. 235, no. 4, 2006, pp. 886–894., doi:10.1002/dvdy.20683.

- Choi, Jayoung, et al. "FoxH1 Negatively Modulates flk1 Gene Expression and Vascular Formation in Zebrafish." *Developmental Biology*, vol. 304, no. 2, 2007, pp. 735–744., doi:10.1016/j.ydbio.2007.01.023.
- Echarri, A., and M. A. Del Pozo. "Caveolae - Mechanosensitive Membrane Invaginations Linked to Actin Filaments." *Journal of Cell Science*, vol. 128, no. 15, 2015, pp. 2747–2758., doi:10.1242/jcs.153940.
- Estrach, Soline, et al. "Laminin-Binding Integrins Induce Dll4 Expression and Notch Signaling in Endothelial Cells." *Circulation Research*, vol. 109, no. 2, 2011, pp. 172–182., doi:10.1161/circresaha.111.240622.
- Fiuza, U.-M., and A. M. Arias. "Cell and Molecular Biology of Notch." *Journal of Endocrinology*, vol. 194, no. 3, 2007, pp. 459–474., doi:10.1677/joe-07-0242.
- Gall, Maude Le, et al. "Molecular Separation of Two Signaling Pathways for the Receptor, Notch." *Developmental Biology*, vol. 313, no. 2, 2008, pp. 556–567., doi:10.1016/j.ydbio.2007.10.030.
- Gerhardt, H., et al., *VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia*. J Cell Biol., 2003. **161**(6): p. 1163-77. Epub 2003 Jun 16.
- Gessler, Manfred. "Dll1 And Dll4: Similar, but Not the Same." *Blood*, vol. 113, no. 22, 2009, pp. 5375–5376., doi:10.1182/blood-2009-02-205468.

- Gordon, W. R., et al. "The Molecular Logic of Notch Signaling - a Structural and Biochemical Perspective." *Journal of Cell Science*, vol. 121, no. 19, 2008, pp. 3109–3119., doi:10.1242/jcs.035683.
- Gordon, Wendy R., et al. "Effects of S1 Cleavage on the Structure, Surface Export, and Signaling Activity of Human Notch1 and Notch2." *PLoS ONE*, vol. 4, no. 8, 2009, doi:10.1371/journal.pone.0006613.
- Guilherme, Adilson, et al. "EHD2 And the Novel EH Domain Binding Protein EHBPI Couple Endocytosis to the Actin Cytoskeleton." *Journal of Biological Chemistry*, vol. 279, no. 11, 2003, pp. 10593–10605., doi:10.1074/jbc.m307702200.
- He, T.-C., et al. "A Simplified System for Generating Recombinant Adenoviruses." *Proceedings of the National Academy of Sciences*, vol. 95, no. 5, 1998, pp. 2509–2514., doi:10.1073/pnas.95.5.2509.
- Hen, G., et al. "Venous-Derived Angioblasts Generate Organ-Specific Vessels during Zebrafish Embryonic Development." *Development*, vol. 142, no. 24, 2015, pp. 4266–4278., doi:10.1242/dev.129247.
- Hoernke, Maria, et al. "EHD2 Restrains Dynamics of Caveolae by an ATP-Dependent, Membrane-Bound, Open Conformation." *PNAS*, vol. 144, no. 22, 30 May 2017, pp. E4360–E4369.

- Hoshijima, K., M.J. Juryneć, and D.J. Grunwald, *Precise Editing of the Zebrafish Genome Made Simple and Efficient*. *Dev Cell*, 2016. 36(6): p. 654-67.
- Kaksonen, Marko, and Aurélien Roux. “Mechanisms of Clathrin-Mediated Endocytosis.” *Nature Reviews Molecular Cell Biology*, vol. 19, no. 5, 2018, pp. 313–326., doi:10.1038/nrm.2017.132.
- Kamen, L., et al., *A novel method for determining antibody-dependent cellular phagocytosis*. *J Immunol Methods*, 2019. 468: p. 55-60.
- Kovtun, O., et al. “Cavin Family Proteins and the Assembly of Caveolae.” *Journal of Cell Science*, vol. 128, no. 7, 2015, pp. 1269–1278., doi:10.1242/jcs.167866.
- Krebs, Luke T, et al. “Endothelial Notch Signaling Is Essential to Prevent Hepatic Vascular Malformations in Mice.” *Genes & Development*, vol. 14, no. 11, 1 June 2000, pp. 1343–1352.
- Liu, Zhao-Jun, et al. “Regulation of Notch1 and Dll4 by Vascular Endothelial Growth Factor in Arterial Endothelial Cells: Implications for Modulating Arteriogenesis and Angiogenesis.” *Molecular and Cellular Biology*, vol. 23, no. 1, 2003, pp. 14–25., doi:10.1128/mcb.23.1.14-25.2003.
- Love, Michael I., et al. “RNA-Seq Workflow: Gene-Level Exploratory Analysis and Differential Expression.” *F1000Research*, vol. 4, 2015, p. 1070., doi:10.12688/f1000research.7035.1.

Mack, Julia J., and M. Luisa Iruela-Arispe. “NOTCH Regulation of the Endothelial Cell Phenotype.” *Current Opinion in Hematology*, vol. 25, no. 3, 2018, pp. 212–218., doi:10.1097/moh.0000000000000425.

Melo, Arthur Alves, et al. “Structural Insights into the Activation Mechanism of Dynamin-like EHD ATPases.” *Proceedings of the National Academy of Sciences*, vol. 114, no. 22, 2017, pp. 5629–5634., doi:10.1073/pnas.1614075114.

Meloty-Kapella, Laurence, et al. “Notch Ligand Endocytosis Generates Mechanical Pulling Force Dependent on Dynamin, Epsins, and Actin.” *Developmental Cell*, vol. 22, no. 6, 2012, pp. 1299–1312., doi:10.1016/j.devcel.2012.04.005.

Morén, Björn, et al. “EHD2 Regulates Caveolar Dynamics via ATP-Driven Targeting and Oligomerization.” *Molecular Biology of the Cell*, vol. 23, no. 7, 2012, pp. 1316–1329., doi:10.1091/mbc.e11-09-0787.

Morris, Kyle L., et al. “Cryo-EM of Multiple Cage Architectures Reveals a Universal Mode of Clathrin Self-Assembly.” *Nature Structural & Molecular Biology*, vol. 26, no. 10, 2019, pp. 890–898., doi:10.1038/s41594-019-0292-0.

Nakatsu, Martin N., et al. “Optimized Fibrin Gel Bead Assay for the Study of Angiogenesis.” *Journal of Visualized Experiments*, no. 3, 2007, doi:10.3791/186.

- Panin, Vladislav M., et al. "Notch Ligands Are Substrates for ProteinO-Fucosyltransferase-1 and Fringe." *Journal of Biological Chemistry*, vol. 277, no. 33, 2002, pp. 29945–29952., doi:10.1074/jbc.m204445200.
- Peacock, et al. "Arteriovenous Malformations in Hereditary Haemorrhagic Telangiectasia: Looking beyond ALK1-NOTCH Interactions." *OUP Academic*, Oxford University Press, 8 Dec. 2015, academic.oup.com/cardiovascres/article/109/2/196/2196662.
- Proulx, Kira, et al. "Cranial Vasculature in Zebrafish Forms by Angioblast Cluster-Derived Angiogenesis." *Developmental Biology*, vol. 348, no. 1, 2010, pp. 34–46., doi:10.1016/j.ydbio.2010.08.036.
- Quillard, Thibaut, and Beatrice Charreau. "Impact of Notch Signaling on Inflammatory Responses in Cardiovascular Disorders." *International Journal of Molecular Sciences*, vol. 14, no. 4, 2013, pp. 6863–6888., doi:10.3390/ijms14046863.
- Šamaj, Jozef, et al. "Endocytosis, Actin Cytoskeleton, and Signaling." *Plant Physiology*, vol. 135, no. 3, 2004, pp. 1150–1161., doi:10.1104/pp.104.040683
- Sankaranarayanan, S., et al., *The use of pHluorins for optical measurements of presynaptic activity*. Biophys J, 2000. 79(4): p. 2199-208.

- Shergill, Bhupinder, et al. “Optical Tweezers Studies on Notch: Single-Molecule Interaction Strength Is Independent of Ligand Endocytosis.” *Developmental Cell*, vol. 22, no. 6, 2012, pp. 1313–1320., doi:10.1016/j.devcel.2012.04.007.
- Therapontos, Christina, and Neil Vargesson. “Zebrafish Notch Signalling Pathway Mutants Exhibit Trunk Vessel Patterning Anomalies That Are Secondary to Somite Misregulation.” *Developmental Dynamics*, vol. 239, no. 10, 2010, pp. 2761–2768., doi:10.1002/dvdy.22410.
- Thisse, Christine, and Bernard Thisse. “High-Resolution in Situ Hybridization to Whole-Mount Zebrafish Embryos.” *Nature Protocols*, vol. 3, no. 1, 2007, pp. 59–69., doi:10.1038/nprot.2007.514.
- Torrino, Stéphanie, et al. “EHD2 Is a Mechanotransducer Connecting Caveolae Dynamics with Gene Transcription.” *The Journal of Cell Biology*, vol. 217, no. 12, 2018, pp. 4092–4105., doi:10.1083/jcb.201801122.
- Sander, Guy R., et al. “Developmental Signaling Networks: The Notch Pathway.” *Physiology of the Gastrointestinal Tract*, 2006, pp. 287–306., doi:10.1016/b978-012088394-3/50013-1.
- Sjöqvist, Marika, and Emma R. Andersson. “Do as I Say, Not(Ch) as I Do: Lateral Control of Cell Fate.” *Developmental Biology*, vol. 447, no. 1, 2019, pp. 58–70., doi:10.1016/j.ydbio.2017.09.032.

Wu, Roland S., et al. "A Rapid Method for Directed Gene Knockout for Screening in G0 Zebrafish." *Developmental Cell*, vol. 46, no. 1, 2018, doi:10.1016/j.devcel.2018.06.003.

Appendix A

Reagent Type (species) or Resource	Designation	Source or Reference	Identifiers	Additional Information
Antibody	Clathrin Heavy Chain Monoclonal Antibody (Mouse)	Thermo Fisher Scientific	MA1-065	Immunohistochemistry: 1/1000 dilution
Antibody	Caveolin 1 Polyclonal Antibody (Rabbit)	Thermo Fisher Scientific	PA1-064	Immunohistochemistry: 1/2000 dilution
Antibody	Dll4 Polyclonal Antibody (Goat)	Thermo Fisher Scientific	PA5-46974	Immunohistochemistry: 1/1000 dilution
Antibody	Dll4 Polyclonal Antibody (Rabbit)	abcam	ab7280	Immunoblotting: 1/500 dilution
Antibody	Dll4 Polyclonal Antibody (Mouse)	Thermo Fisher Scientific	PA5-46974	
Antibody	EHBP1 Polyclonal Antibody (Rabbit)	Thermo Fisher Scientific	PA5-57282	Immunohistochemistry: 1/500 Dilution
Antibody	EHD2 Polyclonal Antibody (Rabbit)	Thermo Fisher Scientific	PA5-61497	Immunohistochemistry: 1/1000 dilution Immunoblotting: 1/500 dilution
Antibody	Recombinant Anti-alpha Tubulin antibody (Rabbit)	Abcam	ab52866	Immunoblotting: 1/5000 dilution
Antibody	Monoclonal Anti- α -Tubulin antibody (Mouse)	Sigma Aldrich	T9026-.2ML	Immunoblotting: 1/5000 dilution
Antibody	VE-cadherin polyclonal antibody	Enzo Life Sciences	ALX-210-232-C100	Immunostaining: 1/200
Antibody	DYKDDDDK Tag polyclonal antibody	Thermo Fisher Scientific	PA1-984B	Immunostaining: 1/100 dilution
Antibody	Donkey anti-Mouse IgG (H+L) Highly Cross-Absorbed Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific	A-31571	Immunostaining: 1/1000 dilution
Antibody	Goat anti-Rabbit IgG (H+L) Highly Cross-Absorbed Secondary Antibody, Alexa Fluor Plus 647	Thermo Fisher Scientific	A32733	Immunostaining: 1/1000 dilution
Antibody	Chicken anti-Rabbit IgG (H+L) Cross-Absorbed Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific	A-21443	Immunostaining: 1/1000 dilution

Antibody	Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP	Thermo Fisher Scientific	31430	Immunoblotting: 1/5000 dilution
Antibody	Goat Anti-Rabbit IgG (HL), HRP-Linked Whole Ab	Genesee Scientific	20-303	Immunoblotting: 1/5000
Cellular Dye	Hoechst 33342, trihydrochloride, trihydrate	Thermo Fisher Scientific	H3570	1/1000 dilution cell culture
Cellular Dye	Alexa Fluor™ 647 Phalloidin	Thermo Fisher Scientific	A22287	1/500 dilution cell culture
Recombinant Protein	Recombinant Human Notch-1 Fc Chimera	R&D Systems	P46531	
Chemical compound	Cas9 protein with NLS, high concentration	PNA Bio	CP02	
Chemical compound	Cytochalasin B	Sigma Aldrich	C6762-5MG	
Chemical compound	Dimethyl sulfoxide ≥99.5%, Ultra Pure Grade	VWR Life Science	97063-136	
Chemical compound	LY-411575	Sigma Aldrich	SML0506-5MG	2 µM final concentration
Chemical compound	Latrunculin A	Sigma Aldrich	428021-100UG	5 µM final concentration
Chemical compound	Methyl-β-cyclodextrin	Sigma Aldrich	M7439-1G	10 mM final dilution
Chemical compound	pHrodo™ iFL Red STP Ester (amine-reactive)	Thermo Fisher Scientific	P36010	
Chemical compound	DAPT	Sigma Aldrich	D5942-5MG	5 µM final concentration
Chemical compound	Dynasore hydrate	Sigma Aldrich	D7693-5MG	100 µM final concentration
Chemical compound	Phenol Red	VWR	470301-974	0.1% final dilution in water
Commercial assay or kit	MEGAscript™ T3 Transcription Kit	Thermo Fisher Scientific	AM1338	
Commercial assay or kit	HiScribe™ SP6 RNA Synthesis Kit	New England BioLabs	E2070S	

Commercial assay or kit	High Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	4368814	
Other	Dynabeads™ Protein G for Immunoprecipitation	Thermo Fisher Scientific	10004D	
Other	Protein A Agarose Resin	Gold Biotechnology	P-400-5	
Other	Poly-D-Lysine	Thermo Fisher Scientific	A3890401	
Other	Laminin Mouse Protein, Natural	Thermo Fisher Scientific	23017015	

Appendix B

	baseMean	log2Fold Change	lfcSE	stat	pvalue	padj	symbol
ENSG000000089723	22.1014999	-4.63E+00		-4.0720217	4.66E-05	NA	OTUB2
ENSG000000183840	27.3967476	-3.15E+00	0.85943631	-3.6656858	0.00024668	3.52E-02	GPR39
ENSG000000157045	171.041544	-1.99E+00	0.36389978	-5.4718281	4.45E-08	2.17E-05	NTAN1
ENSG000000115504	473.845438	-1.73E+00	0.22589519	-7.6578629	1.89E-14	4.80E-11	EHBP1
ENSG000000081041	242.551212	-1.59E+00	0.27410654	-5.7860868	7.20E-09	5.38E-06	CXCL2
ENSG000000169429	764.532758	-1.55E+00	0.1774785	-8.7351553	2.43E-18	1.03E-14	CXCL8
ENSG000000163131	182.37218	-1.54E+00	0.34081567	-4.5207608	6.16E-06	1.78E-03	CTSS
ENSG000000163739	1040.71362	-1.46E+00	0.22843278	-6.385674	1.71E-10	1.97E-07	CXCL1
ENSG00000010319	632.589134	-1.22E+00	0.2171759	-5.6369928	1.73E-08	1.14E-05	SEMA3G
ENSG000000205403	451.260567	-1.18E+00	0.21285323	-5.5305096	3.19E-08	1.69E-05	CFI
ENSG000000120337	1000.91183	-1.03E+00	0.15505798	-6.6528189	2.88E-11	4.56E-08	TNFSF18
ENSG000000165092	3357.86352	-1.02E+00	0.12647914	-8.0326286	9.54E-16	3.03E-12	ALDH1A1
ENSG000000158270	672.056722	-9.71E-01	0.17238019	-5.6302976	1.80E-08	1.14E-05	COLEC12
ENSG000000108691	3071.1138	-9.70E-01	0.16099826	-6.0263488	1.68E-09	1.42E-06	CCL2
ENSG000000141655	271.839886	-9.27E-01	0.25456054	-3.6428714	0.00026961	3.68E-02	TNFRSF11 A
ENSG000000165801	275.612365	-8.97E-01	0.24951248	-3.5952761	0.00032405	4.24E-02	ARHGEF40
ENSG000000111339	449.594481	-8.34E-01	0.20054603	-4.1573446	3.22E-05	7.29E-03	ART4
ENSG000000179044	597.448679	-8.32E-01	0.18207129	-4.5714542	4.84E-06	1.46E-03	EXOC3L1
ENSG000000185339	2045.81482	-8.26E-01	0.13540518	-6.0974007	1.08E-09	9.77E-07	TCN2
ENSG000000205502	819.148073	-8.20E-01	0.22562937	-3.632243	0.00028097	3.79E-02	C2CD4B
ENSG000000151012	4060.63317	-7.76E-01	0.14666717	-5.2879169	1.24E-07	5.61E-05	SLC7A11
ENSG000000137507	1984.79427	-7.73E-01	0.13753493	-5.620421	1.90E-08	1.15E-05	LRRC32
ENSG000000128918	420.821593	-7.60E-01	0.20193262	-3.7650566	0.00016651	2.74E-02	ALDH1A2
ENSG000000005108	803.863033	-7.42E-01	0.1664832	-4.4566431	8.33E-06	2.30E-03	THSD7A
ENSG000000154222	924.924761	-7.37E-01	0.20311171	-3.6263785	0.00028742	3.84E-02	CC2D1B
ENSG000000159640	2833.08572	-7.27E-01	0.13001192	-5.5949959	2.21E-08	1.22E-05	ACE
ENSG000000182621	743.292897	-7.11E-01	0.19503798	-3.6437596	0.00026868	3.68E-02	PLCB1
ENSG000000128052	8404.32115	-7.00E-01	0.1136694	-6.1620562	7.18E-10	7.01E-07	KDR
ENSG000000069122	1349.65009	-6.75E-01	0.14560161	-4.6333794	3.60E-06	1.11E-03	ADGRF5
ENSG000000112096	3514.23599	-6.68E-01	0.17974848	-3.7164804	0.00020202	3.07E-02	SOD2
ENSG000000170323	1881.10706	-6.45E-01	0.1546985	-4.1698611	3.05E-05	7.03E-03	FABP4
ENSG000000189221	824.77486	-6.42E-01	0.16453121	-3.9042183	9.45E-05	1.82E-02	MAOA
ENSG000000185070	3810.78858	-6.05E-01	0.14115872	-4.2865627	1.81E-05	4.70E-03	FLRT2
ENSG000000101445	2880.30759	-5.68E-01	0.12449194	-4.5630315	5.04E-06	1.49E-03	PPP1R16B

ENSG00000100906	1598.15353	-5.52E-01	0.14284274	-3.8665744	0.00011038	2.03E-02	NFKBIA
ENSG00000120885	4198.03674	-5.42E-01	0.12752303	-4.2472826	2.16E-05	5.42E-03	CLU
ENSG00000125266	2397.97689	-5.16E-01	0.1360608	-3.7903846	0.00015041	2.62E-02	EFNB2
ENSG00000173852	3386.38156	-4.96E-01	0.13157754	-3.7689363	0.00016395	2.74E-02	DPY19L1
ENSG00000167671	2455.64308	-4.89E-01	0.13311375	-3.6743535	0.00023845	3.48E-02	UBXN6
ENSG00000140105	6448.63426	-4.87E-01	0.12091255	-4.0288764	5.60E-05	1.17E-02	WARS1
ENSG00000137509	6604.07757	-4.87E-01	0.11862697	-4.1015272	4.10E-05	8.98E-03	PRCP
ENSG00000113555	1551.34199	-4.85E-01	0.13618446	-3.5606847	0.00036989	4.74E-02	PCDH12
ENSG00000130309	11541.5449	-4.65E-01	0.10815847	-4.3006895	1.70E-05	4.60E-03	COLGALT1
ENSG00000091490	2844.23249	-4.64E-01	0.12452639	-3.7229279	0.00019693	3.07E-02	SEL1L3
ENSG00000110799	111048.992	-4.42E-01	0.1029534	-4.2928171	1.76E-05	4.66E-03	VWF
ENSG00000003436	6639.33473	-4.16E-01	0.11516043	-3.6103013	0.00030584	4.04E-02	TFPI
ENSG00000142798	150415.616	-3.89E-01	0.10621257	-3.6659041	0.00024647	3.52E-02	HSPG2
ENSG00000124762	7670.1264	4.15E-01	0.11337507	3.66028578	0.00025193	3.55E-02	CDKN1A
ENSG00000182492	13726.0988	4.53E-01	0.11192617	4.0483092	5.16E-05	1.09E-02	BGN
ENSG00000134250	2345.1412	5.12E-01	0.13528724	3.78209148	0.00015552	2.67E-02	NOTCH2
ENSG00000166073	1558.61309	5.18E-01	0.14084313	3.6758658	0.00023704	3.48E-02	GPR176
ENSG00000149948	4044.85411	5.52E-01	0.11810873	4.67713976	2.91E-06	9.46E-04	HMGA2
ENSG00000152377	14633.1236	5.67E-01	0.12096004	4.69093988	2.72E-06	9.08E-04	SPOCK1
ENSG00000106624	3258.23347	5.68E-01	0.11945835	4.75291138	2.01E-06	7.07E-04	AEBP1
ENSG00000026508	2248.14992	5.73E-01	0.14296751	4.00554371	6.19E-05	1.27E-02	CD44
ENSG00000173597	2273.77964	5.73E-01	0.15704098	3.64937447	0.00026288	3.66E-02	SULT1B1
ENSG00000163453	10657.3348	5.89E-01	0.10847826	5.4309934	5.60E-08	2.63E-05	IGFBP7
ENSG00000137076	15256.9275	6.31E-01	0.11173934	5.64404327	1.66E-08	1.14E-05	TLN1
ENSG00000135596	763.813419	6.64E-01	0.16387353	4.05007799	5.12E-05	1.09E-02	MICAL1
ENSG00000136205	1965.21818	6.70E-01	0.16006608	4.18425307	2.86E-05	6.80E-03	TNS3
ENSG00000102802	1022.89021	6.72E-01	0.16063164	4.18144251	2.90E-05	6.80E-03	MEDAG
ENSG00000137463	591.931053	6.79E-01	0.18210269	3.72801379	0.000193	3.07E-02	MGARP
ENSG00000164237	744.6505	6.87E-01	0.18465221	3.71976233	0.00019941	3.07E-02	CMBL
ENSG00000058729	661.623394	6.92E-01	0.1780192	3.88508294	0.0001023	1.91E-02	RIOK2
ENSG00000104419	813.844009	7.20E-01	0.18821135	3.8233801	0.00013164	2.35E-02	NDRG1
ENSG00000213853	534.861358	7.31E-01	0.20376671	3.58613329	0.00033562	4.34E-02	EMP2
ENSG00000176438	626.176204	7.41E-01	0.18613628	3.98051737	6.88E-05	1.36E-02	SYNE3
ENSG00000042493	1232.92484	7.51E-01	0.16794293	4.47117265	7.78E-06	2.19E-03	CAPG
ENSG00000164176	1846.00677	7.51E-01	0.14914398	5.03662689	4.74E-07	1.82E-04	EDIL3
ENSG00000058085	537.388406	7.53E-01	0.20303128	3.70996898	0.00020729	3.09E-02	LAMC2
ENSG00000110422	1148.78248	7.53E-01	0.20281268	3.71483592	0.00020334	3.07E-02	HIPK3

ENSG000000128567	11224.646	7.54E-01	0.14876757	5.06628173	4.06E-07	1.61E-04	PODXL
ENSG000000170017	4245.17267	7.75E-01	0.13135229	5.9018274	3.59E-09	2.85E-06	ALCAM
ENSG000000128606	463.967334	7.87E-01	0.20841935	3.77529963	0.00015982	2.70E-02	LRRC17
ENSG000000257103	1011.24753	8.03E-01	0.18945659	4.23799976	2.26E-05	5.50E-03	LSM14A
ENSG000000100979	1043.9293	8.26E-01	0.16175581	5.10843024	3.25E-07	1.37E-04	PLTP
ENSG000000167244	672.855837	8.31E-01	0.17640768	4.70840377	2.50E-06	8.56E-04	IGF2
ENSG000000122861	2039.71415	8.49E-01	0.13724269	6.18881744	6.06E-10	6.41E-07	PLAU
ENSG000000070404	907.394343	8.50E-01	0.17565711	4.8402049	1.30E-06	4.84E-04	FSTL3
ENSG000000136928	318.037015	8.82E-01	0.230182	3.8320666	0.00012707	2.30E-02	GABBR2
ENSG000000135480	7541.39705	8.86E-01	0.1274009	6.95684172	3.48E-12	6.31E-09	KRT7
ENSG000000117152	1998.0236	8.94E-01	0.13707658	6.52432894	6.83E-11	9.63E-08	RGS4
ENSG000000134668	1208.86968	9.06E-01	0.16184649	5.59948036	2.15E-08	1.22E-05	SPOCD1
ENSG000000055163	414.539763	9.85E-01	0.20676131	4.76392303	1.90E-06	6.88E-04	CYFIP2
ENSG000000142173	282.89198	1.01E+00	0.25334933	3.97164385	7.14E-05	1.39E-02	COL6A2
ENSG000000115107	406.782247	1.09E+00	0.20634744	5.27535593	1.32E-07	5.80E-05	STEAP3
ENSG000000156466	174.801706	1.13E+00	0.29087678	3.89531747	9.81E-05	1.86E-02	GDF6
ENSG000000166263	300.420972	1.15E+00	0.30746465	3.72566798	0.0001948	3.07E-02	STXBP4
ENSG000000172260	971.078177	1.18E+00	0.15493531	7.59557985	3.06E-14	6.48E-11	NEGR1
ENSG000000169282	136.94956	1.24E+00	0.3281543	3.78999393	0.00015065	2.62E-02	KCNAB1
ENSG000000095303	480.096711	1.27E+00	0.19689882	6.46808397	9.93E-11	1.26E-07	PTGS1
ENSG000000132470	157.422906	1.27E+00	0.31945966	3.98815633	6.66E-05	1.34E-02	ITGB4
ENSG000000162490	283.662047	1.41E+00	0.25686768	5.47408984	4.40E-08	2.17E-05	DRAXIN
ENSG000000149591	1053.8429	1.72E+00	0.18537703	9.25936363	2.06E-20	1.30E-16	TAGLN
ENSG000000198121	81.8514719	1.77E+00	0.41757138	4.24568193	2.18E-05	5.42E-03	LPAR1
ENSG000000111863	103.548945	1.78E+00	0.38418903	4.64077259	3.47E-06	1.10E-03	ADTRP
ENSG000000140092	614.921263	1.89E+00	0.18470883	10.2580904	1.09E-24	1.38E-20	FBLN5
ENSG000000198768	67.17495	2.00E+00	0.48231197	4.14023541	3.47E-05	7.72E-03	APCDD1L
ENSG000000074047	48.9564553	2.55E+00	0.68282283	3.72863732	0.00019252	3.07E-02	GLI2
ENSG000000038427	93.1004752	2.60E+00	0.51313274	5.06633197	4.06E-07	1.61E-04	VCAN